Negative Charges at Protein Kinase C Sites of Troponin I Stabilize the Inactive State of Actin

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ABSTRACT Alterations in the troponin complex can lead to increases or decreases in contractile activity. Most mutations of troponin that cause hypertrophic cardiomyopathy increase the activity of cardiac muscle fibers. In at least some cases these mutants stabilize the active state of regulated actin. In contrast, phosphorylation of troponin I at residues 43, 45, and 144 inhibits muscle contractility. To determine if alterations of troponin I that reduce activity do stabilize the inactive state of actin, we introduced negative charges at residues 43, 45, and 144 of troponin I to mimic a constitutively phosphorylated state. At saturating calcium, all mutants decreased ATPase rates relative to wild-type actin-tropomyosin-troponin. Reduced activation of ATPase activity was seen with a single mutation at S45E and was not further altered by mutating the other two sites. In the presence of low concentrations of NEM-S1, wild-type troponin was more active than the mutants. At high NEM-S1, the rates of wild-type and mutants approached the same limiting value. Changes in Ca^{2+} affinity also support the idea that the equilibrium between states of actin-tropomyosin-troponin was shifted to the inactive state by mutations that mimic troponin I phosphorylation.

INTRODUCTION

Striated muscle contraction is controlled by the actin associated regulatory protein complex of three troponin subunits and tropomyosin (1). Binding of calcium to troponin C exposes a hydrophobic pocket that binds to the switch segment of troponin I (2,3). This interaction moves the inhibitory loop of troponin I away from the actin filament; as a result, there is a cooperative transition of tropomyosin along the actin filament permitting muscle contraction (4,5). Calcium gives partial stabilization of the active state whereas binding of *N*-ethylmaleimide-S1 (NEM-S1) or other ''activating'' forms of S1 produces full activation (6–8). Details of the changes that occur during activation can be found elsewhere (9–13).

The ATPase rate depends on the fraction of myosin S1 that is bound to actin and the fraction of actin that is in the active state. Calcium has little effect on the binding of S1 to actin during ATP hydrolysis (13) but it has a greater effect on the binding of activating states of S1 such as rigor S1 and S1-ADP. Several models have been used to describe actin-myosin binding (14–17). Regulation of the ATPase rate can be described by a model in which two or more states of actin are in equilibrium with each other, creating parallel pathways for activation of ATP hydrolysis (17,18) (Fig. 1). Actin in the active state has a higher k_{cat} for actin-activated ATPase activity (19). This higher rate constant is likely due to a larger rate constant for product release (20,21).

Alterations in the structure of the regulatory proteins can significantly alter muscle regulation. Single point mutations on troponin, such as those associated with hypertrophic cardiomyopathy, can either activate or inhibit ATPase rates by altering the distribution of states (22,23). Physiological modifications of troponin can also alter regulation in normal and pathological states. One important example of this is phosphorylation of troponin I by protein kinase C (PKC), which reduces the calcium sensitivity of force production in skinned fibers and sliding velocity in motility assays (24).

Protein kinase C is one of the two major signaling kinases in the heart and acts downstream of adrenergic receptor stimulation. The phosphorylation sites specific for PKC on troponin I are Ser-43, Ser-45, and Thr-144 (mouse sequence). Fig. 2 shows the locations of residues 43, 45, and 144 in the crystal structure of the core domain of cardiac troponin (25). The core domain of troponin can be divided into two parts: the regulatory head and the IT arm comprised of α-helices from troponin I and troponin T. Thr-144 is located in the middle of the inhibitory region of cardiac troponin I, which connects the regulatory head to the IT arm and is not visible in the crystal structure of the cardiac troponin core domain. Because of this location, modification of Thr-144 could affect the myofilament activity, especially in the resting state. Some studies showed the functional importance of the phosphorylation of Thr-144 (26,27). Residues 43 and 45 are located in the IT arm region and are close to the C-lobe of cardiac troponin C, which is known as a ''structural domain''. Early studies could not positively identify if residues 43 and 45 are both phosphorylated by PKC. Subsequent studies, including those with transgenic animal models, treated residues 43 and 45 as a cluster and indicated that the phosphorylation of residues 43 and 45 has a large impact on myofilament activity (24,28,29). Finley and Rosevear showed that the introduction of negative charges into these two sites destabilized helix G of the C-lobe of cardiac troponin C in the troponin C-troponin

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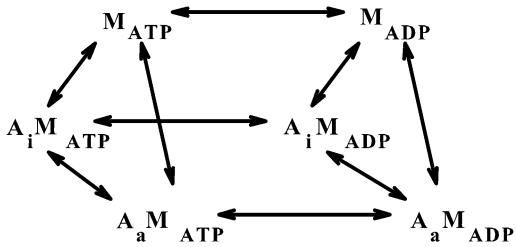

FIGURE 1 Schematic showing the parallel pathway model for muscle regulation. Myosin must be bound to actin in the active state (A_a) for rapid hydrolysis of ATP. Actomyosin in the inactive state (A_i) does not hydrolyze ATP at a sufficient rate to drive muscle contraction and the enzymatic activity of unbound myosin is very low. Ca^{2+} partially shifts the equilibrium between states to favor the active state but cannot fully activate the filament. Tight binding forms of myosin or S1 (rigor myosin, myosin-ADP, NEM-S1) shift the equilibrium to the fully active state. Thus, alterations in troponin I could potentially alter the equilibrium between states, reduce myosin-ATP binding, or alter a step within the active pathway.

I binary complex. They also found that the hydrogen/deuterium exchange rates were altered in Ca^{2+}-binding loops III and IV of cardiac troponin C (30). Recently Ruse et al. found that of these two residues only Ser-45 was phosphorylated when they treated isolated cardiac troponin I with PKC (31). Thus, the obvious question to be answered is whether the phosphorylation of Ser-45 alone is enough to show as significant an effect on myofilament activity as that of both Ser-43 and Ser-45.

About 12 PKC isoforms have been reported so far and they are classified into three subsets by their activation mechanisms: conventional, novel, and atypical. Each isoform may have different predilections for phosphorylating the three sites (32). Some isoforms also phosphorylate residues 22 and 23, which are the protein kinase A phosphorylation sites of troponin I (27,33,34).

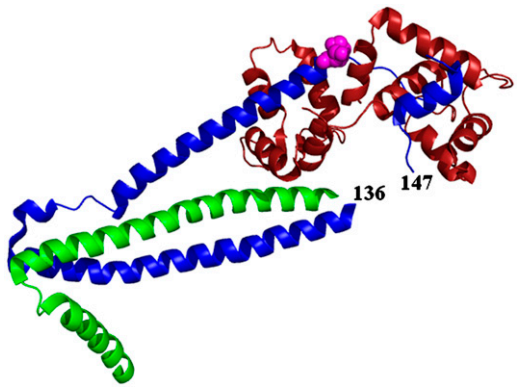

FIGURE 2 Crystal structure of human cardiac troponin complex (Protein Data Bank code, 1J1E). Troponin C is shown in red, troponin I in blue, and troponin T in green. The phosphorylation sites of protein kinase C on mouse troponin I are residues S43, S45, and T144. Residues 43 and 45 are shown as magenta spheres. Residues 136 and 147 (human) are also shown to approximate the position of the T144 site, which has not yet been structurally well defined.

Phosphorylation of troponin I by protein kinase C decreases the maximum force and maximum shortening velocity and decreases Ca^{2+} sensitivities of force production, filament sliding speed, and ATPase rates (24). However, the role of the individual phosphorylation sites has not been totally clarified and the mechanism by which phosphorylation inhibits contraction has not been elucidated.

We examined the effect of introducing charges at specific sites of troponin I on the mechanism of regulation by using a glutamate substitution at the three PKC phosphorylation sites on mouse cardiac troponin I to mimic constitutive phosphorylation. These mutations have been shown to accurately reflect the effects of PKC phosphorylation in muscle fiber studies (24). We examined the effect of these charges on the regulation of ATPase activity and the binding of S1-ADP to actin filaments in solution.

The mutants caused a decrease in ATPase rates at intermediate levels of activity such as at saturating calcium or in EGTA with low levels of NEM-S1. Binding of S1 to actin in the presence of ADP was decreased at low levels of S1. Binding of S1 to actin in the presence of ATP was unaffected. From these results we conclude that the distribution between the inactive and active states of actin is shifted to the inactive state by phosphorylation of troponin I. Neither the binding of S1 to regulated actin by ATP hydrolysis nor the apparent rate constant for the fully active state were affected by these mutations.

MATERIAL AND METHODS

Protein preparation

Actin and myosin were isolated from rabbit back muscle (35). Myosin-S1 was made by digestion of myosin with chymotrypsin (Worthington Biochemical, Freehold, NJ) (36). Tropomyosin was isolated from bovine cardiac tissue. Human cardiac troponin C in pET3d, mouse cardiac troponin I in pET3d, and mouse cardiac troponin T in pSBET were expressed as described previously (37).

Protein concentrations were determined by absorbance measurements at 280 nm, corrected for scattering at 340 nm, using the following extinction coefficients ($\varepsilon^{0.1\%}$) for 280 nm: actin (1.15), myosin-S1 (0.75), tropomyosin (0.23), troponin (0.37).

Measuring ATPase rates

Rates of (γ^{32}P) ATP hydrolysis were determined in the presence and absence of calcium at 25°C, pH 7.0, by measuring the release of ^{32}P (19). Four time points were taken over a 10–15 min period over which the production of ^{32}P was linear with time so that the measured velocities were initial velocities. The buffer generally contained 1 mM ATP, 3 mM $MgCl_2$, 10 mM MOPS, 34 mM NaCl, 1 mM EGTA or 0.5 mM $CaCl_2$, and 1 mM dithiothreitol. When measuring rates in the absence of NEM-S1, 10 μM actin and 0.1 μM S1 was used. The concentration of NEM-S1 was then increased incrementally until the system reached full activation. Virtually all of the NEM-S1 added bound to actin under these conditions (38). To prevent competitive inhibition of S1 binding, the actin concentration was increased

by an equal amount to the NEM-S1 added to maintain the same level of free actin (39). The ratio of actin/tropomyosin/troponin was 7:1.5:1.5. The rates were adjusted by subtracting the low ATPase activity of free S1 and of NEM-S1.

Equilibrium S1-ADP binding

Equilibrium binding was measured by the fractional increase in light scattering when titrating an actin solution with myosin S1. Measurements were made using an Aminco Bowman II luminescence spectrometer (Thermo Electron, Waltham, MA) with excitation wavelength at 340 and emission at 360 nm. Conditions for the binding experiments were: 25°C, pH 7.0, 0.2 mg/mL bovine serum albumin, 14 units/mL hexokinase, 1 mM glucose, 20 μM p^1,p^5-di(adenosine-5′)pentaphosphate (AP5A), 2 mM ADP, 20 mM MOPS, 5 mM $MgCl_2$, 88 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA or 0.5 mM $CaCl_2$. Actin was stored as a 30 μM stock in 4 mM imidazole (pH 7.0), 1 mM dithiothreitol, 2 mM $MgCl_2$, and 30 μM phalloidin. The phalloidin-actin concentration was 0.075 μM and the ratio of actin/tropomyosin/troponin was kept at 1:1:1 to ensure full binding of troponin and tropomyosin at the low actin concentration used. Details of the binding experiments are described elsewhere (39).

Calcium binding

Ca^{2+} binding was measured by changes in fluorescence emission intensity of 2-(4′-iodoacetamido-anilino)-naphthalene-6-sulfonic acid (IAANS) attached to Cys-35 of a single Cys mutant cardiac troponin C (C84S) as described previously (37). The probe was excited at 325 nm. The solution conditions were 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 20 mM MOPS at pH 7.0 and 25°C. The free Ca^{2+} concentration was calculated with the WEBMAXC Standard program (40). Measurements were carried out three to four times for each complex.

Binding during steady-state ATP hydrolysis

Binding was measured by cosedimentation with actin. S1 (0.1 μM) and variable amounts of actin were mixed in a solution containing 3 mM ATP, 10 mM MOPS, 5 mM $MgCl_2$, 14 mM NaCl, 1 mM dithiothreitol, and 0.5 mM $CaCl_2$ at pH 7. Mixtures were centrifuged for 30 min at 25°C at 184,000 $\times$ g. The ammonium-EDTA ATPase rates of the supernatants were measured to determine the fractions of unbound S1 (19). The fractions of bound S1 were determined from conservation of mass considerations. S1 was clarified by centrifugation immediately before use in binding assays.

Statistics

Data in figures are shown as means with error bars showing standard deviation. Statistical significance ($p < 0.05$) was determined using the two-sample significance t-test to compare means.

RESULTS

We examined ATPase rates in the presence and absence of calcium for wild-type (WT) troponin and for mutants that simulate phosphorylation at various sites. Fig. 3 shows that in the presence of Ca^{2+} all of the mutants had repressed ATPase rates relative to wild-type ($p < 0.05$). Ratios of the ATPase rates (wild-type/mutant) were: 3.3 for S45E, 3.4 for S43E/S45E, and 4.2 for S43E/S45E/S144E. The introduction of a single negative charge at position 45 was sufficient to reduce the Ca^{2+} activated ATPase rate. No significant differences were observed in the fully inhibited state where EGTA was added to sequester the free Ca^{2+}.

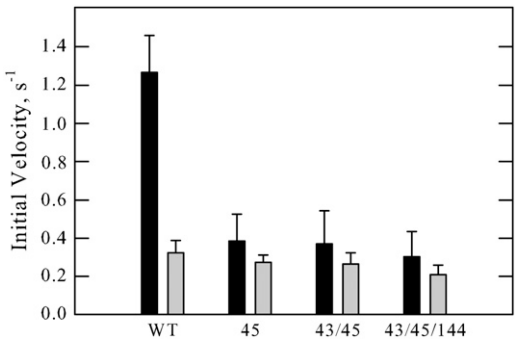

FIGURE 3 Inhibition of actin-activated ATPase activity by mutants in both the presence (*solid bars*) and absence (*shaded bars*) of Ca^{2+} at 0.05 M ionic strength. Initial velocities in units of micromolars ATP/s/μM S1 are shown for wild-type cardiac troponin and the following mutants: S45E, S43E/S45E, and S43E/S45E/T144E. All rates were corrected for the ATPase rate of S1 in the absence of actin. Data are presented as mean values ± SD ($n = 6$). Conditions are: 25°C, pH 7, 0.1 μM S1, 10 μM actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$, or 1 mM EGTA. Actin/tropomyosin/troponin is 7:1.5:1.5.

We did a series of studies to determine if the reduction in ATPase rates by the phosphorylation mimicking mutants resulted from stabilization of the inactive form of actin. One way of probing the distribution between inactive and active states is by an analysis of binding of S1-ADP to actin-tropomyosin-troponin. Fig. 4 shows a series of such binding curves measured as a function of increasing free S1-ADP concentrations. The binding profiles were more sigmoidal for the mutants. This more pronounced ''S'' shape with a greater lag at low concentrations of free S1-ADP is indicative of stabilization of the inactive state of actin. Although the effect was observed for the single mutation at position 45, the effect was greater with increasing numbers of introduced negative charges. This repression of binding suggests that the transition to the active state was made more difficult by the introduction of negative charge on the troponin I. Because the differences between the wild-type and mutants were small we confirmed the result with other approaches.

The relative stabilities of the inactive and active states at any condition can be measured by the degree of activation of ATPase activity relative to the fully inactive state (EGTA, no strongly bound S1) and to the fully active state (either Ca^{2+} or EGTA with optimal amounts of strongly bound S1). NEM-S1 binds to actin but has minimal ATPase activity and therefore serves as a tool to stabilize the active state of actin.

Fig. 5 shows the results of the NEM-S1 dependent acto-S1 ATPase activities in the presence of Ca^{2+}. Rates shown were corrected for the ATPase activities of both unbound S1 and NEM-S1. Without NEM-S1, the ATPase rates of the mutants were 24–30% of the wild-type values. However, at intermediate concentrations of NEM-S1 (i.e., 2 μM NEM-S1) the ATPase rates of the three mutants and the wild-type began to

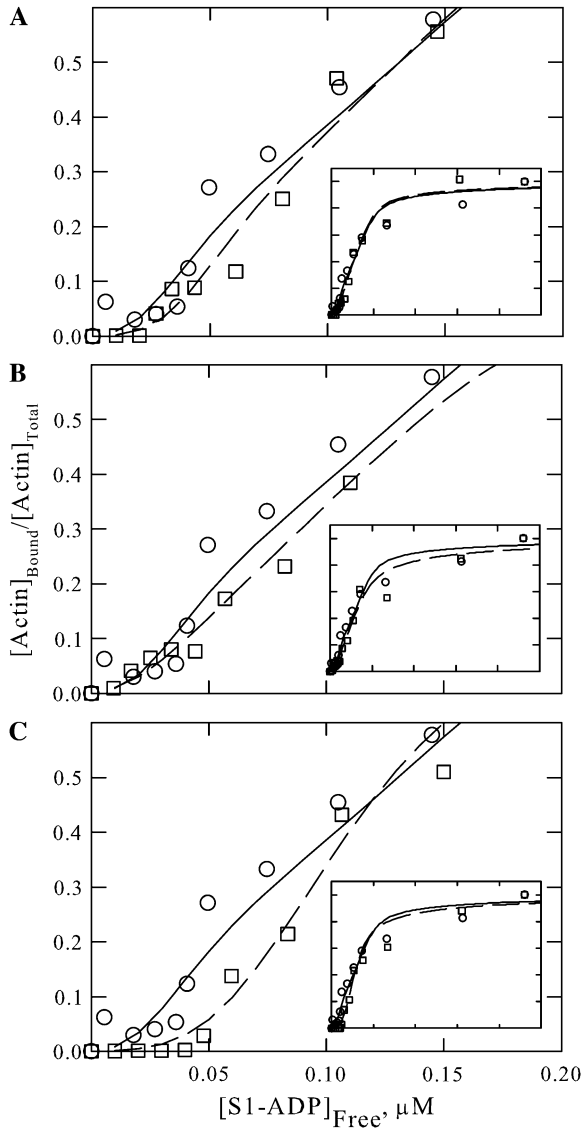

FIGURE 4 Representative curves showing binding of S1-ADP to actin-tropomyosin-troponin in the absence of Ca^{2+} at 120 mM ionic strength. Binding curves for each mutant were repeated three to four times. (*A*) The binding of WT (*circles*) and mutant 45 (*squares*) with increasing S1-ADP concentrations; (*B*) WT (*circles*) and mutant 43/45 (*squares*); (*C*) WT (*circles*) and mutant 43/45/144 (*squares*). Insets show saturation: *x* axis, $[S1\text{-}ADP]_{Free}$ (0–1 μM); *y* axis, $Actin_{Bound}/Actin_{Total}$ (0–1.1). Conditions are: 25°C, pH 7, 0.075 μM phalloidin-actin, 0.2 mg/mL BSA, 14 units/mL hexokinase, 1 mM glucose, 20 μM AP5A, 2 mM ADP, 20 mM MOPS, 5 mM $MgCl_2$, 88 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA. Actin/tropomyosin/troponin was 1:1:1. Light scattering was used to measure the binding with excitation at 340 nm and emission at 360 nm.

converge (Fig. 5, *A–C*). At saturation with NEM-S1, the rates were nearly identical indicating that all troponin types examined produced the same maximum rate. This convergence of rates is easily seen in Fig. 5 *D*, which shows the ratio of ATPase rates as a function of NEM S1 concentration for each of the mutants. The ratio of rates (mutant/wild-type) tended to converge to 1 with increasing concentrations of NEM-S1 for all of the mutants. At 2 μM NEM-S1, the effects of the single mutation at position 45 were reversed to a greater extent than the other mutants. However, at 7 μM NEM-S1, the effect of the triple mutant was most completely reversed. Thus the three mutations showed minor differences but overall behaved similarly.

As shown earlier in Fig. 3, no significant difference was seen between any mutant and wild-type in the absence of both Ca^{2+} and NEM-S1 ($p > 0.05$). One reason for a lack of inhibition in EGTA is that the ATPase activity was already near its minimum value in the case of wild-type troponin. To ascertain whether the depression of ATPase rates also occurred in the absence of Ca^{2+}, we examined the effect of partial activation with NEM-S1. Fig. 6 shows that whereas the ATPase rates were similar in the absence of NEM-S1, the rates were lower for the phosphorylation mimicking mutants in the presence of NEM-S1 ($p < 0.05$ for NEM-S1 $= 4$ μM). Thus, the introduction of negative charges at positions 43, 45, and 144 of cardiac troponin stabilized the inactive state of regulated actin in both the presence and absence of Ca^{2+}.

Ca^{2+} activates thin filaments because it binds more tightly to the active state of thin filaments and produces the conformational changes that were discussed earlier. Thus, one would expect that mutations of troponin I that stabilize the inactive state should weaken Ca^{2+} binding. We measured the Ca^{2+} affinity for the mutants S45E and S43E/S45E/T144E along with the wild-type ternary troponin complex using the fluorescence of an IAANS probe on troponin C (Fig. 7). The normalized data show reductions in calcium affinity for the troponin complexes with phosphorylation mimicking mutations as compared to wild-type ($p < 0.0001$). The association constants $\pm$SD for Ca^{2+} of the troponin complexes were: wild-type $K_a = 5.45 \pm 0.10 \times 10^6$ (M^{-1}), S45E $K_a = 2.12 \pm 0.04 \times 10^6$ (M^{-1}), S43E/S45E/T144E $K_a = 1.76 \pm 0.01 \times 10^6$ (M^{-1}). The S45E mutation was sufficient for producing most of the observed effect on Ca^{2+} affinity. Note that the affinity was not reduced to such an extent that full Ca^{2+} binding under the conditions of Figs. 3 or 5 would be prevented.

Reduced binding of S1 to regulated actin during steady-state ATP hydrolysis could also cause a decreased activation of ATPase activity by actin. Fig. 8 shows that the amount of S1 bound to actin during ATP hydrolysis was similar for wild-type and all mutants at both 20 and 30 μM actin. Differences in binding could not have explained the observed differences in activation of ATPase rates.

DISCUSSION

Mutants of troponin I that constitutively mimic PKC phosphorylation produced a modest reduction in the ability of actin-tropomyosin-troponin to activate the ATPase activity of myosin S1 at intermediate levels of activation with NEM-S1 (Figs. 5 and 6). In the case of activation by Ca^{2+}

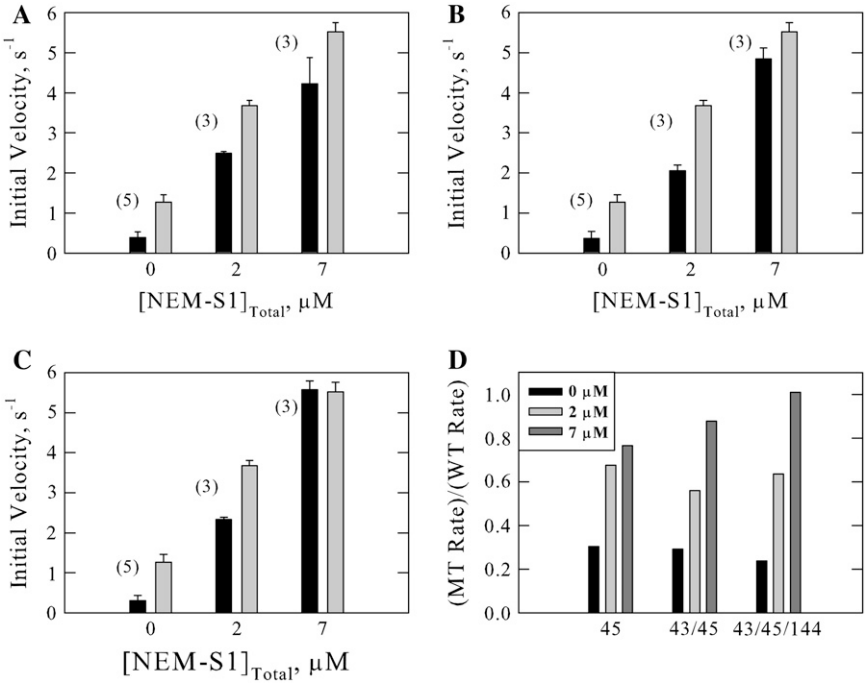

FIGURE 5 Actin-activated ATPase activity of S1 with increasing concentrations of activating NEM-S1 in the presence of Ca^{2+}. The transition of the regulated acto-S1 complex to maximal ATPase activity with NEM-S1 binding was blunted with the mutants. (*A*) The rates of mutant 45 (*solid bars*) and WT (*shaded bars*) with increasing NEM-S1 concentration; (*B*) mutant 43/45 (*solid bars*) and WT (*shaded bars*); (*C*) mutant 43/45/144 (*solid bars*) and WT (*shaded bars*). All rates were corrected for the ATPase rate of S1 and NEM-S1. (*D*) The ratio between the mean rates of the various mutants to the wild-type with (mutant 45)/(WT), (mutant 43/45)/(WT), and (mutant 43/45/144)/(WT) at different NEM-S1 concentrations (0, 2, and 7 μM). Data for panels *A*, *B*, and *C* are presented as mean values ± SD with the number of trials in parentheses. Conditions are: 25°C, pH 7, 0.1 μM S1, 10 μM actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$. Actin concentration was increased by an amount equal to the NEM-S1 added to maintain the same free actin concentration. Actin/tropomyosin/troponin was 7:1.5:1.5.

alone, ATPase rates were reduced to approximately one-third of wild-type values so that there was little activation by Ca^{2+} with the mutants. In the case of partial activation by NEM-S1 in the absence of Ca^{2+}, ATPase rates were reduced to ~50% of wild-type values. These results are consistent with stabilization of the inactive state of actin by the negatively charged troponin I mutants.

The same conclusion may be reached from earlier results with the same mutants and with troponin I that was phosphorylated by PKC (24). In that study the introduction of negative charges at sites 43, 45, and 144 resulted in a reduced Ca^{2+} sensitivity, reduced maximum force in Ca^{2+} and a reduced sliding speed in Ca^{2+} with the actin filament gliding assay. However the magnitude of the results seen here

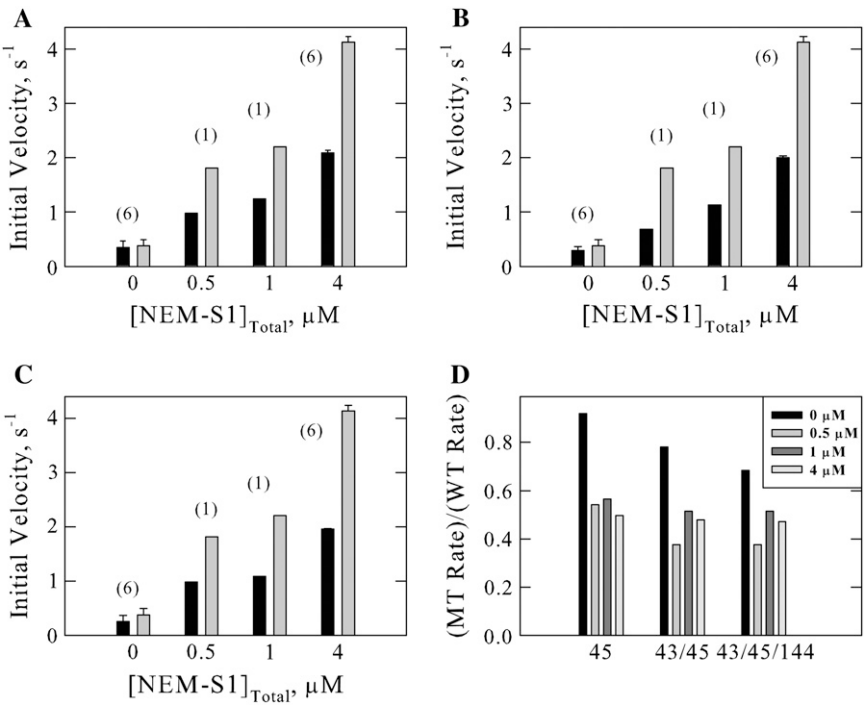

FIGURE 6 Actin-activated ATPase activity in the absence of Ca^{2+} with increasing concentrations of NEM-S1. (*A*) The rates of mutant 45 (*solid bars*) and WT (*shaded bars*); (*B*) mutant 43/45 (*solid bars*) and WT (*shaded bars*); and (*C*) mutant 43/45/144 (*solid bars*) and WT (*shaded bars*). (*D*) The ratio of mean ATPase rates with (mutant 45)/(WT), (mutant 43/45)/(WT), and (mutant 43/45/144)/(WT) at different NEM-S1 concentrations (0, 0.5, 1, 4 μM). All rates were corrected for the ATPase rate of S1 and NEM-S1. Data for panels *A*, *B*, and *C* are presented as mean values ± SD with the number of trials in parentheses. Conditions are: 25°C, pH 7, 0.1 or 0.2 μM S1, 10 μM actin, 1 mM ATP, 10 mM MOPS, 3 mM $MgCl_2$, 33 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA. Actin concentration was increased by an equal amount as the NEM-S1 added to maintain the same number of binding sites. Actin/tropomyosin/troponin was 7:1.5:1.5.

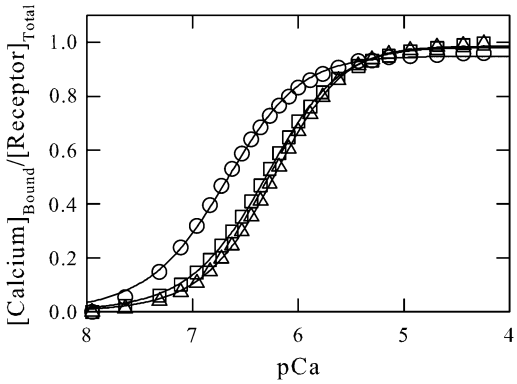

FIGURE 7 Binding of calcium to various troponin complexes including wild-type (*circles*), mutant 45 (*squares*), and mutant 43/45/144 (*triangles*). Binding was decreased for both mutants ($n = 3$–4). The conditions were 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 20 mM MOPS, pH 7.0, and the titration was carried out at 25°C.

for ATPase rates is somewhat different than observed earlier in other assays. ATPase rates in solution are never reduced to zero although there may be essentially no movement of muscle filaments. A greater activity in the absence of Ca^{2+} in ATPase assays than in gliding assays may be due to partial activation of the actin filaments in the ATPase assay. However, the concentration of unmodified S1 present in ATPase assays is too low to stabilize the active state of actin even if all of the S1 bound in a rigor-like state in the presence of ATP. Alternatively, the presence of a small load in the actin gliding assays might result in no movement despite low levels of ATPase activation.

Mutants that mimic phosphorylation of troponin I allow the effects of changes in individual amino acid residues to be examined. We observed that introduction of a negative charge into position S45 of cardiac troponin I near the troponin C

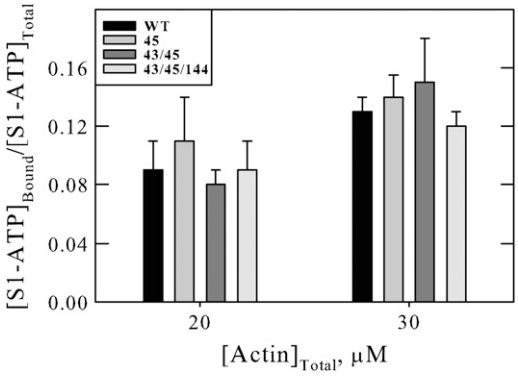

FIGURE 8 Effects of troponin I mutations on binding of S1 to actin in the presence of ATP. Mean values of the fraction of S1-ATP bound with different actin concentrations at 0.04 M ionic strength are shown ± SD ($n = 3$). Binding conditions are: 25°C, pH 7, 0.1 μM S1, 3 mM ATP, 10 mM MOPS, 5 mM $MgCl_2$, 14 mM NaCl, 1 mM dithiothreitol, 0.5 mM $CaCl_2$. There was no significant difference in binding between the wild-type and mutants at the actin concentrations measured.

binding site was sufficient for producing the changes reported here. Introduction of additional negative charges at positions 43 and 144 produced little or no additional inhibition of ATPase activity.

Our ATPase rates were measured at 50 mM ionic strength to obtain reasonable actin activation. The electrostatic shielding in solution is half as great at 50 mM ionic strength as it is at physiological conditions (~180 mM) and one may expect the effects of a single negative charge to less important at physiological conditions. We do not think that this is the case for several reasons. At 50 mM ionic strength a single charge at S45 produced a maximum effect on ATPase activity. At 120 mM ionic strength a single charge at S45 produced a near maximum effect on S1-ADP binding. That is, there may have been a slight attenuation of the charge effect at the higher ionic strength. Should this small attenuation be real, it may be compensated for by the higher charge of a naturally occurring phosphate group compared to an experimental introduced carboxyl group.

The work shown here was designed to determine the reason for the reduced activity of actin filaments containing phosphorylated troponin. A simple scheme of the regulation of ATP hydrolysis is shown in Fig. 1. Myosin S1 hydrolyzes ATP slowly if it is detached from actin. Maximum ATPase activity is obtained when all of the S1 is bound to actin that is in the active state (A_a). Little or no activation of ATPase activity occurs when S1 is bound to actin in the inactive state (A_i). Thus, changes in the regulatory complex may alter the ATPase activity by: 1), inhibiting the binding of S1 to actin during ATP hydrolysis, 2), reducing the rate of a transition along the pathway where S1 is bound to activated actin, or 3), shifting the equilibrium of the actin states toward the inactive state. Several of our observations indicate that the reduced ability of the troponin I mutants to stimulate ATPase activity was the result of stabilization of the inactive state of regulated actin.

First, we eliminated two possible effects of the mutations. We found no differences between the troponin I mutants and wild-type in the amount of S1 bound to actin during steady-state ATP hydrolysis as shown in Fig. 8. Note that because binding of S1-ATP is largely independent of the state of actin-tropomyosin-troponin (19,41) this observation tells us nothing about the distribution among actin states. We also ruled out large changes in other steps of the pathway of the fully activated step. That is, when NEM-S1 was used to stabilize the fully activated state of regulated actin the rates of ATPase activity were similar for all cases examined. These observations indicate that the inhibition of actin activated ATPase activity could be attributed to stabilization of the inactive state, A_i.

Several observations pointed directly to stabilization of the inactive state of actin. The transition from the inactive state to the active state of regulated actin required higher concentrations of free S1-ADP for the mutants. This indicates that the equilibrium between the inactive and active states (A_i

to A_a in Fig. 1) is shifted more toward the inactive states in the case of the mutants.

We confirmed stabilization of the inactive state by using ATPase assays in the presence of NEM-S1 to determine the fraction of maximum activation under each condition. In the absence of bound Ca^{2+} and activating myosin essentially all of actin is in the inactive state, A_i. At saturating concentrations of NEM-S1, the actin filaments have their greatest activity and essentially all of the actin is in the active state A_a. We observed that saturating Ca^{2+} produced a lower fraction of actin in the active state in the case of the phosphorylation mimicking mutants of troponin I than for wild-type troponin. Low levels of NEM-S1 in the absence of Ca^{2+} also failed to be as effective in stabilizing the active state of the mutants. These results confirm that the inactive state was stabilized relative to the active state.

Finally, the lower affinity of Ca^{2+} for the ternary complex is consistent with stabilization of the inactive state. That is, Ca^{2+} binds more tightly to troponin that is the active state than to troponin in the inactive state. Stabilization of the inactive state would be reflected as a lower Ca^{2+} affinity.

These results suggest that alterations of troponin-tropomyosin may change the ATPase activity and Ca^{2+} sensitivity of the actin filament by a relatively simple mechanism. Activation of ATPase rates by some mutations in troponin T have been shown to result from stabilization of the active state of actin (22). We show here that mutations of troponin I that inhibit ATPase activity can act by stabilization of the inactive state. That is, it is possible to have diverse effects on the activity by altering a single transition between the inactive and active states of regulated actin. That mutations of components of a cooperative system should have a primary effect on the transition between the structural states is easy to visualize. The prevailing view of regulation is that troponin serves to stabilize tropomyosin into different states on actin that determine the ability of actin to accelerate ATP hydrolysis (14,15,42). Small changes in troponin or tropomyosin might readily alter the equilibrium between states of actin to stabilize to a greater extent either the less active or more active configuration.

PKC phosphorylation in the heart is a normal adaptive response that is altered in several disease states. PKC activity is increased in ischemic hearts when the calcium levels are raised. This activity has been shown to have a protective effect during myocardial infarction, although the mechanism remains unclear (43). Excessive phosphorylation has also been seen in end stages of hypertrophic cardiomyopathy although it remains unclear whether this is a primary mechanism contributing to the hypertrophy or an adaptive response (44). An adaptive response to the mutations that cause hypertrophy is easily reconciled by the observations that troponin I phosphorylation stabilizes the inactive state of actin, whereas some examined troponin T mutants that produce cardiomyopathy stabilize the active state. Furthermore, as troponin I phosphorylation and mutations of some troponin T residues affect the same transition in regulation this would appear to be an ideal adaptive response. However, activation of PKC in cardiac muscle is complex because of the different signaling pathways available, the presence of several isoforms of PKC and the compartmentation of PKC within cardiac cells (44–47).

This work shows that phosphorylation of troponin I at residues 43, 45, and 144 stabilize the inactive state of regulation but do not prevent full activation by strongly attached cross-bridges. Other changes in troponin, such as with some hypertrophic-cardiomyopathy-causing mutants, stabilize the active state of regulated actin (22,48). Thus, changes in the distribution among actin states may be expected to occur with other changes in the troponin-tropomyosin complex.

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REFERENCES

1. Greaser, M. L., and J. Gergely. 1971. Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* 246:4226–4233.
2. Herzberg, O., and M. N. James. 1985. Structure of the calcium regulatory muscle protein troponin-C at 2.8 A resolution. *Nature.* 313:653–659.
3. Slupsky, C. M., and B. D. Sykes. 1995. NMR solution structure of calcium-saturated skeletal muscle troponin C. *Biochemistry.* 34:15953–15964.
4. Filatov, V. L., A. G. Katrukha, T. V. Bulargina, and N. B. Gusev. 1999. Troponin: structure, properties, and mechanism of functioning. *Biochemistry* (*Mosc.*). 64:969–985.
5. Vinogradova, M. V., D. B. Stone, G. G. Malanina, C. Karatzaferi, R. Cooke, R. A. Mendelson, and R. J. Fletterick. 2005. Ca^{2+}-regulated structural changes in troponin. *Proc. Natl. Acad. Sci. USA.* 102:5038–5043.
6. Williams, D. L., Jr., L. E. Greene, and E. Eisenberg. 1988. Cooperative turning on of myosin subfragment 1 adenosinetriphosphatase activity by the troponin-tropomyosin-actin complex. *Biochemistry.* 27:6987–6993.
7. Nagashima, H., and S. Asakura. 1982. Studies on co-operative properties of tropomyosin-actin and tropomyosin-troponin-actin complexes by the use of N-ethylmaleimide-treated and untreated species of myosin subfragment 1. *J. Mol. Biol.* 155:409–428.
8. Heeley, D. H., B. Belknap, and H. D. White. 2006. Maximal activation of skeletal muscle thin filaments requires both rigor myosin S1 and calcium. *J. Biol. Chem.* 281:668–676.
9. Kobayashi, T., and R. J. Solaro. 2005. Calcium, thin filaments, and the integrative biology of cardiac contractility. *Annu. Rev. Physiol.* 67:39–67.
10. Squire, J. M., and E. P. Morris. 1998. A new look at thin filament regulation in vertebrate skeletal muscle. *FASEB J.* 12:761–771.
11. Gordon, A. M., E. Homsher, and M. Regnier. 2000. Regulation of contraction in striated muscle. *Physiol. Rev.* 80:853–924.
12. Perry, S. V. 2003. What is the role of tropomyosin in the regulation of muscle contraction? *J. Muscle Res. Cell Motil.* 24:593–596.
13. Chalovich, J. M. 1992. Actin mediated regulation of muscle contraction. *Pharmacol. Ther.* 55:95–148.
14. Tobacman, L. S., and C. A. Butters. 2000. A new model of cooperative myosin-thin filament binding. *J. Biol. Chem.* 275:27587–27593.
15. McKillop, D. F., and M. A. Geeves. 1993. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys. J.* 65:693–701.

16. Smith, D. A., and M. A. Geeves. 2003. Cooperative regulation of myosin-actin interactions by a continuous flexible chain II: actin-tropomyosin-troponin and regulation by calcium. *Biophys. J.* 84:3168–3180.

17. Hill, T. L., E. Eisenberg, and L. Greene. 1980. Theoretical model for the cooperative equilibrium binding of myosin subfragment 1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. USA.* 77:3186–3190.

18. Hill, T. L., E. Eisenberg, and J. M. Chalovich. 1981. Theoretical models for cooperative steady-state ATPase activity of myosin subfragment-1 on regulated actin. *Biophys. J.* 35:99–112.

19. Chalovich, J. M., and E. Eisenberg. 1982. Inhibition of actomyosin ATPase activity by troponin-tropomyosin without blocking the binding of myosin to actin. *J. Biol. Chem.* 257:2432–2437.

20. Rosenfeld, S. S., and E. W. Taylor. 1987. The mechanism of regulation of actomyosin subfragment 1 ATPase. *J. Biol. Chem.* 262:9984–9993.

21. Heeley, D. H., B. Belknap, and H. D. White. 2002. Mechanism of regulation of phosphate dissociation from actomyosin-ADP-pi by thin filament proteins. *Proc. Natl. Acad. Sci. USA.* 99:16731–16736.

22. Gafurov, B., S. Fredricksen, A. Cai, B. Brenner, P. B. Chase, and J. M. Chalovich. 2004. The delta 14 mutation of human cardiac troponin T enhances ATPase activity and alters the cooperative binding of S1-ADP to regulated actin. *Biochemistry.* 43:15276–15285.

23. Gomes, A. V., J. Liang, and J. D. Potter. 2005. Mutations in human cardiac troponin I that are associated with restrictive cardiomyopathy affect basal ATPase activity and the calcium sensitivity of force development. *J. Biol. Chem.* 280:30909–30915.

24. Burkart, E. M., M. P. Sumandea, T. Kobayashi, M. Nili, A. F. Martin, E. Homsher, and R. J. Solaro. 2003. Phosphorylation or glutamic acid substitution at protein kinase C sites on cardiac troponin I differentially depress myofilament tension and shortening velocity. *J. Biol. Chem.* 278:11265–11272.

25. Takeda, S., A. Yamashita, K. Maeda, and Y. Maeda. 2003. Structure of the core domain of human cardiac troponin in the Ca^{2+}-saturated form. *Nature.* 424:35–41.

26. Westfall, M. V., A. M. Lee, and D. A. Robinson. 2005. Differential contribution of troponin I phosphorylation sites to the endothelin-modulated contractile response. *J. Biol. Chem.* 280:41324–41331.

27. Wang, H., J. E. Grant, C. M. Doede, S. Sadayappan, J. Robbins, and J. W. Walker. 2006. PKC-betaII sensitizes cardiac myofilaments to Ca^{2+} by phosphorylating troponin I on threonine-144. *J. Mol. Cell. Cardiol.* 41:823–833.

28. Montgomery, D. E., B. M. Wolska, W. G. Pyle, B. B. Roman, J. C. Dowell, P. M. Buttrick, A. P. Koretsky, P. Del Nido, and R. J. Solaro. 2002. Alpha-adrenergic response and myofilament activity in mouse hearts lacking PKC phosphorylation sites on cardiac troponin I. *Am. J. Physiol. Heart Circ. Physiol.* 282:H2397–H2405.

29. Pyle, W. G., M. P. Sumandea, R. J. Solaro, and P. P. De Tombe. 2002. Troponin I serines 43/45 and regulation of cardiac myofilament function. *Am. J. Physiol. Heart Circ. Physiol.* 283:H1215–H1224.

30. Finley, N. L., and P. R. Rosevear. 2004. Introduction of negative charge mimicking protein kinase C phosphorylation of cardiac troponin I. effects on cardiac troponin C. *J. Biol. Chem.* 279:54833–54840.

31. Ruse, C. I., B. Willard, J. P. Jin, T. Haas, M. Kinter, and M. Bond. 2002. Quantitative dynamics of site-specific protein phosphorylation determined using liquid chromatography electrospray ionization mass spectrometry. *Anal. Chem.* 74:1658–1664.

32. Jideama, N. M., T. A. Noland, Jr., R. L. Raynor, G. C. Blobe, D. Fabbro, M. G. Kazanietz, P. M. Blumberg, Y. A. Hannun, and J. F. Kuo. 1996. Phosphorylation specificities of protein kinase C isozymes for bovine cardiac troponin I and troponin T and sites within these proteins and regulation of myofilament properties. *J. Biol. Chem.* 271: 23277–23283.

33. Noland, T. A., Jr., X. Guo, R. L. Raynor, N. M. Jideama, V. Averyhart-Fullard, R. J. Solaro, and J. F. Kuo. 1995. Cardiac troponin I mutants. phosphorylation by protein kinases C and A and regulation of Ca^{2+}-stimulated MgATPase of reconstituted actomyosin S-1. *J. Biol. Chem.* 270:25445–25454.

34. Kobayashi, T., X. Yang, L. A. Walker, R. B. Van Breemen, and R. J. Solaro. 2005. A non-equilibrium isoelectric focusing method to determine states of phosphorylation of cardiac troponin I: identification of Ser-23 and Ser-24 as significant sites of phosphorylation by protein kinase C. *J. Mol. Cell. Cardiol.* 38:213–218.

35. Kielley, W. W., and W. F. Harrington. 1960. A model for the myosin molecule. *Biochim. Biophys. Acta.* 41:401–421.

36. Weeds, A. G., and R. S. Taylor. 1975. Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin. *Nature.* 257:54–56.

37. Kobayashi, T., and R. J. Solaro. 2006. Increased Ca^{2+} affinity of cardiac thin filaments reconstituted with cardiomyopathy-related mutant cardiac troponin I. *J. Biol. Chem.* 281:13471–13477.

38. Schnekenbuhl, S. 1994. Kalzium-unabhangige aktivierung membran-freier muskelpraparate. ist die muskelkontraktion uber zwei verschiedene mechanismen reguliert? [in German]. PhD thesis. Universitat Ulm, Ulm, Germany.

39. Gafurov, B., Y. D. Chen, and J. M. Chalovich. 2004. Ca^{2+} and ionic strength dependencies of S1-ADP binding to actin-tropomyosin-troponin: regulatory implications. *Biophys. J.* 87:1825–1835.

40. Patton, C., S. Thompson, and D. Epel. 2004. Some precautions in using chelators to buffer metals in biological solutions. *Cell Calcium.* 35:427–431.

41. Chalovich, J. M., P. B. Chock, and E. Eisenberg. 1981. Mechanism of action of troponin. tropomyosin. inhibition of actomyosin ATPase activity without inhibition of myosin binding to actin. *J. Biol. Chem.* 256:575–578.

42. Chalovich, J. M. 2002. Regulation of striated muscle contraction: a discussion. *J. Muscle Res. Cell Motil.* 23:353–361.

43. Node, K., M. Kitakaze, H. Sato, T. Minamino, K. Komamura, Y. Shinozaki, H. Mori, and M. Hori. 1997. Role of intracellular Ca^{2+} in activation of protein kinase C during ischemic preconditioning. *Circulation.* 96:1257–1265.

44. Dorn, G. W., 2nd, and T. Force. 2005. Protein kinase cascades in the regulation of cardiac hypertrophy. *J. Clin. Invest.* 115:527–537.

45. Sabri, A., and S. F. Steinberg. 2003. Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure. *Mol. Cell. Biochem.* 251:97–101.

46. Shirai, Y., and N. Saito. 2002. Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. *J. Biochem.* (*Tokyo*). 132:663–668.

47. Mochly-Rosen, D., and A. S. Gordon. 1998. Anchoring proteins for protein kinase C: a means for isozyme selectivity. *FASEB J.* 12:35–42.

48. Burhop, J., M. Rosol, R. Craig, L. S. Tobacman, and W. Lehman. 2001. Effects of a cardiomyopathy-causing troponin T mutation on thin filament function and structure. *J. Biol. Chem.* 276:20788–20794.