Basic residues within the cardiac troponin T C-terminus are required for full inhibition of muscle contraction and limit activation by calcium

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ABSTRACT

Striated muscle is activated by myosin- and actin-linked processes, with the latter being regulated through changes in the position of tropomyosin relative to the actin surface. The C-terminal region of cardiac troponin T (TnT), a tropomyosin-associated protein, is required for full TnT inactivation at low Ca²⁺ and for limiting its activation at saturating Ca²⁺. Here, we investigated whether basic residues in this TnT region are involved in these activities, whether the TnT C terminus undergoes Ca²⁺-dependent conformational changes, and whether these residues affect cardiac muscle contraction. We generated a human cardiac TnT variant in which we replaced seven C-terminal Lys and Arg residues with Ala and added a Cys residue at either position 289 or 275 to affix a fluorescent probe. At pCa 3.7, actin filaments containing high-alanine TnT had an elevated ATPase rate like that obtained when the last TnT 14 residues were deleted. Acrylodan-tropomyosin fluorescence changes and S1–actin binding kinetics revealed that at pCa 8, the high-alanine TnT-containing filaments did not enter the first inactive state. Förster Resonance Energy Transfer analyses indicated that the C-terminal TnT region approached Cys-190 of tropomyosin as actin filaments transitioned to the inactive B state; that transition was abolished with high-alanine TnT. High-alanine TnT–containing cardiac muscle preparations had increased Ca²⁺ sensitivity of both steady-state isometric force and sinusoidal stiffness as well as increased maximum steady-state isometric force and sinusoidal stiffness. We conclude that C-terminal basic residues in cardiac TnT are critical for the regulation of cardiac muscle contraction.

Activation of striated muscle occurs both through myosin-linked (1,2) and actin-linked processes (3-5). The actin-linked portion of regulation works primarily through changes in
the position of tropomyosin relative to the surface of actin (6-8). Tropomyosin is in an inactivating state when Ca\textsuperscript{2+} levels are low (9,10). Saturation of the regulatory binding site(s) of TnC (troponin C) with Ca\textsuperscript{2+} opens a hydrophobic cleft to which the switch region of TnI (troponin I) can bind (11) permitting detachment of the inhibitory region of TnI from actin. Tighter binding of the switch region to TnC has been associated with Ca\textsuperscript{2+} sensitization (12) and with movement of tropomyosin into a mixture of the C or Ca\textsuperscript{2+} state and M or myosin stabilized states. Fig. 1 defines the various states. The C state, like the B state appears to be inactive (13). Full activation occurs when in addition to Ca\textsuperscript{2+} a fraction of actin protomers has tightly bound myosin that is either nucleotide free or contains only bound ADP (14,15). Stimulation of S1 ATPase activity by Ca\textsuperscript{2+} and “activating” forms of myosin S1 (rigor and S1-ADP) are adequately described by changes in the population of the states of regulated actin (15).

The degree to which Ca\textsuperscript{2+} stimulates the ATPase rate depends on the equilibrium between the C and M states and that depends on the composition of the actin filament. For example, substitution of acanthamoeba actin into skeletal thin filaments enhances the degree of activation by Ca\textsuperscript{2+} (16,17). An enhanced activation by Ca\textsuperscript{2+} occurs in some disease-causing mutations of TnT, most notably with deletion of the last 14 C-terminal residues of TnT (\(\Delta^{14}\) TnT) (18,19). When both \(\Delta^{14}\) TnT and A8V TnC were present in the same thin filaments, there was full activation with Ca\textsuperscript{2+} alone (no myosin-ADP binding required) (19). Other changes in the thin filament, such as protein kinase C phosphomimetic mutants of TnI (20), decrease the response to Ca\textsuperscript{2+}. Stabilization of the intermediate C state of actin filaments can also occur with another mutation that causes familial hypertrophic cardiomyopathy (13). The C-terminus of TnT is particularly interesting because deletion of the last 14 residues has dual effects of eliminating the inactive B state at very low Ca\textsuperscript{2+} and enhancing the active M state at saturating Ca\textsuperscript{2+} (19,21).

The preponderance of positively charged residues in the C-terminus of TnT raises the possibility that electrostatic interactions are responsible for the functions of this region. We produced a construct of human cardiac TnT that maintained the length of wild type TnT but had the terminal 7 basic amino acids replaced with Ala. We call this construct HAHA TnT (High Ala, High Activation). Both the HAHA TnT and wild type contained either an added Cys at position 289 as a terminal residue or a Ser to Cys mutation at position 275 for placement of a fluorescent probe.

By measuring rates of ATP hydrolysis, changes in fluorescence of a probe on tropomyosin, kinetics of S1 binding to actin filaments and Förster Resonance Energy Transfer studies, we show that the C-terminal basic residues of TnT are critical for proper regulation. Actin filaments containing HAHA TnT were unable to enter the inactive B state at low Ca\textsuperscript{2+} conditions. At saturating Ca\textsuperscript{2+} levels, those same filaments moved more fully to the M state and had enhanced activity. The effects of HAHA TnT on muscle fiber mechanics were consistent with the solution results. Furthermore, the C-terminal region of TnT underwent a Ca\textsuperscript{2+} dependent conformational change that was eliminated in the case of HAHA TnT. These results suggest that the C-terminal region of TnT, or its binding partners, are potential targets for manipulating cardiac contractility and improving the function of diseased hearts.

**RESULTS**

Saturating Ca\textsuperscript{2+} normally gives about 30% of the maximum possible ATPase activity (19) but deletion of the last 14 C-terminal residues of human cardiac TnT gives a further doubling of activity (i.e. doubling of the population of actin in the active M state). Because the C-terminal region of TnT is rich in basic amino acids, replacing the basic residues with uncharged Ala residues was expected to increase the fraction of actin filaments in the active state at saturating Ca\textsuperscript{2+}.

Fig. 2 shows the effect of different types of TnT on the actin activated ATPase rate of S1 at saturating Ca\textsuperscript{2+} and sub-saturating actin concentrations. In the absence of tropomyosin or troponin the rate was 1.2/s; this is typical for these
Basic residues within the cardiac troponin T C-terminus

conditions (19). Human cardiac troponin normally increases the activity of actin-tropomyosin and it increased the rate here to 2.2/s. When wild type troponin was replaced with TnT having either S275C or 289C mutations the rates remained similar to wild type (2.0 and 2.1/s for S275C and S289C, respectively). In contrast, 289C-HAHA TnT increased the rate to 3.8/s. That is similar to the increase from 1.9/s to 4/s noted earlier when TnT missing the last 14 residues was analyzed (19). The fraction of actin in the active state increased from 34% in wild type to 69% in 289C-HAHA TnT based on the results of Fig. 2.

We showed earlier that stepwise truncation of the 14 C-terminal residues of TnT progressively decreases the population of the inactive B state of regulated actin (21). We have now measured changes in the distribution of the inactive B that occur with the 289C-HAHA mutant of TnT. The first method used is based on the change in the rate of binding of ATP-free S1 to actin-tropomyosin-troponin at low free Ca²⁺ relative to the rate at high Ca²⁺ (10,22). In studies reported here, low Ca²⁺ is 10⁻⁸ M and high Ca²⁺ is generally 0.2 mM free. The rate of binding at high Ca²⁺, where the C and M states are populated, is normally about 3 times that rate in the virtual absence of Ca²⁺ where the B state predominates.

Fig. 3 shows binding isotherms for rigor S1 binding to actin-tropomyosin-troponin at a very low free Ca²⁺ concentration. Curve 1 is for actin filaments containing wild type human cardiac TnT. S1 binding to pyrene labeled actin occurred with a mono-exponential decrease in fluorescence with an apparent rate constant of 1.0/s. The apparent rate constant of binding for filaments containing C289-TnT was similar at 1.2/s (curve 2). Curve 3 was obtained using actin filaments containing 289C-HAHA TnT. The apparent rate constant of binding was 4.1/s. That value is similar to the wild type rate at saturating Ca²⁺ where there is no B state.

Similar measurements were made at a saturating Ca²⁺ concentration and are shown in Fig. 4. Mono-exponential traces were obtained for wild type (curve 1), 289C-TnT (curve 2), and 289C-HAHA TnT. The apparent rate constants for wild type and 289C-TnT were similar (3.7 and 3.9/s, respectively). These rate constants were faster than those measured in the virtual absence of Ca²⁺ and are generally regarded to represent the maximum possible rate (10). However, the apparent rate constant of binding of S1 to actin filaments containing 289C-HAHA TnT was approximately double that rate (6.8/s). A similar rate of binding was observed with Δ14 TnT (21). This suggests that the rate of rigor S1 binding to the M state is faster than to the C state.

We also measured changes in the occupancy of the inactive B state by monitoring changes in the fluorescence of acrylodan-labeled tropomyosin (23). The active M and inactive B states have high fluorescence while the inactive C state has low fluorescence. Actin-acrylodan tropomyosin-troponin is maintained in the M state by rigor S1 binding even at very low free Ca²⁺ giving rise to a high fluorescence state. Upon mixing rapidly with ATP, the S1 dissociates and the regulated actin transitions rapidly to the C state and then more slowly to the B state. Because the transition to the C state is very rapid and virtually complete, the magnitude of the final fluorescence rise is proportional to the occupancy of the B state.

Fig. 5 shows acrylodan fluorescence increases for wild type (curve 1), 289C-TnT (curve 2) and 289C-HAHA TnT (curve 3). Part of the initial fluorescence decrease from state M to state C can be seen in the curves. The transition of wild type actin filaments to the blocked state had an amplitude of 0.28 relative fluorescence units with an apparent rate constant of 32/s. Similarly, 289C-TnT had an amplitude of 0.28 relative fluorescence units and an apparent rate constant of 29/s. However, eliminating the basic residues in the C-terminal region of TnT produced a large change. No increase in fluorescence was observed in the case of 289C-HAHA TnT (curve 3). That curve is similar to that observed previously for ∆14 TnT (23,24) indicating absence of the B state.

Because the C-terminal region of TnT appears to be involved in regulation we expected to see changes in the location or orientation of that region with changes in the state of activation. A donor molecule, IAEDANS, was placed on a thiol group engineered into the C-terminal region of TnT. Förster resonance energy transfer was measured between an IAEDANS donor and a DABMI probe on Cys 190 of tropomyosin during
the transition from the active M state to the inactive C and B states using the ATP chase protocol used in Fig. 5. The time courses of FRET were monitored in the stopped flow apparatus and are shown in Figs. 6, 8 and 9.

Curve 1 in Fig 6A shows 289C-TnT IAEDANS (wild type with an added Cys for labeling) fluorescence in the absence of an acceptor as the filaments transitioned from the M state to the C state and finally to B state at 10° C. Curve 2 shows the same transition in the presence of the acceptor DABMI on tropomyosin. Here a single exponential decay was observed with an apparent rate constant of 6.2 ± 0.5 /s. This rate is similar to that measured in Fig. 5 for the transition from the C to the B state. The same measurement was made at 18° (Panel B) and 25° C (Panel C). Panels D-F show the calculated changes in FRET efficiency with time for the three temperatures. The increase in FRET efficiency indicates that in going from the C to the B state, the distance from the C-terminus of TnT to Cys 190 of tropomyosin decreased. Table 1 shows the calculated distance changes in going from the C state to a mixture of the C and B states at a very low concentration of Ca²⁺.

Table 2 gives values of the anisotropy of several of the probes at different free Ca²⁺ concentrations. The anisotropies of the donor molecules changed only slightly with changes in free Ca²⁺. The values were near 0.1 which is between the limits for free rotation (-0.2) and for immobile probes (0.4) (25). Because the acceptor probe, DABMI, is non fluorescent, we measured the anisotropy of a fluorescein probe on Cys 190 of tropomyosin which was also near 0.1.

We know from earlier work that the transition between the inactive C and B states, is temperature dependent (23). The apparent rate constants for the distance change attributed to the transition from the C to the B state are shown in the form of an Arrhenius plot in Fig. 7 along with those earlier acrylodan-tropomyosin data. The similarity of the temperature dependences is evidence that the energy transfer observed corresponds to the C to B transition.

Fig. 8 shows that no energy transfer occurred in the presence of IAEDANS-labeled 289C-HAHA TnT where the C-terminal basic residues have been substituted with Ala. The transfer efficiency, and thus the distance between the probes, remained constant at a value similar to that of the wild type in the C state. In the case of 289C-HAHA TnT there is no transition to the B state. Furthermore, the movement shown in Fig. 6 appears to require the presence of the basic residues in the C-terminal region of TnT.

In order to further define the movement of the C-terminal region of TnT, we measured the change in FRET efficiency with IAEDANS placed on an engineered Cys at position 275 of human cardiac TnT. The IAEDANS probe on Cys 275 of TnT produced an increase in FRET efficiency in going to the B state (Fig. 9 A & B). This change differed in two respects from that observed with Cys 289 of TnT. First, the difference in efficiency between the C and B states was smaller. Second, the initial and final values of the efficiency were larger than with 289C-TnT indicating that residue 275 is closer to Cys 190 of tropomyosin than is residue 289 of TnT. The transition was slower (3.5±0.2/s) possibility due to the probe at position 275.

Panels C and D of Fig. 9 show energy transfer between IAEDANS at position 143 of TnI and Cys 190 of tropomyosin. This was measured because it is well known that TnI moves toward the actin-tropomyosin filament in the relaxed B state. Panel C shows the traces in the absence and presence of the donor on tropomyosin while panel D shows the time course of the efficiency change. These data shown movement of the inhibitory region of TnI toward Cys 190 of tropomyosin as actin filaments progress from the C to the B state. Table 1 summarizes the results of Figs. 6, 8 & 9.

Our earlier work showed that deletion of the last 14 residues of human cardiac TnT increased the Ca²⁺ sensitivity of muscle fibers (18). We exchanged the native troponin in permeabilized cardiac muscle preparations (CMPs) with recombinant human cardiac wild type TnT or with human cardiac 289C-HAHA TnT. To determine the efficiency of the exogenous TnT to displace the native troponin complex, we measured the unregulated tension at low Ca²⁺ concentration (pCa 8) after TnT incubation as described in the methods section (26,27). We did not observe a significant change in the ability of the 289C-HAHA TnT to displace the native troponin complex compared to its respective control (86.96 ± 1.13% vs 81.80 ±
Basic residues within the cardiac troponin T C-terminus

3.79%, Table 3). This finding indicates that the 289C-HAHA TnT does not display an altered affinity for the thin filament since its incorporation to the CMPs were comparable to the wild type TnT.

Fig. 10A is a plot of relative force against pCa for CMPs containing wild type or 289C-HAHA TnT. Replacement of basic residues with neutral Ala in the C-terminal region of TnT produced myofilament Ca$^{2+}$ sensitization measured by steady-state isometric force with a shift of 0.24 pCa units in the midpoint of activation from $F_{pCa_{50}}$ 5.38 ± 0.03 to 5.62 ± 0.02 (Table 3). No changes in cooperativity of thin filament activation ($n_{Hill}$) were observed in CMPs containing 289C-HAHA vs wild type TnT (Fig 10 and Table 3). The other notable feature of these curves is that the maximum force recovery was increased 24.6% in CMPs containing 289C-HAHA TnT (Fig. 10B and Table 3). Table 3 reports the absolute steady-state isometric force values before (P) and after (P$_0$) recombinant troponin incorporation.

**DISCUSSION**

We describe here the production of a mutant of human cardiac TnT that eliminated the B state, the major state that actin-tropomyosin-troponin occupies in the virtual absence of Ca$^{2+}$. That mutant also caused an increase in activation by Ca$^{2+}$ in both solution and in permeabilized cardiac muscle preparations and an enhanced response to Ca$^{2+}$. Fluorescent probes on Cys residues placed within the C-terminal region of TnT reported conformational changes occurring in response to changes in the state of activation. The HAHA mutation of TnT mimicked all of the effects of deleting the C-terminal region of TnT (19,21,24). This study demonstrates the importance of positive charges in the C-terminal region of TnT for normal regulation of contraction and for a conformational change in the C-terminal region of TnT that occurs in moving between the two inactive states (state C to B). Cys labeled HAHA TnT is a useful tool for studying contractile regulation.

It is well known that rigor myosin binding to actin can stabilize the active M state and increase ATPase activity at both low and high Ca$^{2+}$. It now appears that troponin can give the same kind of activation of ATPase activity as occurs with rigor myosin binding. Understanding how the C-terminal region of TnT modulates the degree of activation by Ca$^{2+}$ could have important health implications as this is a previously unrecognized target.

HAHA TnT has Ala in place of the Lys and Arg residues within the last 16 residues of TnT. We used a 16 rather than a 14 residue stretch in an attempt to increase the Ca$^{2+}$ activation beyond that seen with Δ14 TnT. We were limited to the last 16 residues by the presence of the I-T helix. Extending the modification beyond 14 residues did not result in complete activation.

Elimination of the inactive B state by the HAHA mutation was demonstrated here by the disappearance of the acrylodan tropomyosin signal. We showed earlier that an increase in acrylodan fluorescence occurs as the B state becomes populated (19,23,24). The absence of a signal indicates that the B state was virtually eliminated with 289C-HAHA TnT.

Additional evidence for loss of the B state comes from an increase in the rate of binding of S1 to pyrene labeled actin in the virtual absence of Ca$^{2+}$, from 1/s to 4.1/s (Fig. 3), a rate
Basic residues within the cardiac troponin T C-terminus

equal to that observed at saturating Ca\(^{2+}\) (4.1/s in Fig. 4).

Strong support for the idea that the C-terminal region of TnT is critical for forming the B state came from the observation that the conformational change associated with forming the B state was eliminated when the basic residues in the C-terminal region of TnT were eliminated (see below).

The primary evidence for stabilization of the M state by the basic residues within the C-terminal region of TnT comes from ATPase measurements. Replacement of wild type TnT with HAHA TnT doubled the actin activated ATPase activity at saturating Ca\(^{2+}\). Although this is a large effect it does not represent complete stabilization of the active state. At saturating Ca\(^{2+}\), activation by N-ethylmaleimide labeled S1 or by a combination of two troponin mutants (A8V TnC and ∆14 troponin) tripled the activation of ATPase activity by Ca\(^{2+}\) (19). At saturating Ca\(^{2+}\), HAHA TnT gave approximately 70% of that maximum ATPase rate. Put another way, deleting the basic residues in the C-terminal region of TnT increased the population of actin in the active M state, as saturating Ca\(^{2+}\), from <35% to about 70% (see also Baxley (19)).

HAHA TnT has advantages over the use of rigor S1 to stabilize the active state. HAHA TnT allows measurements to be made at saturating ATP. Also, corrections for changes in available myosin binding sites (19) are not necessary. Furthermore, when one of the Cys containing varieties of HAHA TnT is used, both the probe and the function can be monitored. As we show here, HAHA TnT can be used in fiber studies as well as in solution.

Rigor S1 stabilizes the active state by displacing tropomyosin on actin (8). An interesting question is whether troponin accomplishes this activation also by repositioning tropomyosin in the same manner as occurs with rigor S1 binding to actin. It is interesting that the ∆28 deletion of TnT does not appear to cause changes in the position of tropomyosin (28). The ∆28 deletion extends into the I-T helix and weakens binding of troponin to actin-tropomyosin.

Another indication that basic residues within the C-terminal region of TnT impact the function of regulated actin at saturating Ca\(^{2+}\) comes from the rate of rigor S1 binding to actin. The rate of binding to wild type actin filaments has been assumed to be at its maximum at saturating Ca\(^{2+}\) (10). That is, the rates of rigor S1 binding to the active M and inactive C states are assumed to be equal. At saturating Ca\(^{2+}\), HAHA TnT shifts the equilibrium between the C and M states toward the active M state. Thus, replacing HAHA TnT for wild type should not affect the kinetics of S1 binding at high Ca\(^{2+}\). However, we observed an increase from the wild type rate of 3.7/s to 6.8/s with HAHA TnT (Fig. 4). We observed a similar behavior with deletion mutants of TnT (21). The simplest explanation is that the rate of rigor S1 binding is >2 fold faster to the M state than to the C state with cardiac regulatory proteins. Alternatively, these TnT mutants could produce a state that is normally unpopulated. If the latter is true, then that state must be identical to the M state in stimulating ATP hydrolysis.

In situations where the rate of rigor S1 binding to the M state is greater than to the C state, one cannot calculate the fraction of actin in the B state by comparing the rates of binding in the presence and absence of Ca\(^{2+}\) as described by others (10).

Despite this limitation, measurements of S1 binding kinetics does give an accurate measure of the change in occupancy of the B state; the magnitude of acrylodan-tropomyosin fluorescence gives a similar measure of the change (21). Quantitation by those methods requires a standard that is 100% in the B state. Regulated actin filaments containing S45E TnI had an actin activated ATPase rate, at low Ca\(^{2+}\), equal to 0.61x the wild type rate (20). The acrylodan tropomyosin fluorescence amplitude for S45E TnI containing filaments was 1.3x that of wild type (24). This is a useful benchmark although it is unclear that it represents 100% B state.

To begin to understand how the C terminal basic residues of TnT function, we measured changes in position of that region relative to Cys 190 of tropomyosin in the transition from the inactive C state to the inactive B state. The location of Cys 190 relative to the core region of troponin is shown in Fig. 13 which is based on Takeda et al. (29). Earlier evidence suggested that Cys 190 of tropomyosin was near TnT residues 197-239 (30) or residues 272-288.
Basic residues within the cardiac troponin T C-terminus

(31,32). That location is not certain, however, as a fragment of TnT (residues 262-288) was shown to interact with whole TnI (33).

In moving from the C state to the B state, probes on positions 275 and 289 of TnT moved toward Cys 190 of tropomyosin. Fig. 13 illustrates the location of the donor probe on position 289 of TnT (d1) and the acceptor on tropomyosin in that relaxed or B state. Positions 289 and 275 moved similarly relative to Cys 190 of tropomyosin.

As a control we also measured changes in a probe on position 143 of TnI, shown as d2 in Fig. 13. That probe is within the inhibitory region of TnI that binds to actin in the B state. That inhibitory region was seen to move closer to actin in going from the C state to the B state as expected.

The results seen here point to interesting structural differences between the two inactive states, B and C. Although neither state supports productive actomyosin interactions they differ in their orientations of both TnI and TnT relative to Cys 190 of tropomyosin. We are currently exploring these changes, and those that occur between the active M state and the inactive C state.

No change in distance between the C-terminal region of TnT and cys 190 of tropomyosin was observed in the case of 289C-HAHA TnT. This strong evidence that the B state does not form in the absence of a basic patch at the C-terminal end of TnT. The distance between residue 289 of HAHA TnT and 190 of tropomyosin remained at approximately 45 Å which seems to be the distance characteristic of the C state. The basic residues within the C-terminal region of TnT appear to be required for tropomyosin movement into the B state.

The changes in Förster Resonance Energy Transfer reported here have been interpreted in terms of changes in distance between the donor and acceptor probes. Changes in energy transfer efficiency can also occur as a result of changes in the quantum yield of the donor or probe orientation (κ² term of Equation 4). In the present case, changes in the quantum yields between high and low Ca²⁺ were insignificant. Anisotropy measurements showed that there were no large changes in probe mobility so the value of κ² was apparently unchanged. It seems likely that the changes in transfer efficiency reported here are due to distance changes.

Actin filaments containing human cardiac 289C-TnT and 289C-HAHA TnT behaved, in solution, like wild type and Δ14 TnT containing filaments, respectively. The 289C-HAHA troponin construct also behaved like Δ14 TnT when introduced into CMPs. In solution the ATPase activity was increased and the B state was eliminated when the basic residues were removed from the C-terminal region of TnT. Similarly, in CMPs the sinusoidal stiffness was increased at low Ca²⁺ concentrations. Eliminating those basic residues also caused an increase in ATPase activity and a faster rate of rigor S1 binding at high Ca²⁺. Similarly, cardiac muscle preparations containing 289C-HAHA TnT produced a greater level of steady-state isometric force and had an increased number of force producing crossbridges at all Ca²⁺ levels.

289C-HAHA TnT produced a shift of 0.24 pCa units in the myofilament Ca²⁺ sensitivity measured by steady-state isometric force in skinned CMPs. This compares well with the 0.2 pCa leftward shift observed with Δ14 TnT in skinned trabeculae strips (18). The increase in force was associated with an increase in the number of force producing crossbridges (Fig 10).

One may argue that the state of actin obtained by using HAHA TnT, or other such mutants (21,24), could be different from the M state. The M state is defined as the state obtained when actin-tropomyosin-troponin has sufficient rigor type myosin bound to achieve full activity at saturating Ca²⁺. We described earlier the ATPase activity of regulated actin in the presence of N-ethylmaleimide labeled S1 with various types of regulatory proteins bound to actin (19). The use of various mutants of troponin achieved the same level of activity as obtained with modified S1 (19,21). This is evidence that the same state is stabilized in both cases. The change in pCa-Force relationship observed here is also expected if the M state is stabilized. We analyzed other mutants of troponin and found them to affect the equilibria among the B, C and M states (34). So the idea that mutations alter the distribution of actin states seems reasonable. The most straightforward explanation
is that HAHA TnT, as well as other mutants of TnT, stabilize the M state.

All of the results shown here, both in solution and in organized muscle preparations, support the idea that the C-terminal region of TnT serves a critical role in contraction. Elimination of the basic residues in the C-terminal region of TnT eliminates one of the inactive states (the B state). The other function of the basic residues is to limit the extent of activation by Ca\(^{2+}\). This leads to the possibility that the extent of activation may be regulated by an unknown mechanism in addition to Ca\(^{2+}\) binding to TnC. Fig. 10 shows that altering the C-terminal region of TnT can produce increases in cardiac muscle force of contraction at physiological Ca\(^{2+}\) concentrations, e.g., pCa 5.8 – 5.4. If these same changes can be created in a wild type fiber using a drug or other intervention, it could be possible to improve the performance of a diseased heart.

**EXPERIMENTAL PROCEDURES**

**Proteins.** Actin was prepared from a bulk dissection of the back muscles of a rabbit (35). New Zealand White rabbits were sacrificed in accordance with NIH guidelines and animal care protocol approved by the Animal Care and Use Committee of East Carolina University. F-Actin was labeled with pyrene-iodoacetamide (36,37). The modification buffer was 1 mM Tris pH 8, 0.1 mM CaCl\(_2\) and 0.5 mM ATP. The reaction was stopped with excess dithiothreitol and the actin was centrifuged in a Ti 50 rotor for 20 minutes at 30,000 rpm to remove precipitated protein and excess probe. Pyrene labeled actin was dialyzed against a minimum of 3 changes of 4 mM MOPS, 2 mM MgCl\(_2\), 1 mM DTT buffer at 4\(^{\circ}\) C. The extent of pyrene labeling was determined using an extinction coefficient of 22,000 M\(^{-1}\)cm\(^{-1}\) at 344 nm. The extent of labeling with pyrene was generally 70%.

Rabbit skeletal myosin was prepared from back muscle (38) and was digested with chymotrypsin to prepare the soluble catalytic fragment, S1 (39). Tropomyosin was prepared from bovine cardiac left ventricles (40) and was labeled at Cys 190 with acrylodan using a 10:1 ratio of acrylodan to tropomyosin (24). The extent of labeling was approximately 70% using an extinction coefficient of 14400 M\(^{-1}\) cm\(^{-1}\) at 372 nm for acrylodan (41). Tropomyosin was labeled at Cys 190 with DABMI using a similar procedure. The molar ratio of DABMI to tropomyosin was 5:1 and the extent of labeling was 68% using an extinction coefficient of 24800 M\(^{-1}\) (42). Human cardiac troponin components were expressed in E. coli and purified as described earlier (19). TnT (isoform 2) was expressed in pSBETa, TnI and TnC in pET3d. The purified troponin components were reconstituted and purified by ion exchange chromatography (19).

The mutants of human cardiac TnT used in this study are shown in Table 4. They were synthesized and cloned into a pMK vector by the Invitrogen GeneArt Gene Synthesis Service. Two restriction enzyme sites: NdeI and BamHI, were added to the 5’ and 3’ termini, respectively. Each cloned DNA sequence was verified and excised from the recombined plasmid by digestion with appropriate restriction enzymes and subcloned into the NdeI-BamHI site of the vector pSBETa (a kind gift from Dr. H.-H. Steinbiss) to yield an expression plasmid designated pSBET-289C-HAHA TnT. Clones containing the correct recombinant plasmid were confirmed by NdeI and BamHI double digestion and DNA sequencing. The following primers were used for TnT sequencing. TNNT2FW: ATCCGGAATGAGCGGGAGAA, TNNT2RV: ATTCAGGTCCTTCTCCATGCG.

BL21(DE3)pLysS strain was used as the expression host for 289C-HAHA TnT. The masses of tryptic fragments of the 289C-HAHA TnT were analyzed by liquid chromatography mass spectrometry. The masses obtained were consistent with the sequences.

The concentration of actin, and S1 were determined by absorbance at 280 nm after correction for light scattering at 340 nm using the following extinction coefficients (\(\varepsilon^{0.1%}\): 1.15 for actin and 0.75 for S1. Tropomyosin and troponin subunits were quantified by the Lowry protein assay using a bovine serum albumin standard. Molecular weights were assumed to be 120000 for myosin S1, 68000 for tropomyosin, 42000 for actin, 35923 for TnT, 24000 for TnI and 18400 for TnC.

**ATPase Assays.** The rate of liberation of \(^{32}\)Pi from [\(\gamma\)-\(^{32}\)P]ATP was measured as described.
Basic residues within the cardiac troponin T C-terminus

earlier (43). The phosphate concentration was determined from a 0.05 ml aliquot taken at 3, 6 and 9 min to ensure linearity of the reaction. Initial rates were determined from a linear least-squares analysis. All reactions were run at 25°C at conditions indicated in the Fig. legends. The fraction of actin in the active M state was calculated from the ATPase rate (19).

Rapid Kinetic Measurements. Pre-steady state rates were measured using a SF20 sequential mixing stopped-flow spectrometer equipped with an LED light source (Applied Photophysics, Leatherhead, UK). Excitation wavelengths were determined by the wavelength of the LED light source. The emission wavelength was set with a high pass filter. Reactions were generally run at high Ca²⁺ (approximately 0.2 mM free) and low Ca²⁺ (approximately 10⁻⁸ M free).

The formation of the B state was determined by monitoring acrylodan-tropomyosin fluorescence (21,23). Acrylodan was excited with a 390 nm LED and emission was measured with a 451 nm high pass filter. The B state was also determined by comparing the rate of binding of rigor S1 to actin filaments in the virtual absence of Ca²⁺ to that at saturating Ca²⁺ (10). Pyrene probes on actin were excited using a 360 nm LED and fluorescence was monitored through a 400 nm midpoint high pass filter.

For Förster Resonance Energy Transfer studies the single Cys residue of donor proteins 289C of TnT or 275C of both TnT and HAHA TnT or 143C of TnI were labeled with IAEDANS. The acceptor molecule DABMI was placed on Cys 190 of tropomyosin. A 340nm LED was used for IAEDANS excitation and emission was monitored through a high pass filter with a cut-on midpoint of 451 nm.

IAEDANS emission was measured in both the absence and presence of the DABMI acceptor probe. The absorbance of all solutions at 336 nm were < 0.01 to avoid inner filter effects. The traces were corrected using a baseline generated from the same experiment in the absence of donor and acceptor probes. The initial and final fluorescence was recorded for the donor with (ID+A) and without acceptor (ID). The efficiency of transfer, E, was calculated from Equation 1:

\[ E = 1 - \frac{(ID+A(t))/ID(t)}{FA} \]

Where \( ID+A(t) \) is the time course of the fluorescence intensity of the donor in the presence of the acceptor, \( ID \) is the time course of the donor alone and \( FA \) is the fraction of the acceptor protein, tropomyosin, labeled with DABMI.

The distance (Å) between the donor and acceptor probes, \( r \), was calculated with Mathematica (Wolfram Research Inc., Champaign, IL) using Equations 2-4 (44):

\[ E = \frac{R_0^6}{R_0^6 + r^6} \]

\[ Q_D = Q_S * A_S/A_D * F_D/F_S \]

\[ R_0^6 = 8.79 \times 10^{-5}(\kappa^2 n - 4Q_J(\lambda)) \]

where \( R_0 \) is the Förster distance for the donor-acceptor pair on their respective proteins.

\( R_0^6 \), in units of Å⁶, was calculated from Equation 3. \( \kappa^2 \), the orientation factor, was assumed to be 2/3 for random orientations of the probes. An alternate assumption of 0.476 for rigid probes and a random ensemble (45) resulted in distances approximately 10% smaller. The refractive index of the medium, \( n \) was assumed to be 1.33. The quantum yield of the donor probe on the reconstituted troponin complex, \( Q_D \) was measured using a standard of 1 µM quinine sulfate in 0.1 N H₂SO₄ (46). \( Q_S \), in Equation 3, is the quantum yield of the standard and equals 0.55. \( A_S/A_D \) is the ratio of absorbance values of the standard and the donor and \( F_D/F_S \) is the corresponding ratio of the integrated fluorescence intensities of the donor and the quinine sulfate standard, respectively. The overlap integral, \( J(\lambda) \), was calculated from the emission spectrum of the donor (IAEDANS) normalized to a maximum value of 1 and the absorption spectrum of the acceptor (DABMI) normalized to a maximum value equal to the extinction coefficient of DABMI (24800 M⁻¹) (42) using the program a|e (FluorTools, www.fluortools.com).

Fluorescence emission spectra were measured with a Fluoromax-4 spectrofluorometer (Horiba Scientific, Edison, NJ) and corrected for lamp output and radiometric corrections. The baseline was corrected, prior to integration, using the Horiba software or by fitting 2 or more Gaussian curves to the data using Mathematica.
Basic residues within the cardiac troponin T C-terminus

(Wolfram Research Inc., Champaign, IL). The absorbance of all solutions at 336 nm were < 0.01 to avoid inner filter effects.

Anisotropy measurements were made on the same instrument at the same conditions used for distance measurements.

ATPase measurements at high Ca²⁺ showed that the effects of the probes were modest. The actin activated ATPase rate in the presence of tropomyosin was 0.46 ± 0.06/s. That rate reduced to 0.37 ± 0.02/s when tropomyosin was labeled. The rate measured with actin-tropomyosin-troponin was 1.9 ± 0.08/sec. That compares with 1.4 ± 0.02/s when TnI was labeled, 1.8 ± 0.07 when TnT was labeled at Cys 275 and 1.6 ± 0.04/s when TnT was labeled at Cys 289.

Mathematical analysis of the temperature dependence of apparent rate constants, k(T), was done with MLAB (Civilized Software, Bethesda, MD). The Arrhenius equation is shown in Equation 5 where A is the pre-exponential factor, $E_A$ is the energy of activation with units of $R^*T$, R is the gas constant 8.314 joules/(mole*K) and T is in degrees K.

Equation 5. $k(T) = A*exp(-E_A/R^*T)$

Muscle Mechanics Measurements.

Cardiac Muscle Preparations (CPMs) – Cardiac papillary muscles were isolated from left ventricles of freshly slaughtered pigs obtained from a nearby abattoir. The papillary muscles were further dissected, skinned and stored as described (26, 27).

Native troponin complex displacement and reconstitution – To displace the endogenous troponin complex, CPMs were incubated with 1.3 mg/ml wild type or 289C-HAHA TnT in a solution containing 400 mM KCl, 20 mM MOPS, 5 mM MgCl₂, 5 mM EGTA, 10 mM DTT, pH 6.2 for ~1.5 h at room temperature after a brief 10-min exposure to a preincubation buffer without protein (same buffer as above, however, with 250 mM KCl). The TnT-treated preparations were then washed with preincubation buffer to remove unbound exogenous TnT. Subsequently, the efficiency of endogenous troponin displacement was evaluated by the level of unregulated force development obtained by the ratio of steady-state tension generated at pCa 8 and pCa 4 solutions. The Ca²⁺ regulation of force was restored using a pre-formed human cardiac TnI-TnC binary complex. The CPMs were incubated with 21 μM of the binary complex in pCa 8.0 solution for ~1 h at room temperature until the force returns to low force levels and reaches a steady-state. The Ca²⁺ dependence of steady-state isometric force and sinusoidal stiffness (SS) were determined after performing the displacement and reconstitution as previously described (26).

pCa vs Force – Force was measured using a force transducer (Aurora Scientific Inc. Model 403A) and length was controlled using a high-speed servomotor (Aurora Scientific Inc. Model 322C). Free Ca²⁺ concentrations were calculated using a pCa Calculator (47). Reconstituted CPMs were exposed to Ca²⁺ solutions ranging from 10⁻⁸ mol/L to 10⁻⁴ mol/L and expressed as pCa (-log [Ca²⁺]) (48). To minimize compliance, CPMs were chemically edge-fixed with 1% glutaraldehyde. Sarcomere length was set to 2.1 μm using HeNe laser diffraction under resting condition (pCa 8). All experiments were carried out at room temperature (~21°C). Steady-state, isometric force data were analyzed and fit using a sigmoidal Hill equation as previously described (48).

Sinusoidal Stiffness – Determined by measuring changes in sarcomere length and their respective changes in force, sinusoidal stiffness (SS) was measured after force reached steady-state levels in each pCa solution. CMP was oscillated ~ 0.2% peak-to-peak of its initial length at frequency of 100 Hz with a sampling rate of 1 kHz. Sinusoidal stiffness measurements were performed and the data were analyzed and fit as previously described (48).
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Author Contributions: JMC and DJ designed the solution studies, JRP and ML-V designed the mechanical studies, LZ produced the mutants. DJ and ML-V conducted most of the solution and mechanical studies, respectively. JMC wrote the manuscript with contributions from all others.
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Basic residues within the cardiac troponin T C-terminus

FOOTNOTES
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The abbreviations used are: CMPs, Cardiac Muscle Preparations; DABMI, (4-(dimethylamino)phenylazophenyl-4′-maleimide); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; IAEDANS, N-(Iodoacetaminoethyl)-1-naphthylamine-5-sulfonic acid; MOPS, 3-(N-Morpholino]-propanesulfonic acid; pyrene iodoacetamide, N-(1-pyrene)iodoacetamide; regulated actin, actin-tropomyosin-troponin; S1, myosin subfragment 1.
Basic residues within the cardiac troponin T C-terminus

Tables

Table 1. Transitions of IAEDANS probes at the C-terminal region of TnT relative to DABMI at cys 190 of tropomyosin in going from the inactive state C to the inactive B state.

|                  | Efficiency initial | Efficiency final | Distance initial \(^1,2,3\) | Distance final \(^1,2,3\) |
|------------------|--------------------|------------------|-----------------------------|---------------------------|
| IAEDANS 275C-TnT 10° C | 0.68               | 0.75             | 38 ± 2                      | 35 ± 1                    |
| IAEDANS 289C-TnT 10° C | 0.37               | 0.48             | 46 ± 5                      | 43 ± 4                    |
| IAEDANS 289C-TnT 18° C | 0.37               | 0.45             | 46 ± 6                      | 44 ± 3                    |
| IAEDANS 289C- TnT 25° C | 0.44               | 0.49             | 44 ± 1                      | 42 ± 5                    |
| IAEDANS 289C-HAHA TnT 18° C | 0.35             | 0.34             | 47 ± 4                      | 47 ± 4                    |
| IAEDANS T143C-TnT 10° C | 0.27               | 0.35             | 50 ± 3                      | 47 ± 2                    |

1Distances are given in Å; Quantum yields were 0.510, 0.523, and 0.537 at 25°, 18°, and 10° C, respectively.
2Distances were calculated assuming \(\kappa^2 = 2/3\). To estimate distances with \(\kappa^2 = 0.467\), multiply value by 0.945.
3Errors in the calculation based on the Förster equations are shown. Other uncertainties exist such as the value of \(\kappa^2\).

Table 2. Ca\(^2+\) - dependence of anisotropy of probes on troponin-tropomyosin-actin

|                  | Anisotropy 10\(^{-8}\) M Ca\(^{2+}\) | Anisotropy 2 mM Ca\(^{2+}\) |
|------------------|--------------------------------------|-----------------------------|
| IAEDANS 289 C-TnT | 0.120 ± 0.001                        | 0.113 ± 0.001               |
| IAEDANS 289 C-HAHA TnT | 0.083 ± 0.007                      | 0.071 ± 0.009               |
| IAEDANS 143 C-TnI  | 0.111 ± 0.008                        | 0.111 ± 0.007               |
| Fluorescein 190 C-tropomyosin | 0.131 ± 0.001                  | 0.131 ± 0.001               |
Table 3. Contractile Parameters for CMPs containing wild type or 289C-HAHA troponin T.

|                      | wild type       | 289C-HAHA       |
|----------------------|-----------------|-----------------|
| % Displacement       | 81.80 ± 3.79    | 86.96 ± 1.13    |
| FpCa\textsubscript{50} | 5.38 ± 0.03     | 5.62 ± 0.02*    |
| F\textsubscript{Hill}   | 1.43 ± 0.21     | 1.00 ± 0.07     |
| P (\text{mN/mm}\textsuperscript{2}) | 13.40 ± 1.03 | 17.41 ± 1.17"  |
| P\text{0} (\text{mN/mm}\textsuperscript{2}) | 22.62 ± 2.27 | 20.77 ± 0.05   |
| SSpCa\textsubscript{50} | 5.44 ± 0.03     | 5.63 ± 0.07"    |
| SS\text{max} (MPa)     | 0.82 ± 0.03     | 0.96 ± 0.03"    |
| #CMP                  | 5               | 5               |

*p<0.001, unpaired student’s t-test 289C-HAHA vs wild type.

*p<0.05, unpaired student’s t-test 289C-HAHA vs wild type.

The maximum steady-state isometric force values are reported as average of absolute force normalized to the cross-sectional area of each preparation where P\text{0} and P values were obtained prior and after troponin complex replacement, respectively.

FpCa\textsubscript{50} = pCa necessary to reach 50% of the maximum steady-state isometric force; F\textsubscript{Hill} = cooperativity of thin filament activation; SSpCa\textsubscript{50} = pCa necessary to reach 50% of the maximum steady-state sinusoidal stiffness; SS\text{max} = maximum steady-state sinusoidal stiffness.

Table 4. C-terminal mutants of human cardiac TnT

| Wild type TnT       | NH\textsubscript{2}--...KVSKT RGKAK VTGRW K--COOH |
|---------------------|---------------------------------------------------|
| S275C TnT           | NH\textsubscript{2}--...KVCKT RGKAK VTGRW K--COOH |
| 289C TnT            | NH\textsubscript{2}--...KVSKT RGKAK VTGRW KC--COOH |
| 289C-HAHA TnT       | NH\textsubscript{2}--...AVSAT AGAAA VTGAW AC--COOH |
**Figures and Legends**

**Fig. 1.** The three states of actin in the McKillop & Geeves model. The B state, equivalent to state 1 in the Hill model Hill (9), has no bound Ca$^{2+}$ and is inactive. State C, equivalent to state 1$_{Ca}$ in the Hill model, has bound Ca$^{2+}$ but is inactive. State M (equivalent to state 2) is active and is stabilized by binding of both Ca$^{2+}$ and rigor myosin to regulated actin filaments. Note that states B, C and M are equivalent to states 1$_{0}$, 1$_{Ca}$ and 2 of the earlier Hill et al. model.

![Diagram showing the three states of actin with states B, C, and M connected by arrows labeled $K_B$, $K_T$.]

**Fig. 2:** ATPase rates of myosin S1 in the presence of actin and actin-tropomyosin with different troponin variants at saturating Ca$^{2+}$. Measurements were made at 25°C and pH 7.0 in solutions containing 1 mM ATP, 4 mM MgCl$_2$, 31 mM KCl, 10 mM MOPS, 1 mM dithiothreitol and 0.1 mM CaCl$_2$. The concentrations of S1, actin, tropomyosin, and troponin were 0.1, 10, 2.2, and 2.2 µM, respectively. Data are shown as mean ± standard error of the mean. The data shown for HAHA are for 289C-HAHA TnT.
Fig. 3: Rate of binding of S1 to an excess of pyrene labeled actin filaments containing tropomyosin and troponin in the absence of ATP at low Ca\textsuperscript{2+}. Plots are averages of five traces of binding to actin-tropomyosin containing wild type (curve 1); 289C (curve 2); and 289C-HAHA TnT (curve 3). Dashed lines are mono-exponential fits. The apparent rate constants were 1.0 ± 0.3, 1.2 ± 0.4, and 4.1 ± 0.4 per second for wild type, 289C, and 289C-HAHA TnT respectively. Conditions: 25 °C, 4 μM pyrene-actin (40% labeled), 0.86 μM tropomyosin and 0.86 μM troponin was rapidly mixed with 0.4 μM myosin S1 in 20 mM MOPS, 152 mM KCl, 4 mM MgCl\textsubscript{2}, 1 mM dithiothreitol and 2 mM EGTA.
Fig. 4: Rate of binding of S1 to an excess of pyrene labeled actin filaments containing tropomyosin and troponin in the absence of ATP at high Ca²⁺. Plots are averages of 5 traces of binding to actin-tropomyosin containing wild type (curve 1); 289C (curve 2); and 289C-HAHA TnT (curve 3). Dashed lines are single exponential fits. The apparent rate constants were 3.7 ± 0.6, 3.9 ± 0.6, and 6.8 ± 0.8 per second for wild type, 289C, and 289C-HAHA TnT respectively. Conditions: same as Fig. 3 except 0.2 mM CaCl₂ was substituted for EGTA.
Basic residues within the cardiac troponin T C-terminus

Fig. 5: Time courses of acrylodan tropomyosin fluorescence changes as regulated actin moved from the active M state to the B state at low Ca\textsuperscript{2+}. At time zero the mixture of regulated actin and S1 was rapidly mixed with an excess of ATP. The major transition observed is the C to the B state. Wild type (curve 1), 289C (curve 2) and 289C-HAHA TnT (curve 3). Traces shown are averages of five measurements. The amplitudes of the C to B transition were 0.28 for both wild type and 289C-TnT. The apparent rate constants were, 32 ± 4 and 29 ± 6 per sec for wild type and 289C-TnT, respectively. No C to B transition was observed for filaments containing 289C-HAHA TnT. Conditions: 2 µM actin, 0.43 µM tropomyosin, 0.43 µM troponin and 2 µM S1 in 20 mM MOPS, 152 mM KCl, 4 mM MgCl\textsubscript{2}, 1 mM dithiothreitol and 2 mM EGTA was rapidly mixed with 2 mM ATP, 20 mM MOPS, 152 mM KCl, 8 mM MgCl\textsubscript{2}, 1 mM dithiothreitol and 2 mM EGTA at 10 °C.
Fig. 6: Time courses of IAEDANS 289C TnT fluorescence change in the absence and presence of a DABMI fluorescence acceptor probe on Cys 190 of tropomyosin following the rapid detachment of myosin S1 from actin at low Ca\(^{2+}\). Traces shown are averages of at least five measurements. Traces labeled 1 are in the absence of acceptor DABMI. Traces labeled 2 are in the presence of acceptor DABMI. Panels A, B and C are at 10\(^\circ\), 18\(^\circ\) and 25\(^\circ\) C and panels D, E and F are the corresponding transfer efficiencies (not corrected for the extent of probe labeling). The rate constants for the transients in D, E and F are 6.2 ± 0.52, 10.9 ± 3.04 and 21.4 ± 5.8/s, respectively. Conditions: 2 µM actin, 0.51 µM tropomyosin, 0.43 µM troponin and 2 µM S1 in 20 mM MOPS, 152 mM KCl, 4 mM MgCl\(_2\), 1 mM dithiothreitol and 2 mM EGTA was rapidly mixed with 2 mM ATP, 20 mM MOPS, 152 mM KCl, 8 mM MgCl\(_2\), 1 mM dithiothreitol and 2 mM EGTA.
Basic residues within the cardiac troponin T C-terminus

Fig. 7. Apparent rate constant for transition from the C to the B state. Measurements of FRET from IAEDANS 289C-TnT to DABMI tropomyosin (open circles). Measurement of acrylodan tropomyosin fluorescence transition from Fig. 5 (open triangle). Measurements of acrylodan tropomyosin fluorescence (23) (solid circles). The curve is the best fit of the Arrhenius equation with $A = 8 \times 10^{12} \text{s}^{-1}$ and $E_A = 65,634 \text{joules*K/mole}$.

Fig. 8: Time courses of IAEDANS 289C-HAHA TnT fluorescence change in the absence and presence of a DABMI fluorescence acceptor probe on C190 of tropomyosin following the rapid detachment of myosin S1 from actin at low Ca$^{2+}$. Traces shown are averages of seven measurements. Panel A, upper trace, donor alone; lower trace donor + acceptor. Panel B, transfer efficiency (not corrected for the extent of probe labeling). Conditions: same as Fig. 6 but at 18 °C.
Fig. 9: Time courses of Förster resonance energy transfer between either IAEDANS 275C TnT (panels A & B) or IAEDANS 143C TnI (panels C & D) and DABMI C190 of tropomyosin following the rapid detachment of myosin S1 from actin at low Ca\textsuperscript{2+}. Traces shown are averages of at least five different measurements. Panel A, upper trace: TnT donor alone; lower trace: donor + DABMI acceptor on tropomyosin. Panel B, calculated efficiency of transfer between the probes on TnT and tropomyosin with an apparent rate constant of 3.5 ± 0.17/s. Panel C, upper trace: TnI donor alone; lower trace: donor + DABMI acceptor on tropomyosin. Panel D, efficiency of transfer between probes on TnI and tropomyosin with an apparent rate constant of 8.9/s. The efficiencies in B and D are not corrected for the extent of probe labeling. Conditions: same as Fig. 6 but at 10° C.
Fig. 10. Normalized pCa-force relationship in CMPs containing wild type (solid circles) or 289C-HAHA human cardiac TnT (open circles). Panel A shows normalized force values to the maximum force produced by each individual CMPs. Panel B shows the force values normalized to the wild type to illustrate the increase in force produced by the 289C-HAHA mutant. The average absolute value of the 289C-HAHA TnT maximum steady-state isometric force was normalized to the average absolute value of the wild type TnT maximum steady-state isometric force value, where the wild type maximum value was called 100%. Conditions: To determine the Ca^{2+} sensitivity of force development, the CMPs were gradually exposed to solutions of increasing Ca^{2+} concentration from pCa 8.0 to 4.0 (10^{-8} – 10^{-4} M [Ca^{2+}]_{free}, 1 mM [Mg^{2+}]_{free}, 7 mM EGTA, 2.5 mM MgATP^{2+}, 20 mM MOPS, pH 7.0, 15 mM creatine phosphate, and 15 units/ml creatine phosphokinase, I = 150 mM) at 21°C. Data are shown as mean ± standard error of the mean.
Fig. 11. Sinusoidal stiffness analysis in CMPs containing wild type (solid circles) or 289C-HAHA human cardiac TnT (open circles). Panel A shows pCa vs sinusoidal stiffness and panel B shows normalized force to wild type vs sinusoidal stiffness. In panel B, the average absolute value of the 289C-HAHA TnT maximum steady-state isometric force was normalized to the average absolute value of the wild type TnT maximum steady-state isometric force value, where the wild type maximum value was called 100%. Conditions were similar to Fig. 10. Data are shown as mean ± standard error of the mean.
Fig. 12. Normalized pCa-sinusoidal stiffness relationship in CMPs containing wild type (solid circles) or 289C-HAHA human cardiac TnT (open circles). Sinusoidal stiffness values were normalized to the maximum sinusoidal stiffness produced by each individual CMPs. Conditions were similar to Fig. 10. Data are shown as mean ± standard error of the mean.
Fig. 13. Schematic of the key interaction sites between troponin and actin (yellow) and tropomyosin (black) in the relaxed state based on Takeda et al. (29). TnC (blue) is positioned between two helices, one from TnI (red) and the IT helix involving both TnI and TnT (green). In the relaxed state TnI interacts with actin-tropomyosin through the inhibitory region (IR) and the C-terminal region (C-TnI). Between these regions is the switch region (SW) that binds to TnC at saturating Ca²⁺. TnT is shown in green. Adjacent to the IT helix is the C-terminal region of TnT shown as a thin green line. That C-terminal region has a fluorescent donor (d1). An acceptor of fluorescence (a) is present on Cys 190 of tropomyosin. The distance between d1 and a is at its minimum in the relaxed B state. The black arrow shows a type of change in the C-terminal region of TnT when Ca²⁺ binds to TnC. Ca²⁺ binding also increases the distance between the donor in the IR region of TnI (d2) as shown by the red arrow. The interactions among troponin and actin-tropomyosin, shown by the columns of black dashes, are also disrupted and sw ultimately binds to TnC. For clarity, the donor at position 275 is not shown.
Basic residues within the cardiac troponin T C-terminus are required for full inhibition of muscle contraction and limit activation by calcium
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