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# Effect of Hypertrophic Cardiomyopathy-Linked Troponin C Mutations on the Response of Reconstituted Thin Filaments to Calcium upon Troponin I Phosphorylation

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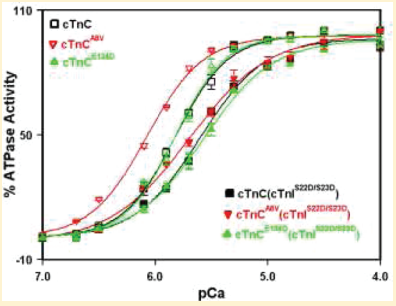
**ABSTRACT:** The objective of this work was to investigate the effect of hypertrophic cardiomyopathy-linked A8V and E134D mutations in cardiac troponin C (cTnC) on the response of reconstituted thin filaments to calcium upon phosphorylation of cardiac troponin I (cTnI) by protein kinase A. The phosphorylation of cTnI at protein kinase A sites was mimicked by the S22D/S23D double mutation in cTnI. Our results demonstrate that the A8V and E134D mutations had no effect on the extent of calcium desensitization of reconstituted thin filaments induced by cTnI pseudophosphorylation. However, the A8V mutation enhanced the effect of cTnI pseudophosphorylation on the rate of dissociation of calcium from reconstituted thin filaments and on the calcium dependence of actomyosin ATPase. Consequently, while the A8V mutation still led to a slower rate of dissociation of calcium from reconstituted thin filaments upon pseudophosphorylation of cTnI, the ability of the A8V mutation to decrease the rate of calcium dissociation was weakened. In addition, the ability of the A8V mutation to sensitize actomyosin ATPase to calcium was weakened after cTnI was replaced by the phosphorylation mimetic of cTnI. Consistent with the hypothesis that the E134D mutation is benign, it exerted a minor to no effect on the rate of dissociation of calcium from reconstituted thin filaments or on the calcium sensitivity of actomyosin ATPase, regardless of the cTnI phosphorylation status. In conclusion, our study enhances our understanding of how cardiomyopathy-linked cTnC mutations affect the response of reconstituted thin filaments to calcium upon cTnI phosphorylation.




Familial hypertrophic cardiomyopathy (HCM) is an inherited cardiovascular disorder, characterized by the thickening of the heart muscle and diastolic dysfunction. HCM may lead to a variety of symptoms, such as shortness of breath, chest pain, fatigue, fainting, heart palpitations, and sudden cardiac death (for reviews, see refs 1−3). HCM has been attributed to mutations in a number of genes encoding sarcomeric proteins, including $\beta$-myosin heavy chain, myosin binding protein C, actin, tropomyosin, cardiac troponin I (cTnI), and cardiac troponin T (cTnT) (for reviews, see refs 4−6). Until recently, the gene encoding cardiac troponin C (cTnC) was not considered to be associated with inherited cardiomyopathies. However, recent discoveries linked a number of mutations in cTnC to both HCM and dilated cardiomyopathy (DCM) (for reviews, see refs 5 and 7).

The $Ca^{2+}$ sensor subunit of the cTn complex, cTnC, is a member of the EF-hand (helix−loop−helix motif) family of $Ca^{2+}$ binding proteins. cTnC consists of the N- and C-terminal globular domains connected by an $\alpha$-helical linker (for reviews, see refs 8 and 9). Each domain of cTnC contains a pair of EF-hand motifs numbered I−IV, but the first EF-hand of cTnC is unable to bind $Ca^{2+}$ because of several loop residue substitutions.[10] Therefore, exchange of $Ca^{2+}$ with the second EF-hand of cTnC plays a direct role in the regulation of muscle contraction and relaxation. The third and fourth C-domain EF-hands are believed to be play a structural role in anchoring cTnC into the cTn complex (for a review, see ref 11). The $\alpha$-helices within cTnC are denoted A−H, with an additional 14-residue N-helix at the N-terminus.

Both the intrinsic $Ca^{2+}$ binding properties of cTnC and its interactions with other regulatory muscle proteins play an important role in controlling $Ca^{2+}$ binding and exchange with myofilaments. Numerous studies have focused on elucidating the interactions between cTnC and cTnI, which play a crucial role in the regulation of cardiac muscle contractility. cTnI, an inhibitory subunit of the cTn complex, is a rodlike flexible molecule that contains an ∼30-residue N-terminal extension region, which is absent in both skeletal and slow skeletal isoforms of TnI. The N-extension region of cTnI includes $Ser^{22}$ and $Ser^{23}$ residues that are targets of phosphorylation by protein kinase A (PKA). A number of studies demonstrated that phosphorylation of cTnI at PKA sites during $\beta$-adrenergic stimulation induces myofilament $Ca^{2+}$ desensitization and accelerates cardiac relaxation (for reviews, see refs 12 and 13). While the additional phosphorylation sites are present in

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cTnI, their functional significance remains unclear and controversial (for a review, see ref 14).

Recently, a number of HCM- and DCM-linked cTnC mutations were shown to blunt or abolish myofilament $Ca^{2+}$ desensitization induced by phosphorylation of cTnI by PKA.[15−17] We wanted to determine whether blunting of the myofilament $Ca^{2+}$ desensitization induced by cTnI phosphorylation was a common mechanism among cardiomyopathy-linked cTnC mutations. The objective of this study was to examine whether recently discovered HCM-linked A8V and E134D cTnC mutations[18] affect the response of reconstituted thin filaments to $Ca^{2+}$ upon cTnI phosphorylation by PKA.

## EXPERIMENTAL PROCEDURES

**Materials.** Phenyl-Sepharose CL-4B, $CaCl_2$, and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). IAANS and phalloidin were purchased from Invitrogen (Carlsbad, CA). Affi-Gel 15 affinity media were purchased from Bio-Rad (Hercules, CA). Malachite green oxalate and poly(vinyl alcohol) were purchased from Fisher Scientific (Pittsburgh, PA)

**Protein Mutagenesis and Purification.** The pET3a plasmid encoding human cTnC was a generous gift from L. B. Smillie (University of Alberta, Edmonton, AB). The cTnC construct used in this work (with the exception of actomyosin ATP assays) contained C35S, T53C, and C84S substitutions, to allow fluorescent labeling of cTnC at $Cys^{53}$.[19,20] The HCM-linked cTnC mutants were generated as previously described and confirmed by DNA sequencing.[19,20] Expression and purification of cTnC and its mutants were conducted as previously described.[19−21] The pET3d plasmids encoding human cTnI and human cTnT were generated by GenScript USA (Piscataway, NJ). The cTnI and cTnT subunits were bacterially expressed, purified, and quantified as described previously.[20] The $cTnI^{S22D/S23D}$ mutant was generated from the pET3d cTnI plasmid by primer-based site-directed mutagenesis and verified by DNA sequencing. Rabbit fast skeletal actin and myosin S1 and bovine cTm were isolated, purified, and quantified as described previously.[19]

**Labeling of cTnC and Its Mutants.** cTnC and its mutants were labeled with the environmentally sensitive thiol-reactive fluorescent probe IAANS at $Cys^{53}$ as previously described.[19,20]

**Reconstitution of the cTn Complexes.** The cTn complexes were prepared and reconstituted as previously described.[19,20]

**Reconstitution of Thin Filaments.** After exhaustive dialysis against reconstitution buffer [10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT (pH 7.0)], actin was mixed with an equal molar ratio of phalloidin to stabilize actin filaments. Thin filaments were reconstituted as previously described.[19,20] Briefly, actin−phalloidin (4 $\mu$M) and cTm (0.57 $\mu$M) were mixed in reconstitution buffer and kept on ice for ∼15 min. The cTn complexes (0.5 $\mu$M) were subsequently added, and reconstituted thin filaments were kept on ice for ∼15 min prior to being used. Therefore, the stoichiometry of reconstituted thin filaments was 7:1:0.88 (actin:cTm:cTn).

**Determination of $Ca^{2+}$ Binding Sensitivities.** All steady-state fluorescence measurements were performed using a Perkin-Elmer LS55 fluorescence spectrometer at 15 °C. IAANS fluorescence was excited at 330 nm and monitored at 450 nm as microliter amounts of $CaCl_2$ were added to 2 mL of reconstituted thin filaments in titration buffer [200 mM MOPS (to prevent changes in pH upon addition of $Ca^{2+}$), 150 mM KCl, 2 mM EGTA, 1 mM DTT, and 3 mM $MgCl_2$ (pH 7.0)] at 15 °C with constant stirring. $[Ca^{2+}]_{free}$ was calculated using the computer program EGCA02 developed by Robertson and Potter.[22] The $Ca^{2+}$ sensitivities of conformational changes were reported as a dissociation constant $K_d$, representing a mean of at least three titrations ± the standard error (SE). The data were fit with a logistic sigmoid function (mathematically equivalent to the Hill equation), as previously described.[23]

**Determination of $Ca^{2+}$ Dissociation Kinetics.** All kinetic measurements were performed utilizing an Applied Photophysics Ltd. (Leatherhead, U.K.) model SX.18 MV stopped-flow instrument with a dead time of ∼1.4 ms at 15 °C. The rates of conformational changes induced by EGTA removal of $Ca^{2+}$ from reconstituted thin filaments were measured following IAANS fluorescence. The IAANS fluorescence was excited at 330 nm. The IAANS emission was monitored through a 510 nm BrightLine Basic filter from Semrock (Rochester, NY). Stopped-flow buffer consisted of 10 mM MOPS, 150 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT (pH 7.0). The data were corrected for scattering artifacts as described previously.[19,20] The data were fit using a program (by P. J. King, Applied Photophysics Ltd.) that utilizes the nonlinear Levenberg−Marquardt algorithm. Each $k_{off}$ represents an average of at least three separate experiments ± SE, each averaging at least five traces fit with a single-exponential equation.

**Actomyosin S1 ATPase Assay.** Reconstituted thin filaments (5 $\mu$M actin, 1.0 $\mu$M cTm, 1.5 $\mu$M cTn, and 0.3 $\mu$M myosin S1) were formed at 25 °C in a buffer consisting of 50 mM MOPS and 5 mM $MgCl_2$ (pH 7.0). EGTA (to a final concentration of 0.5 mM) and various amounts of $CaCl_2$ were added to the 100 $\mu$L reaction mixture aliquots to achieve the desired pCa values. The ATPase reaction was initiated by addition of ATP (to a final concentration of 1 mM), and 10 $\mu$L aliquots were removed and added to 90 $\mu$L of 0.2 M ice-cold perchloric acid to terminate the reaction. For determination of the $Ca^{2+}$ dependence of actomyosin ATPase, the ATPase rates were measured at a single time point at which the reaction was still linear with time. To measure minimal and maximal specific actomyosin ATPase activities, 10 $\mu$L aliquots were terminated at 3 min intervals (up to 12 min time course) with 90 $\mu$L of 0.2 M ice-cold perchloric acid. Actomyosin ATPase activity was determined by the amount of phosphate released. The amount of phosphate released was quantified using the malachite green method, as previously described.[20]

**Statistical Analysis.** Statistical significance was determined by an unpaired two-sample $t$ test using the statistical analysis software Minitab. The two means were considered to be significantly different when the $p$ value was <0.05. All data are shown as means ± SE.

## RESULTS

**Location of the A8V and E134D Mutations within cTnC.** Figure 1 shows that the A8V mutation is located in the N-helix of the N-domain of cTnC, while the E134D mutation is located in the C-domain of cTnC, between $Ca^{2+}$ binding sites III and IV.

**Effect of the A8V and E134D cTnC Mutations on the $Ca^{2+}$ Sensitivities of Reconstituted Thin Filaments.** The $Ca^{2+}$-induced increases in IAANS fluorescence, occurring when $Ca^{2+}$ binds to the regulatory N-domain of the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex reconstituted into thin filaments, are shown in Figure 2A and summarized in Table 1. Thin filaments reconstituted with the cTn complex exhibited a half-maximal $Ca^{2+}$-dependent increase in IAANS fluorescence with a $pCa_{50}$


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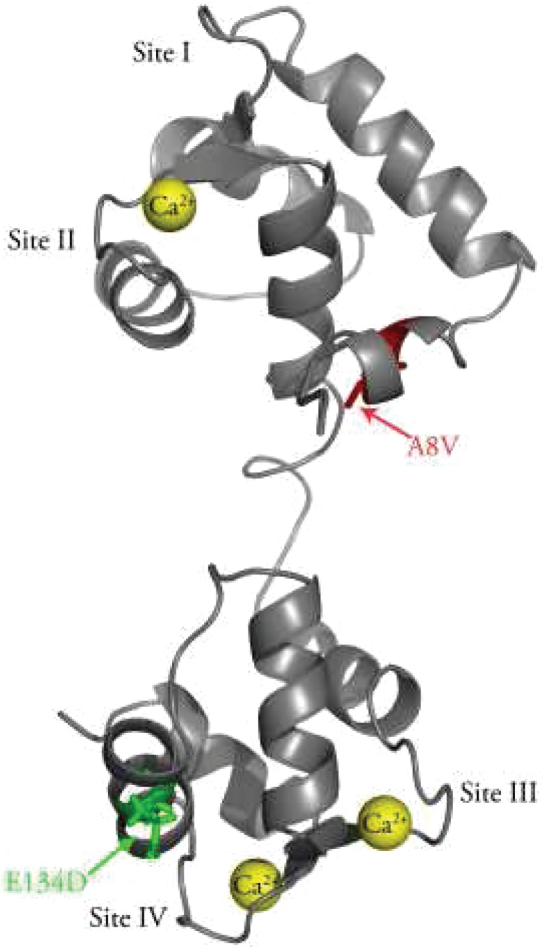


**Figure 1.** Location of the A8V and E134D mutations in the N- and C-domains of cTnC. The figure shows a ribbon representation of cTnC in the $Ca^{2+}$-bound state (Protein Data Bank entry 1AJ4[37]). The A8V mutation (red) is located in the N-helix of the N-domain, while the E134D mutation (green) is located between $Ca^{2+}$ binding sites III and IV. This figure was generated using PyMOL (http://www.pymol.org).

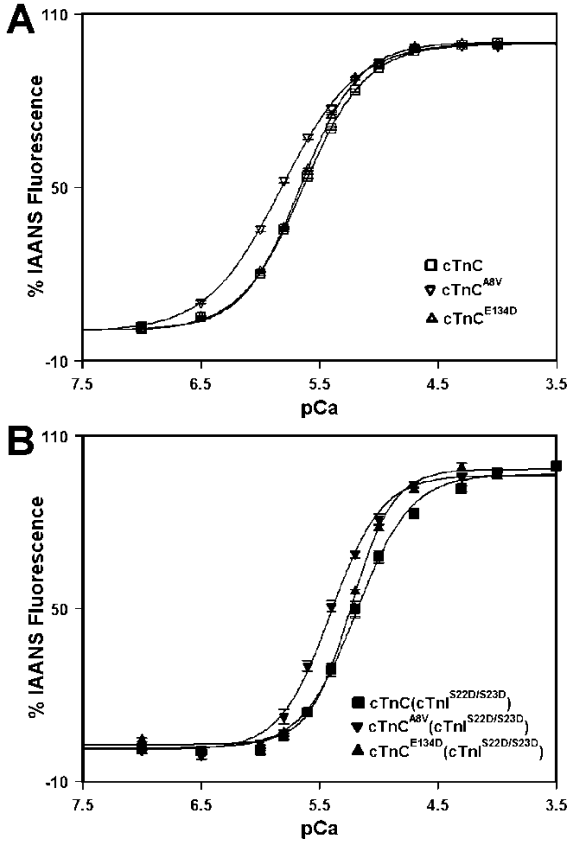


**Figure 2.** Effect of the A8V and E134D cTnC mutations on the $Ca^{2+}$ sensitivities of reconstituted thin filaments in the absence and presence of cTnI pseudophosphorylation. (A) Increases in IAANS fluorescence, which occur as $Ca^{2+}$ binds to the regulatory N-domain of the cTn (□), $cTn^{A8V}$ (▽), or $cTn^{E134D}$ (△) complex reconstituted into thin filaments in the absence of cTnI pseudophosphorylation. (B) Increases in IAANS fluorescence, which occur as $Ca^{2+}$ binds to the regulatory N-domain of the cTn (■), $cTn^{A8V}$ (▼), or $cTn^{E134D}$ (▲) complex, containing the phosphomimetic of cTnI, reconstituted into thin filaments. Each data point represents the mean ± SE of at least three titrations fit with a logistic sigmoid function. The IAANS fluorescence was excited at 330 nm and monitored at 450 nm.

of 5.64 ± 0.02. Thin filaments reconstituted with the $cTn^{A8V}$ complex exhibited a half-maximal increase in IAANS fluorescence with a $pCa_{50}$ of 5.82 ± 0.02, while thin filaments reconstituted with the $cTn^{E134D}$ complex exhibited a half-maximal increase in IAANS fluorescence with a $pCa_{50}$ of 5.660 ± 0.007. The A8V mutation affected the steepness of the curve, indicated by a slightly decreased Hill coefficient ($n_H$), while the E134D mutation had no significant effect on $n_H$ (Table 1). Thus, our results demonstrate that the A8V mutation led to a statistically significant increase in the $Ca^{2+}$ sensitivity of reconstituted thin filaments, while the E134D mutation did not significantly affect the $Ca^{2+}$ sensitivity of reconstituted thin filaments. Furthermore, the A8V mutation decreased the cooperativity of binding of $Ca^{2+}$ to reconstituted thin filaments (indicated by a lower $n_H$), while the E134D mutation had no effect on the cooperativity of binding of $Ca^{2+}$ to reconstituted thin filaments.

**Effect of the A8V and E134D cTnC Mutations on the $Ca^{2+}$ Sensitivities of Reconstituted Thin Filaments in the Presence of cTnI Pseudophosphorylation.** The $Ca^{2+}$-induced increases in IAANS fluorescence, occurring when $Ca^{2+}$ binds to the regulatory N-domain of the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex, containing the phosphomimetic of cTnI ($cTnI^{S22D/S23D}$), reconstituted into thin filaments, are shown in Figure 2B and summarized in Table 1. Thin filaments reconstituted with the cTn complex, containing the phosphomimetic of cTnI, exhibited a half-maximal $Ca^{2+}$-dependent increase in IAANS fluorescence with a $pCa_{50}$ of 5.18 ± 0.04.

**Table 1. Effect of cTnC Mutations on the $Ca^{2+}$ Binding Properties of Reconstituted Thin Filaments in the Absence and Presence of cTnI Pseudophosphorylation**

| protein | $pCa_{50}$ | $n_H$ | $Ca^{2+}$ $k_{off}$ ($s^{-1}$) |
|---|---|---|---|
| cTnC | 5.64 ± 0.02 | 1.63 ± 0.05 | 93 ± 1 |
| $cTnC^{A8V}$ | 5.82 ± 0.02[a] | 1.38 ± 0.03[a] | 42.0 ± 0.4[a] |
| $cTnC^{E134D}$ | 5.660 ± 0.007 | 1.76 ± 0.02 | 96 ± 1 |
| cTnC and $cTnI^{S22D/S23D}$ | 5.18 ± 0.04 | 2.0 ± 0.1 | 310 ± 5 |
| $cTnC^{A8V}$ and $cTnI^{S22D/S23D}$ | 5.41 ± 0.02[b] | 2.0 ± 0.2 | 237 ± 8[b] |
| $cTnC^{E134D}$ and $cTnI^{S22D/S23D}$ | 5.23 ± 0.01 | 2.4 ± 0.1 | 280 ± 6[b] |

[a]Significantly different from their respective cTnC values ($p < 0.05$). [b]Significantly different from their respective cTnC and $cTnI^{S22D/S23D}$ values ($p < 0.05$).


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Thin filaments reconstituted with the $cTn^{A8V}$ complex, containing the phosphomimetic of cTnI, exhibited a half-maximal increase in IAANS fluorescence with a $pCa_{50}$ of 5.41 ± 0.02, while thin filaments reconstituted with the $cTn^{E134D}$ complex, containing the phosphomimetic of cTnI, exhibited a half-maximal increase in IAANS fluorescence with a $pCa_{50}$ of 5.23 ± 0.01. In the presence of cTnI pseudophosphorylation, the $n_H$ values for thin filaments reconstituted with the cTn complexes were not significantly affected by either the A8V or E134D mutation (Table 1). Our results show that thin filaments reconstituted with the cTn complex underwent a substantial $Ca^{2+}$ desensitization upon cTnI pseudophosphorylation ($\Delta pCa_{50}$ = −0.46 ± 0.04). The A8V mutation did not significantly affect the extent of $Ca^{2+}$ desensitization induced by cTnI pseudophosphorylation ($\Delta pCa_{50}$ = −0.41 ± 0.02). The E134D mutation also did not significantly affect the extent of $Ca^{2+}$ desensitization ($\Delta pCa_{50}$ = −0.43 ± 0.01). Therefore, our results demonstrate that neither the A8V nor the E134D mutation significantly blunted the extent of the decrease in the $Ca^{2+}$ sensitivity of reconstituted thin filaments induced by cTnI pseudophosphorylation. In addition, our results show that in the presence of cTnI pseudophosphorylation, the A8V mutation led to a statistically significant increase in the $Ca^{2+}$ sensitivity of reconstituted thin filaments, while the E134D mutation exerted no significant effect on the $Ca^{2+}$ sensitivity of reconstituted thin filaments. Furthermore, our results show that neither the A8V nor the E134D mutation significantly affected the cooperativity of binding of $Ca^{2+}$ to reconstituted thin filaments containing the phosphomimetic of cTnI.

**Effect of the A8V and E134D cTnC Mutations on the Rates of Dissociation of $Ca^{2+}$ from Reconstituted Thin Filaments.** Fluorescence stopped-flow measurements, utilizing IAANS fluorescence, were conducted to determine the effect of the A8V and E134D cTnC mutations on the kinetics of dissociation of $Ca^{2+}$ from the regulatory N-domain of the cTn complex reconstituted into thin filaments. The results are summarized in Table 1. Figure 3A shows that excess EGTA removed $Ca^{2+}$ from the regulatory N-domain of the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex reconstituted into thin filaments at a rate of 93 ± 1, 42.0 ± 0.4, or 96 ± 1 $s^{-1}$, respectively. Therefore, our results show that the A8V mutation led to an ∼2.2-fold slower rate of dissociation of $Ca^{2+}$ from the regulatory N-domain of the cTn complex reconstituted into thin filaments, while the E134D mutation had no significant effect on the rate of $Ca^{2+}$ dissociation.

**Effect of the A8V and E134D cTnC Mutations on the Rates of Dissociation of $Ca^{2+}$ from Reconstituted Thin Filaments in the Presence of cTnI Pseudophosphorylation.** Fluorescence stopped-flow measurements, utilizing IAANS fluorescence, were conducted to determine the effect of the A8V and E134D cTnC mutations on the kinetics of dissociation of $Ca^{2+}$ from the regulatory N-domain site of the cTn complex, containing the phosphomimetic of cTnI, reconstituted into thin filaments. The results are summarized in Table 1. Figure 3B shows that excess EGTA removed $Ca^{2+}$ from the regulatory N-domain of the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex, containing the phosphomimetic of cTnI, reconstituted into thin filaments at a rate of 310 ± 5, 237 ± 8, or 280 ± 6 $s^{-1}$, respectively. Thus, replacement of cTnI by the phosphomimetic of cTnI led to ∼3.3-, 5.6-, or 2.9-fold acceleration of the rate of dissociation of $Ca^{2+}$ from thin filaments reconstituted with the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex, respectively. These results indicate that the A8V mutation enhanced the extent of acceleration of the rate of dissociation of $Ca^{2+}$ from reconstituted thin filaments upon cTnI pseudophosphorylation. On the other hand, the E134D mutation had only a minor effect on the extent of acceleration of the $Ca^{2+}$ dissociation rate associated with pseudophosphorylation of cTnI by PKA. These results also show that after cTnI was replaced by the phosphomimetic of cTnI, the A8V mutation led to an ∼1.3-fold slower rate of dissociation of $Ca^{2+}$ from the N-domain site of cTnC reconstituted into thin filaments. In addition, the E134D mutation exerted a statistically significant, albeit minor, effect on the rate of dissociation of $Ca^{2+}$ from reconstituted thin filaments in the presence of cTnI pseudophosphorylation.

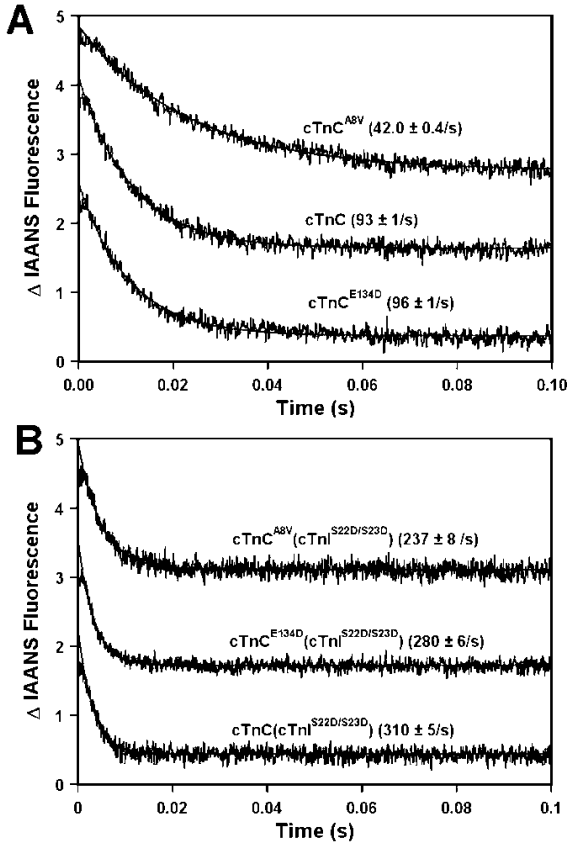


**Figure 3.** Effect of the A8V and E134D cTnC mutations on the rates of dissociation of $Ca^{2+}$ from reconstituted thin filaments in the absence and presence of cTnI pseudophosphorylation. (A) Time course of decreases in IAANS fluorescence as $Ca^{2+}$ was removed by excess EGTA from the regulatory N-domain of the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex reconstituted into thin filaments in the absence of cTnI pseudophosphorylation. (B) Time course of decreases in IAANS fluorescence as $Ca^{2+}$ was removed by excess EGTA from the regulatory N-domain of the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex, containing the phosphomimetic of cTnI, reconstituted into thin filaments. The data traces have been normalized and staggered for the sake of clarity. Each trace is an average of at least five traces fit with a single-exponential equation. The IAANS fluorescence was excited at 330 nm and monitored through a 510 nm bandpass filter.

**Effect of the A8V and E134D cTnC Mutations on the $Ca^{2+}$ Sensitivities of Actomyosin ATPase in the Absence and Presence of cTnI Pseudophosphorylation.** To examine the functional effect of the A8V and E134D mutations, the $Ca^{2+}$ dependence of actomyosin ATPase activity was measured after reconstitution of thin filaments with the cTn, $cTn^{A8V}$, or $cTnC^{E13D}$ complex. CTnC proteins used for the


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**Table 2. Effect of cTnC Mutations on the Properties of Actomyosin ATPase in the Absence and Presence of cTnI Pseudophosphorylation**

| protein | activity at pCa 9.0 [mol of $P_i$ $s^{-1}$ (mol of S1)$^{-1}$] | activity at pCa 4.0 [mol of $P_i$ $s^{-1}$ (mol of S1)$^{-1}$] | $pCa_{50}$ | $n_H$ |
|---|---|---|---|---|
| cTnC | 0.0125 ± 0.0003 | 0.0601 ± 0.0003 | 5.83 ± 0.01 | 2.0 ± 0.1 |
| $cTnC^{A8V}$ | 0.010 ± 0.0003[a] | 0.0742 ± 0.0002[a] | 6.074 ± 0.006[a] | 1.6 ± 0.1 |
| $cTnC^{E134D}$ | 0.0088 ± 0.0001[a] | 0.0553 ± 0.001[a] | 5.82 ± 0.02 | 1.98 ± 0.04 |
| cTnC and $cTnI^{S22D/S23D}$ | 0.0097 ± 0.0002 | 0.0451 ± 0.0001 | 5.60 ± 0.02 | 1.5 ± 0.1 |
| $cTnC^{A8V}$ and $cTnI^{S22D/S23D}$ | 0.0095 ± 0.0003 | 0.0599 ± 0.0003[b] | 5.70 ± 0.01[b] | 1.24 ± 0.08 |
| $cTnC^{E134D}$ and $cTnI^{S22D/S23D}$ | 0.0093 ± 0.0002 | 0.0438 ± 0.0001[b] | 5.57 ± 0.04 | 1.42 ± 0.04 |

[a]Significantly different from their respective cTnC values ($p < 0.05$). [b]Significantly different from their respective cTnC and $cTnI^{S22D/S23D}$ values ($p < 0.05$).

actomyosin ATPase assay were unlabeled and did not carry the C35S, T53C, and C84S mutations. First, we evaluated the effect of the A8V and E134D mutations on the specific activities of actomyosin ATPase at pCa 9.0 and 4.0. The results are summarized in Table 2 and are shown in Figure 4A. Our results demonstrate that at pCa 9.0, neither the A8V nor the E134D mutation impaired the ability of the cTn complex to inhibit actomyosin ATPase, regardless of the cTnI phosphorylation status. Our results also show that in the presence of a saturating level of $Ca^{2+}$ (pCa 4.0), the A8V mutation substantially enhanced the ability of the cTn complex to activate actomyosin ATPase, regardless of the cTnI phosphorylation status. In addition, the E134D mutation led to a significant, albeit minor, weakening of the ability of the cTn complex to activate actomyosin ATPase in the presence of a saturating level of $Ca^{2+}$, regardless of the cTnI phosphorylation status.

For thin filaments reconstituted with the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex, half-maximal $Ca^{2+}$ activation occurred with a $pCa_{50}$ of 5.83 ± 0.01, 6.074 ± 0.006, or 5.82 ± 0.02, respectively (Figure 4B and Table 2). These results indicate that the A8V mutation led to a statistically significant increase in the $Ca^{2+}$sensitivity of actomyosin ATPase, while the E134D mutation had no significant effect on the $Ca^{2+}$ sensitivity of actomyosin ATPase. The $n_H$ values for actomyosin ATPase curves were not significantly affected by either the A8V or E134D mutation (Table 2). For thin filaments reconstituted with the cTn, $cTn^{A8V}$, or $cTn^{E134D}$ complex, containing the phosphomimetic of cTnI, the $Ca^{2+}$ half-maximal activation occurred with a $pCa_{50}$ of 5.60 ± 0.02, 5.70 ± 0.01, or 5.57 ± 0.04, respectively (Figure 4B). Thus, the actomysoin ATPase activity of thin filaments reconstituted with the cTn complex underwent a substantial $Ca^{2+}$ desensitization upon cTnI pseudophosphorylation ($\Delta pCa_{50} = -0.23 \pm 0.02$). The A8V mutation significantly enhanced the extent of $Ca^{2+}$ desensitization induced by cTnI pseudophosphorylation ($\Delta pCa_{50} = -0.38 \pm 0.01$), while the E134D mutation did not significantly affect the extent of $Ca^{2+}$ desensitization ($\Delta pCa_{50} = -0.25 \pm 0.04$) (Figure 4C). In the presence of cTnI pseudophosphorylation, the $n_H$ values for actomyosin ATPase curves were not significantly affected by either the A8V or the E134D mutation (Table 2). Our results show that after cTnI was replaced by the phosphomimetic of cTnI, the A8V mutation still resulted in a statistically significant increase in the $Ca^{2+}$ sensitivity of actomyosin ATPase, while the E134D mutation had no significant effect on the $Ca^{2+}$ sensitivity of actomyosin ATPase. Thus, our results indicate that the A8V mutation significantly increased the $Ca^{2+}$ sensitivity of actomyosin ATPase regardless of the cTnI phosphorylation status. On the other hand, the E134D mutation exerted no significant effect on the $Ca^{2+}$ sensitivity of actomyosin ATPase, regardless of the cTnI phosphorylation status. In addition, our results show that neither the A8V nor the E134D cTnC mutation significantly affected the cooperativity of $Ca^{2+}$ activation of actomoysin ATPase, regardless of the cTnI phosphorylation status. Furthermore, our results show that the A8V mutation enhanced the extent of $Ca^{2+}$ desensitization of actomyosin ATPase induced by cTnI pseudophosphorylation, while the E134D mutation did not affect the extent of $Ca^{2+}$ desensitization.

## DISCUSSION

Recently, several mutations of cTnC, including A8V and E134D, were linked to HCM.[18] Compared to those of wild-type cTnC, the A8V mutation led to a higher force recovery and increased $Ca^{2+}$ sensitivity of force development in skinned fibers.[18] The E134D mutation did not affect either the extent of force recovery or the $Ca^{2+}$ sensitivity of force generation[18] and was hypothesized to be a polymorphism.[5] The main objective of this study was to examine whether HCM-linked A8V and E134D cTnC mutations affect the response of reconstituted thin filaments to $Ca^{2+}$ upon phosphorylation of cTnI by PKA. To mimic phosphorylation of cTnI by PKA, we substituted $Ser^{22}$ and $Ser^{23}$ residues of cTnI with Asp. It is important to note that substitutions of Ser with Asp do not always recapitulate the effects of phosphorylation on the properties of the protein. Thus, use of pseudophosphorylation to elucidate the effects of phosphorylation should be carefully considered. However, a number of studies demonstrated that the effects of cTnI pseudophosphorylation on the properties of cTnI were similar to the effect of phosphorylation. For example, NMR analysis of cTnC−cTnI complexes demonstrated that pseudo-phosphorylated cTnI provided a good structural mimetic for cTnI phosphorylated by PKA.[24] In addition, the effects of the S22D/S23D cTnI double mutation on the $Ca^{2+}$ sensitivity of reconstituted thin filaments and on the $Ca^{2+}$ sensitivity of myofibrillar ATPase were shown to be very similar to that of actual PKA phosphorylation.[25,26] Thus, we are confident that pseudophosphorylated cTnI is able to mimic the properties of phosphorylated cTnI.

The A8V mutation is located within the 14-residue N-helix of cTnC, a region known to modulate $Ca^{2+}$ binding and exchange with the regulatory N-domain site.[27,28] Consistent with previous studies,[29,30] the A8V mutation increased the $Ca^{2+}$ sensitivity of reconstituted thin filaments by slowing the rate of $Ca^{2+}$ dissociation. The slower rate of dissociation of $Ca^{2+}$ from reconstituted thin filaments could potentially result in diastolic dysfunction, a hallmark of HCM. In addition, in the absence of cTnI pseudophosphorylation, the A8V mutation led to a slight decrease in the cooperativity of binding of $Ca^{2+}$ to reconstituted

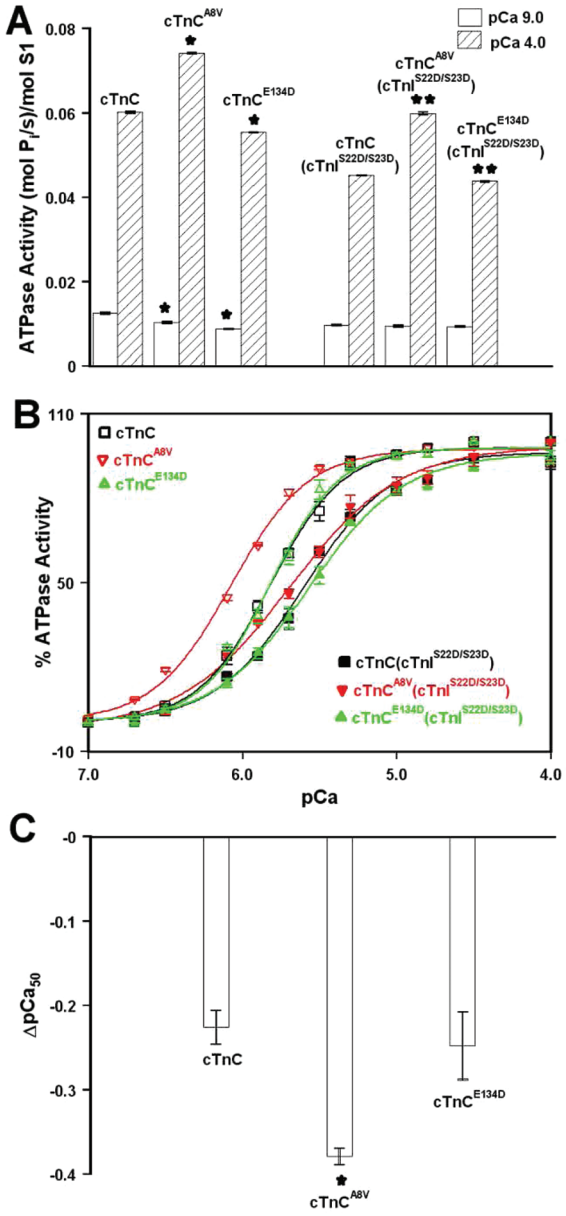


**Figure 4.** Effect of the A8V and E134D cTnC mutations on the $Ca^{2+}$ sensitivity of actomyosin ATPase in the absence and presence of cTnI pseudophosphorylation. (A) Specific actomyosin ATPase activities of thin filaments reconstituted with the cTn, cTn$^{A8V}$, or cTn$^{E134D}$ complex at pCa 9.0 (empty bars) or pCa 4.0 (hatched bars) in the absence and presence (indicated by cTnI$^{S22D/S23D}$) of cTnI pseudophosphorylation. Each data point represents a mean ± SE of three separate experiments (each conducted in triplicate). Values marked with one asterisk are significantly different from their respective cTnC values ($p < 0.05$). Values marked with two asterisks are significantly different from their respective cTnC and cTnI$^{S22D/S23D}$ values ($p < 0.05$). (B) $Ca^{2+}$-dependent activity of actomyosin ATPase in the absence of cTnI pseudophosphorylation for thin filaments reconstituted with the cTn (□), cTn$^{A8V}$ (red ▽), or cTn$^{E134D}$ (green △) complex as a function of pCa. Panel B also shows the $Ca^{2+}$-dependent activity of actomyosin ATPase in the presence of cTnI pseudophosphorylation for thin filaments reconstituted with the cTn (■), cTn$^{A8V}$ (red ▼), or cTn$^{E134D}$ (green ▲) complex as a function of pCa. Each data point represents a mean ± SE of at least three separate experiments (each conducted in triplicate). Data sets were individually normalized for each mutant cTn complex and fit with a logistic sigmoid. The experimental conditions were as described in Experimental Procedures. (C) $\Delta pCa_{50}$ for the $Ca^{2+}$ dependence of actomyosin ATP activity of thin filaments reconstituted with the cTn complexes [mutant cTn $pCa_{50}$ (with cTnI$^{S22D/S23D}$) − mutant cTn $pCa_{50}$ (without cTnI$^{S22D/S23D}$)]. The value marked with one asterisk is significantly different from its control value ($p < 0.05$).

thin filaments, as indicated by an altered slope of the IAANS fluorescence−pCa relationship. It is possible that the A8V mutation alters interactions between cTnC and other subunits of the cTn complex, ultimately affecting near-neighbor regulatory unit interactions along the thin filament.

Our results indicate that the A8V mutation did not significantly affect the extent of the decrease in the $Ca^{2+}$ sensitivity of the cTn complex reconstituted into thin filaments, associated with pseudophosphorylation of cTnI by PKA. Furthermore, the A8V mutation did not abolish the acceleration of the rate of dissociation of $Ca^{2+}$ from the regulatory N-domain site of the cTn complex reconstituted into thin filaments, associated with pseudophosphorylation of cTnI by PKA. In fact, the rate of dissociation of $Ca^{2+}$ from the cTn$^{A8V}$ complex reconstituted into thin filaments was accelerated by a greater extent upon cTnI pseudophosphorylation.

To examine the functional effect of the A8V mutation on the response of reconstituted thin filaments to $Ca^{2+}$ induced by cTnI pseudophosphorylation, we measured the $Ca^{2+}$ dependence of the actomyosin ATPase. Consistent with the effect of the A8V mutation on the $Ca^{2+}$ sensitivity of reconstituted thin filaments and with a previous report,[29] the A8V mutation sensitized actomyosin ATPase to $Ca^{2+}$. In addition to its $Ca^{2+}$ sensitizing effect, the A8V mutation led to a substantial improvement in the ability of cTnC to activate actomyosin ATPase in the presence of a saturating level of $Ca^{2+}$, regardless of the cTnI phosphorylation status. However, the A8V mutation did not blunt the extent of $Ca^{2+}$ desensitization of actomyosin ATPase induced by cTnI pseudophosphorylation. On the contrary, the $Ca^{2+}$ sensitizing effect of the A8V mutation on actomyosin ATPase was diminished upon cTnI pseudophosphorylation because of a larger decrease in the $Ca^{2+}$ sensitivity caused by cTnI pseudophosphorylation.

Numerous studies demonstrated that abnormal response of myofilaments to $Ca^{2+}$ can lead to severe pathophysiological consequences (for reviews, see refs 5 and 31). Available experimental evidence shows that correcting the abnormal $Ca^{2+}$ sensitivity can rescue hypertrophic and restrictive phenotypes in transgenic mouse models.[32,33] Because the A8V mutation did not abolish the response of reconstituted thin filaments to $Ca^{2+}$ upon cTnI pseudophosphorylation, drugs that mimic the effects of cTnI phosphorylation could be potentially designed to correct the pathophysiological consequences caused by the $Ca^{2+}$ sensitizing effect of the A8V and perhaps other cardiomyopathy-linked mutations. Alternatively, regulatory proteins that desensitize reconstituted thin filaments to $Ca^{2+}$, such as the N-terminally truncated cTnI,[32,34−36] might themselves be used as therapeutic tools to treat hypertrophy and diastolic dysfunction associated with the $Ca^{2+}$ sensitizing HCM-linked mutations.

We also examined the effect of the E134D mutation on the response of reconstituted thin filaments to $Ca^{2+}$ upon pseudophosphorylation of cTnI by PKA. Earlier studies demonstrated that the E134D mutation did not affect the $Ca^{2+}$ binding properties of reconstituted thin filaments.[18,29,30]


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However, the E134D mutation could have led to alterations in the $Ca^{2+}$ binding properties of cTnC reconstituted into thin filaments when cTnI was phosphorylated, as was previously observed with several cardiomyopathy-linked cTnC mutations.[15−17] Thus, we decided to determine whether the E134D mutation altered the $Ca^{2+}$ binding properties of reconstituted thin filaments upon pseudophosphorylation of cTnI by PKA. Our results indicate that in the absence of pseudophosphorylation, the E134D mutation exerted no effect on the $Ca^{2+}$ sensitivity or the rate of dissociation of $Ca^{2+}$ from reconstituted thin filaments. However, in the presence of cTnI pseudophosphorylation, the E134D mutation led to a statistically significant, albeit minor, decrease in the rate of dissociation of $Ca^{2+}$ from reconstituted thin filaments. In addition, the E134D mutation had no significant effect on the $Ca^{2+}$ sensitivity of actomyosin ATPase, regardless of the cTnI phosphorylation status. However, the E134D mutation led to a statistically significant, albeit minor, decrease in the ability of the cTn complex to activate actomyosin ATPase in the presence of a saturating level of $Ca^{2+}$, regardless of the cTnI phosphorylation status. Because the effects of the E134D mutation on the $Ca^{2+}$ binding properties of reconstituted thin filaments were rather modest, these results are consistent with the idea that the E134D mutation is a rare polymorphism. However, the possibility remains that the E134D mutation exerts its effect not via changes in $Ca^{2+}$ sensitivity but through a different yet unknown mechanism. These questions can be answered by developing an animal model bearing the E134D mutation.

In summary, we examined the effect of HCM-linked A8V and E134D cTnC mutations on the response of reconstituted thin filaments to $Ca^{2+}$ upon pseudophosphorylation of cTnI by PKA. Our results show that neither the A8V nor the E134D mutation significantly affected the extent of $Ca^{2+}$ desensitization of reconstituted thin filaments induced by cTnI pseudophosphorylation. In fact, the A8V mutation enhanced the effect of cTnI pseudophosphorylation on the rate of dissociation of calcium from reconstituted thin filaments. Consequently, while the A8V mutation still led to a slower rate of dissociation of $Ca^{2+}$ from reconstituted thin filaments upon cTnI pseudophosphorylation, the ability of the A8V mutation to decrease the rate of $Ca^{2+}$ dissociation was diminished. In addition, the ability of the A8V mutation to sensitize actomyosin ATPase to $Ca^{2+}$ was diminished after cTnI was replaced by the phosphomimetic of cTnI. Consistent with the hypothesis that the E134D mutation is a rare polymorphism, it exerted a minor to no effect on the $Ca^{2+}$ binding properties of cTnC reconstituted into thin filaments or on the $Ca^{2+}$ dependence of actomyosin ATPase, regardless of the cTnI phosphorylation status. In conclusion, this study enhances our understanding of how cardiomyopathy-linked cTnC mutations affect the response of reconstituted thin filaments to $Ca^{2+}$ upon cTnI phosphorylation.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; cTnC, cardiac troponin C; $cTnC^{A8V}$, cTnC with the A8V mutation; $cTnC^{E134D}$, cTnC with the E134D mutation; cTnI, cardiac troponin I; phosphomimetic of cTnI, cTnI with the S22D/S23D double mutation; cTnT, cardiac troponin T; cTn, cardiac troponin complex (cTnC−cTnI−cTnT); $cTn^{A8V}$, cTn complex containing $cTnC^{A8V}$ ($cTnC^{A8V}$−cTnI−cTnT); $cTn^{E134D}$, cTn complex containing $cTnC^{E134D}$ ($cTnC^{E134D}$−cTnI−cTnT); IAANS, 2-[4′-(iodoacetamido)anilino]-naphthalene-6-sulfonic acid; EGTA, ethylene glycol bis(2-amino ethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $K_d$, dissociation constant; $k_{off}$, dissociation rate.

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