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Differential pH Effect on Calcium-Induced Conformational Changes of Cardiac Troponin C Complexed with Cardiac and Fast Skeletal Isoforms of Troponin I and Troponin T

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The aim of this study is to investigate the molecular events associated with the deleterious effects of acidosis on the contractile properties of cardiac muscle as in the ischemia of heart failure. We have conducted a study of the effects of increasing acidity on the Ca^{2+} induced conformational changes of pyrene labelled cardiac troponin C (PIA-cTnC) in isolation and in complex with porcine cardiac or chicken pectoral skeletal muscle TnI and/or TnT. The pyrene label has been shown to serve as a useful fluorescence reporter group for conformational and interaction events of the N-terminal regulatory domain of TnC with only minimal fluorescence changes associated with C-terminal domain. Results obtained show that the significant decreases at pH 6.0 of site II Ca^{2+} affinity of PIA-cTnC when complexed as a binary complex with either cTnI or cTnT are significantly reduced when cTnI is replaced with sTnI or cTnT with sTnT. However, this effect is appreciably diminished when the cTnI and cTnT in the ternary complex are replaced by sTnI and sTnT. The smaller effects in the ternary complex of replacing both cTnI and cTnT by their skeletal counterparts on depressing the Ca^{2+} affinity from pH 7.0 to 6.0 arise from TnI replacement. Thus, changes in TnC conformation resulting from isoform-specific interactions with TnI and TnT could be an integral part of the effect of pH on myofilament Ca^{2+}sensitivity.

Key words: acidosis, Ca^{2+} sensitivity, fluorescence, ischemia, pyrene label.

Protons have a marked depressant effect on myofilament Ca^{2+} sensitivity in vertebrate striated muscle, depending on muscle type in the order of cardiac>fast skeletal>slow skeletal (*1–3*). It has been suggested that the muscle-type–dependent effect of acidic pH on the Ca^{2+} sensitivity is a function of differential isoform expression of troponin subunits (*4–6*). Troponin (Tn) is composed of three subunits: troponin C (TnC), a Ca^{2+}-binding subunit; troponin I (TnI), an inhibitory subunit; and troponin T (TnT), a subunit which binds the complex to tropomyosin (TM) (*7*).

Two TnC isoforms are expressed in fast skeletal (sTnC) and slow skeletal/cardiac muscles (cTnC). Both sTnC and cTnC contain four putative Ca^{2+}-binding sites. Due to some amino acid substitutions (D29L, D31A, and V28 insertion) at Ca^{2+}-binding loop I (*8*), cTnC contains three functional Ca^{2+}-binding sites. The two C-terminal sites (III and IV) bind Ca^{2+} with high affinity ($K \sim 10^7\ M^{-1}$) and also Mg^{2+} ($K \sim 10^3\ M^{-1}$), while the other N-terminal site II binds Ca^{2+} exclusively but with lower affinity ($K \sim 10^5\ M^{-1}$). A study of the ectopic expression of the sTnC in the heart of transgenic mice has shown that TnC is a determinant of the differential pH sensitivity of fast skeletal and cardiac muscles (*9*).

There are three different TnI isoforms in fast skeletal (sTnI), slow skeletal (ssTnI), and cardiac muscles (cTnI). Studies with the exchange of the muscle type-specific TnI isoforms in the fast skeletal and cardiac muscle showed that the Ca^{2+} binding affinity of TnC and the effect of acidic pH on myofilament Ca^{2+} sensitivity are significantly modified by the TnI isoform in either muscle (*10–12*). More recent evidence has confirmed the role of TnI isoforms in the determination of Ca^{2+} sensitivity and has localized this to a single amino acid substitution in the C-terminal region distal to the inhibitory segment (*13–15*).

There are multiple muscle-type-specific TnT isoforms encoded by three homologous genes. Each gene undergoes alternative splicing during development, leading to the expression of a variety of TnT isoforms in the different muscles (*16–19*). Among multiple TnT isoforms, the C-terminal and central regions of TnT are largely conserved, but the N-terminal domain is highly variable (*20–21*). Thus far no information is available on whether the muscle type-specific TnT isoforms could contribute to the differential effect of acidic pH on myofilament Ca^{2+} sensitivity in either muscle.

Tn complex is composed of a long tail TnT1 (N-terminus of TnT) and a globular core domain including TnT C-terminus, TnI, and TnC (*22–23*). More recently, Taketa *et al.* (*24*) have resolved this core structure of human cardiac Tn complex (a TnT1-deleted $TnT_{203–271}$, $TnC_{1–161,}$ and $TnI_{31–163}$ /$TnI_{31–210}$) at 2.6 and at 3.3 Å resolution. Based on this crystal structure, the Tn complex contains several functional domains: a regulatory head ($TnC_{N\text{-}end}$ and $TnI_{94–115,IR}$); an IT arm ($TnC_{C\text{-}end}$; $TnI_{1–94,H1+H2}$; $TnT_{205–255}$); a $TnT1_{1–181}$ tail; C-$TnT_{270–288}$; and $TnIreg_{\ 117–183}$. Conformational changes of the regulatory head induced by Ca^{2+} binding to TnC are transmitted through IT arm to TM-actin, thereby facilitating interactions between

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actin and myosin. It appears that the N-terminus of TnT is responsible for anchoring the Tn complex to Tm independently of the Ca^{2+} concentration, whereas the C-terminal half forms a complex with TnC, TnI, and Tm. However, it has been shown that structural differences in the N-terminal region of TnT can contribute to the modulation of the global conformation of TnT (*25*) and the affinity of TnI, TnC, and Tm for TnT (*26*), and the tolerance of the thin filament regulatory system to acidosis (*27*). In addition, a recent study with transgenic mouse hearts expressing human TnT mutant (R92Q) also showed a decreased sensitivity to acidic pH of myofilament Ca^{2+} activation in comparison with the wild type (*6*). This mutation is in the tail region of troponin, which is not known to involve interactions between TnT and the other Tn components. Thus, it will be of interest to examine further if varying the N-terminal domain of TnT isoform as cTnT versus sTnT would confer the differential pH effect on Ca^{2+}-binding affinity to TnC.

In this study, we investigate the effects of varying pH from 8.0 to 7.0 to 6.0 on the Ca^{2+}-induced conformational changes of pyrene-labeled cTnC in isolation and in complex with porcine cardiac or chicken pectoral muscle TnI and/or TnT. The N-terminal domain of cTnC includes two cysteines (Cys-35 and Cys-84) (*28*), thus allowing for the attachment of conformational probes to this region. Here, we labeled cTnC with a sulfhydryl-reactive pyrene-containing fluorophore [*N*-(1-pyrene)-iodoacetamide; PIA]. This compound was shown to be suitable for the study of proximity relations between labeled cysteine residues and to cause minimal disturbance to protein conformation (*29–31*). The fluorescence spectrum of PIA-cTnC exhibited peaks characteristic of pyrene in its monomeric form (monomer) and an additional red-shifted peak due to the formation of an excited dimmer (excimer). A change in monomer fluorescence reports environmental changes to the sites of attached fluorescent compounds; whereas, a change in excimer fluorescence indicates a separation of the two pyrene-labeled cysteine residues. Consistent with previously published observations from several laboratories (*10–15*), the data reported in this study indicated that the replacement of cTnI by sTnI in both the binary and ternary complexes has a blunting effect on the acidic depression (pH 7.0 to 6.0) of Ca^{2+} affinity to PIA-cTnC. In addition, we found that the depressant effect of replacing cTnT with sTnT in the binary complex on the acidic decrease of Ca^{2+} affinity. Together, our results suggest that the conformational change induced by Ca^{2+} through isoform-specific interactions among troponin subunits is important in the differential response of skeletal and cardiac myofilament to acidosis.

MATERIALS AND METHODS

Reagents—Unless otherwise specified, all reagents used were ACS grade. *N*-(1-pyrene)iodoacetamide (PIA) was purchased from Molecular Probes. Chromatographic reagents (DEAE-sephadex A-50 and CM-sephadex C-50) were purchased from Amersham Pharmacia Biotech Asia Pacific. Bicinchoninic acid (BCA) protein assay reagent was from Pierce Chemicals.

Protein Preparation and Modification—Troponin subunits were isolated from an ether powder prepared from chicken pectoral muscle and porcine ventricular muscle according to the method of Potter (*32*). CTnC was also prepared from the left ventricle of porcine hearts according to Szynkiewicz *et al.* (*33*). Purified proteins were stored freeze-dried in a freezer (–80°C).

Based on published data, we confirmed the sequences of purified sTnC (*34*), sTnI (*35*), and sTnT (*27*) by means of mRNA extraction from chicken pectoral muscle, followed by RT-PCR, gene cloning, and cDNA alignment. Two sTnT isoforms of different sizes were found in chicken pectoral muscle. The larger isoform contains 287 amino acids (~33.8 kDa) while the smaller TnT contains 272 amino acids (~32.0 kDa). These two isoforms have conserved sequences in the C-terminal and central regions. The larger sTnT contains seven repeated His pairs ($[H\text{-}(A/E)\text{-}E\text{-}A\text{-}H]_n$) in the hypervariable region of the N-terminal domain, while the smaller isoform has four repeated His pairs. Based on the molecular weight of the purified sTnT, we assumed that the larger sTnT was the major sTnT isoform used in this study. For porcine cardiac Tn components, cTnC (*28*) is the only component with known sequence.

Before use, the freeze-dried cTnC was dissolved in a solution containing 8 M urea, 3 mM dithiothreitol (DTT), 100 mM MOPS, pH 7.0, 2 mM EGTA, and 50 mM KCl to reduce the two thiol groups at room temperature for 4 h. Following exhaustive dialysis against the same buffer at 4°C, the reduced cTnC was reacted at room temperature for four hours with a 2- to 3-fold molar excess of PIA, following the method of Liou and Fuchs (*30*). The reaction was terminated with excess DTT. The latter was removed by solvent exchange with Centricon 10 ultrafiltration cells (Amicon). The proteins were then dialyzed against 6 M urea, 25 mM MOPS, pH 7.0, and 0.1 M KCl at 4°C. Dialysis against the same buffer without urea was repeated twice. The protein was then dialyzed against 10 mM MOPS, pH 7.0, and 0.1 M KCl. The amount of bound pyrene was determined on the basis of an extinction coefficient at 344 nm of 28,000 M^{-1} cm^{-1} for PIA-cTnC (*30*). Concentrations of labeled proteins were measured with the BCA protein assay reagent (*27*). The labeling ratio of pyrene to cTnC was 1.6–1.9 mol fluorophore per mole protein.

Reconstitution of the binary or ternary troponin complexes was performed by mixing equimolar amounts of each subunit in denaturing solution containing 6 M urea, 1 M KCl, 50 mM MOPS, pH 7.0, 5 mM $CaCl_2$, and 5 mM DTT, followed by renaturation by dialysis to remove urea and to lower the salt concentration. The reconstituted protein complex in a buffer solution containing 10 mM MOPS, pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, and 0.25 M KCl was stored at –80°C before use.

ATPase Activity Assay—The biological activities of the labelled proteins were assayed by determining Ca^{2+}-regulated myofibrillar ATPase activity. Porcine cardiac myofibrils were prepared at 4°C according to Liou *et al.* (*31*). The endogenous cTnC of porcine cardiac myofibrils was extracted with 40 mM Tris buffer, pH 8.4, containing 5 mM CDTA (*trans*-1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid), and the degree of extraction was deter-

mined by SDS-PAGE and by loss of Ca^{2+}-activated ATPase activity, as described previously (*30*).

Unlabeled or PIA-cTnC was incubated with CDTA-extracted myofibrils in a ratio of 7 μg cTnC per mg myofibrillar protein. The myofibrils were suspended for 1 h at 25°C in 100 mM MOPS, pH 7.0, 90 mM KCl, 5 mM $MgCl_2$, 0.1 mM free Ca^{2+}, then centrifuged at 2,000 × *g* to remove free cTnC. The pellet was resuspended in the same buffer and centrifugation repeated twice more.

The actomyosin ATPase activity was measured by suspending the intact, extracted, and reconstituted myofibrils (0.4 mg/ml) in 100 mM MOPS, pH 7.0, 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and various additions as indicated. The reaction mixtures were shaken in a water bath at controlled temperature (30°C) for 10 min. The reaction was initiated by the addition of MgATP to a concentration of 1 mM and terminated after 10 min by the addition of malachite green reagent (33% malachite green, 16.7% polyvinyl alcohol, and 16.7% ammonium molybdate). The mixture was then analysed for inorganic phosphate release (*31*).

Fluorescence Measurements—Corrected fluorescence spectra for intrinsic tyrosine fluorescence and pyrene fluorescence were recorded with a Perkin-Elmer LS 50 B spectrofluorimeter (Beaconsfield, Buckinghamshire, England) at constant temperature of 25°C.

Tyrosine fluorescence of isolated cTnC was elicited by excitation at 276 nm and an emission spectrum was scanned from 295 to 320 nm with slit widths of 3 and 10 nm for excitation and emission. Purified cTnC was dissolved at a concentration of 1 μM in different pH buffers (100 mM MOPS, pH 7.0; 100 mM Tris-OH, pH 8.0; and 100 mM MES, pH 6.0) containing 0.1 mM KCl, and 2 mM EGTA, with Ca^{2+} additions as indicated.

For measurements of pyrene fluorescence spectra, 1–2 μM PIA-cTnC, either alone or complexed with other troponin subunits, was dissolved in the same pH buffers as described above, except that 5 mM $MgCl_2$ was included. The solution was excited at 344 nm, and the fluorescence emission was scanned from 370 to 570 nm, with slit widths of 3 and 10 nm for excitation and emission.

Ca^{2+} Titration—Both cTnC and PIA-cTnC were placed in a semi-micro cell (1 ml) and Ca^{2+} titrations were carried out by adding with a digital micropipette aliquots of a commercial standard solution (Radiometer) of 0.1 M $CaCl_2$. Total volume change with Ca^{2+} addition did not exceed 2%. Tyrosine fluorescence emitted at 306 nm was measured as a function of pCa (–log[Ca^{2+}]). For pyrene fluorescence spectra, the emitted fluorescence at 385 nm and/or 485 nm was taken for data analysis on the Ca^{2+}-dependent fluorescence changes in PIA-cTnC in isolation and in complex with cardiac or skeletal muscle TnI and/or TnT.

The measured fluorescence at the actual pCa was subtracted from that at pCa 8. The subtracted fluorescence (F_x) was normalized to the value (F_0) at saturating pCa. If normalized fluorescence ($U = F_x/F_0$) is used, then a straight line is obtained with the expression of log [$U/(1 - U)$] versus the logarithm of the Ca^{2+} concentration. This plot was fitted data to the Hill equation:

$$\log[U/(1 - U)] = n(\log[Ca_x]) + \log k$$

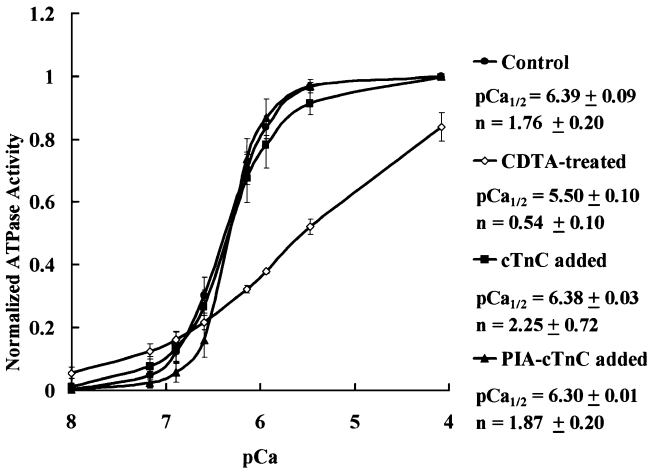


Fig. 1. **Ca^{2+}-regulated myofibrillar ATPase activity of CDTA-extracted, control, cTnC re-incorporated, and PIA-cTnC re-incorporated cardiac myofibrils.** The biological activities of PIA-cTnC were assayed by determining Ca^{2+}-regulated myofibrillar ATPase activity, as described in the Methods section. The actomyosin ATPase activity was measured by suspending the intact, CDTA-extracted, and reconstituted myofibrils (0.4 mg/ml) in 100 mM MOPS, pH 7.0, 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and various Ca^{2+} concentrations. The half-maximal Ca^{2+} concentrations ($pCa_{1/2}$), and Hill coefficient are as indicated. Each point is the mean ± SEM of five measurements.

where [Ca_x] is the actual Ca^{2+} concentration, n (Hill coefficient) is the slope, and k is the *x*-axis intercept of the fitted line. By using the constants derived from the Hill equation, the curves of the normalized fluorescence changes (F_x/F_0) versus pCa were fitted by computer with the equation:

$$(F_x/F_0) = [Ca_x]^n/((EC_{50})^n + [Ca_x]^n)$$

where EC_{50} is the Ca^{2+} concentration giving 50% activation of fluorescence changes.

Free Ca^{2+} concentrations in EGTA buffers were calculated on the basis of constants tabulated by Fabiato and Fabiato (*36*). The pCa values were calculated by the computer program EQCAL (Biosoft, Cambridge, UK).

Statistics—Quantitative values are expressed as mean ± SEM. Comparisons of statistics were performed by a Student's *t*-test where *P* values less than 0.05 were considered as being significant.

RESULTS

Biological Activity of PIA-cTnC—With the low ionic strength CDTA solutions, 60–90% of cTnC was removed from porcine cardiac myofibrils. This CDTA extraction significantly reduces the Ca^{2+}-dependent regulation of myofibrillar ATPase (Hill coefficient, *n*: 0.54 ± 0.10; $pCa_{1/2}$: 5.5 ± 0.1), as shown in Fig. 1. Re-insertion of either the native (*n*: 2.25 ± 0.72; $pCa_{1/2}$: 6.38 ± 0.03) or PIA-cTnC (*n*: 1.87 ± 0.20; $pCa_{1/2}$: 6.30 ± 0.01) restored the Ca^{2+}-activated ATPase activity as effectively as that in the control (*n*: 1.76 ± 0.20; $pCa_{1/2}$: 6.39 ± 0.09). It seems reasonable to assume that the conformational changes which promote

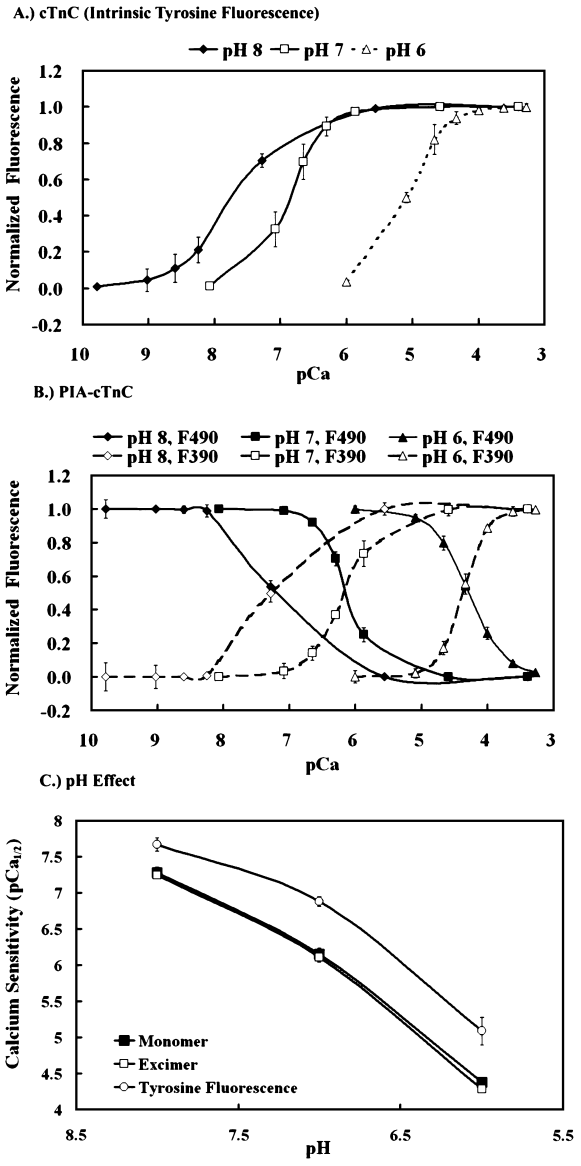


Fig. 2. **The effect of pH on the Ca^{2+}-induced changes in intrinsic tyrosine fluorescence of unlabeled cTnC and PIA-cTnC.** (A) Tyrosine fluorescence of unlabeled cTnC as a function of pCa (–log[Ca^{2+}]) determined at pH 8.0, 7.0, and 6.0. (B) Ca^{2+}-dependent changes in monomer and excimer fluorescence of PIA-cTnC at pH 8.0, 7.0, and 6.0. (C) pH-decreased Ca^{2+} sensitivity ($pCa_{1/2}$) for fluorescence changes in unlabeled cTnC and PIA-cTnC. Purified cTnC or PIA-cTnC was dissolved at a concentration of 1 μM in different pH buffers (100 mM MOPS, pH 7.0; 100 mM Tris-OH, pH 8.0; and 100 mM MES, pH 6.0) containing 0.1 mM KCl and 2 mM EGTA, with Ca^{2+} additions as indicated. Measurements of fluorescence spectra for unlabeled cTnC and for PIA-cTnC were described as in the "MATERIALS AND METHODS" section. Each point or histogram is the mean ± SEM of five measurements.

Ca^{2+} activation occur to the same extent in both native and labeled cTnC.

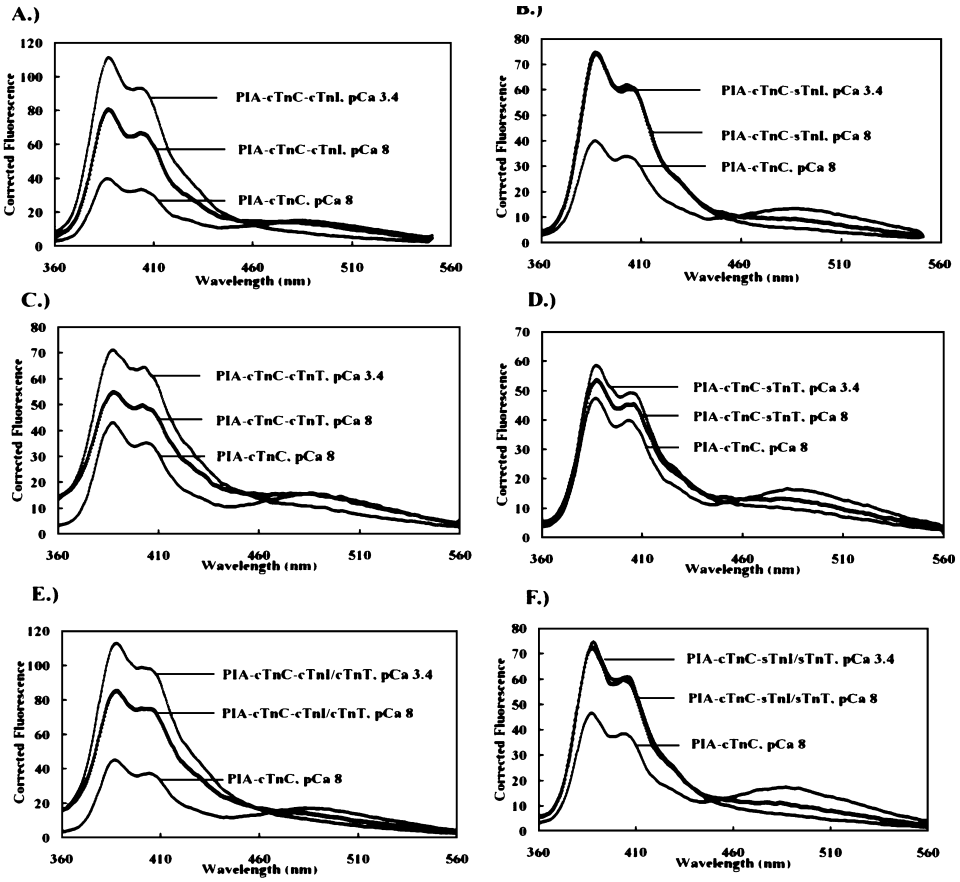
pH Effect on Ca^{2+}-Binding to Unlabeled cTnC and PIA-cTnC—To distinguish between the pH effects on Ca^{2+} binding to high-affinity and low-affinity sites, the Ca^{2+}-induced changes in intrinsic tyrosine fluorescence of cTnC and PIA-cTnC were determined at pH 8.0, 7.0, and 6.0. CTnC contains three tyrosine residues (Tyr-5, Tyr 111, and Tyr-150). It was shown that Ca^{2+} binding to the high-affinity sites (sites III and IV) caused a significant increase in tyrosine fluorescence, while binding at the low-affinity site (site II) caused no further change (*37*). In contrast, studies with pyrene labels at the two cysteines (Cys-35 and Cys-84) in the N-terminal domain of cTnC showed that Ca^{2+} binding to sites III and IV caused a small change in a pyrene fluorescence spectrum, while the binding to site II caused an increase in monomer fluorescence and a decrease in excimer fluorescence (*30*). Figure 2 shows that the Ca^{2+}-dependent curves for the fluorescence changes in cTnC and PIA-cTnC were shifted to the higher Ca^{2+} concentrations (lower pCa) as pH decreased from 8.0 to 6.0. The half-maximal Ca^{2+} concentrations ($pCa_{1/2}$) for these fluorescence changes are summarized in Table 1. These data indicate that protons can interfere with the Ca^{2+} binding to the C-terminal sites (III/IV) and the N-terminal site II of cTnC.

Table 1. **Effects of pH on the half-maximal Ca^{2+} concentrations ($pCa_{1/2}$) for fluorescence changes of purified cTnC or PIA-labeled cTnC in complex with cardiac or skeletal TnI and/or TnT.** Each value is the mean ± SEM of five measurements.

	pH 8.0	pH 7.0	pH 6.0
CTnC	7.67 ± 0.09	6.88 ± 0.07	5.09 ± 0.19
PIA*cTnC			
Monomer	7.28 ± 0.08	6.15 ± 0.09	4.38 ± 0.02
Excimer	7.25 ± 0.02	6.11 ± 0.07	4.29 ± 0.05
PIA*cTnC-cTnI	7.56 ± 0.02	6.50 ± 0.05	4.80 ± 0.02
PIA*cTnC-sTnI	7.44 ± 0.02	6.46 ± 0.01	5.50 ± 0.08
PIA*cTnC-cTnT	7.34 ± 0.01	6.16 ± 0.01	4.67 ± 0.07
PIA*cTnC-sTnT	7.37 ± 0.01	6.17 ± 0.01	5.26 ± 0.03
PIA*cTnC-cTnI-cTnT	7.57 ± 0.01	6.42 ± 0.01	4.58 ± 0.01
PIA*cTnC-sTnI-sTnT	7.54 ± 0.14	6.54 ± 0.01	4.98 ± 0.04
PIA*cTnC-sTnI-cTnT	–	6.52 ± 0.12	4.95 ± 0.05
PIA*cTnC-cTnI-sTnT	–	6.55 ± 0.05	4.70 ± 0.06

Differential Effects of Cardiac and Skeletal TnI and/or TnT, and Ca^{2+}-Binding—When PIA-cTnC was complexed with cTnI and/or cTnT, monomer fluorescence was increased but there was only a small decrease in excimer fluorescence (Fig. 3, A, C, and E). Replacing cTnI and/or cTnT by sTnI and/or sTnT in both binary and ternary complex also caused an increase in monomer fluorescence (Fig. 3, B, D, and F). However, a greater reduction in excimer fluorescence was found in the binary and ternary complex with sTnI and/or sTnT than with their cardiac counterparts. The quantitative analyses are summarized in Fig. 3G. This result suggests that binding of isoform-specific TnI and TnT to cTnC has differential effects on conformational changes in the N-terminal regulatory domain of cTnC.

In agreement with a previous study (*30*), Mg^{2+} or Ca^{2+} binding to the high affinity sites of cTnC had no significant effect on the PIA-cTnC alone or complexed with other troponin subunits. Some experiments performed in this study have 5 mM Mg^{2+} present in the buffer solution to block the Ca^{2+} binding to the C-terminal site of PIA-cTnC. In addition, the presence of 5 mM Mg^{2+} to occupy the high-affinity sites of cTnC will stabilize the complex formed with TnI and/or TnT. When PIA-cTnC formed a complex with cTnI and/or cTnT (Fig. 3, A, C, and E), Ca^{2+}



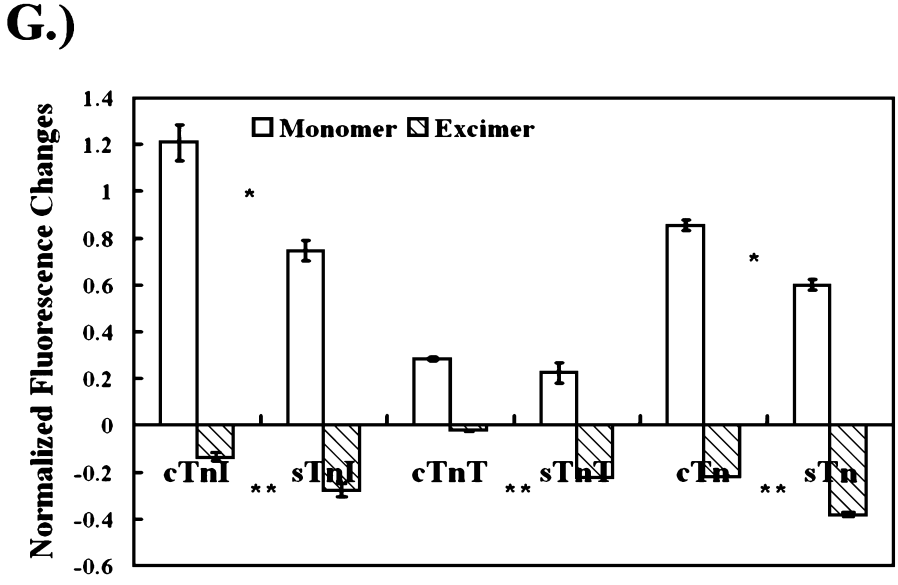


Fig. 3. **Differential effects of Ca^{2+}-induced changes in fluorescence spectra of PIA-cTnC in binary or ternary complexes with cardiac (A, C, E) or skeletal (B, D, F) TnI and/or TnT.** (G) Effect of complexation with cardiac or skeletal TnI and/or TnT on monomer and excimer fluorescence changes in PIA-labeled cTnC. PIA-cTnC (1 μM) alone or complexed with 1 μM TnI and/ or 1 μM TnT in a buffer solutions containing 100 mM MOPS, pH 7.0, 0.1 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, and varying pCa. Fluorescence changes were calculated by subtracting the fluorescence of monomer (at 385 nm) and excimer (at 485 nm) for the binary or ternary complex from each intensity for the PIA-cTnC alone, and then normalizing the difference of each to the value for the PIA-cTnC alone.

binding to the N-terminal specific site II of cTnC elicited an increase in monomer fluorescence and a decrease in excimer fluorescence. In contrast, the Ca^{2+}-decreased excimer fluorescence with no changes or a small increase in monomer fluorescence was found in the PIA-cTnC-sTnI (Fig. 3B), PIA-cTnC-sTnI-sTnT (Fig. 3F), and PIA-cTnC-sTnT (Fig. 3D) complexes. These results suggest that the isoform-specific protein-protein interactions among troponin components impose differential Ca^{2+} effects on conformational changes in the N-terminal regulatory domain of cTnC.

Ca^{2+}-Titration at pH 8.0, 7.0, and 6.0—Figure 4 shows the Ca^{2+}-induced changes in fluorescence spectra of PIA-cTnC in the binary complex with cTnT and sTnT at pH 8.0 (Fig. 4A), pH 7.0 (Fig. 4B), and pH 6.0 (Fig. 4C). At pH 8.0, the Ca^{2+}-dependent changes in fluorescence spectra

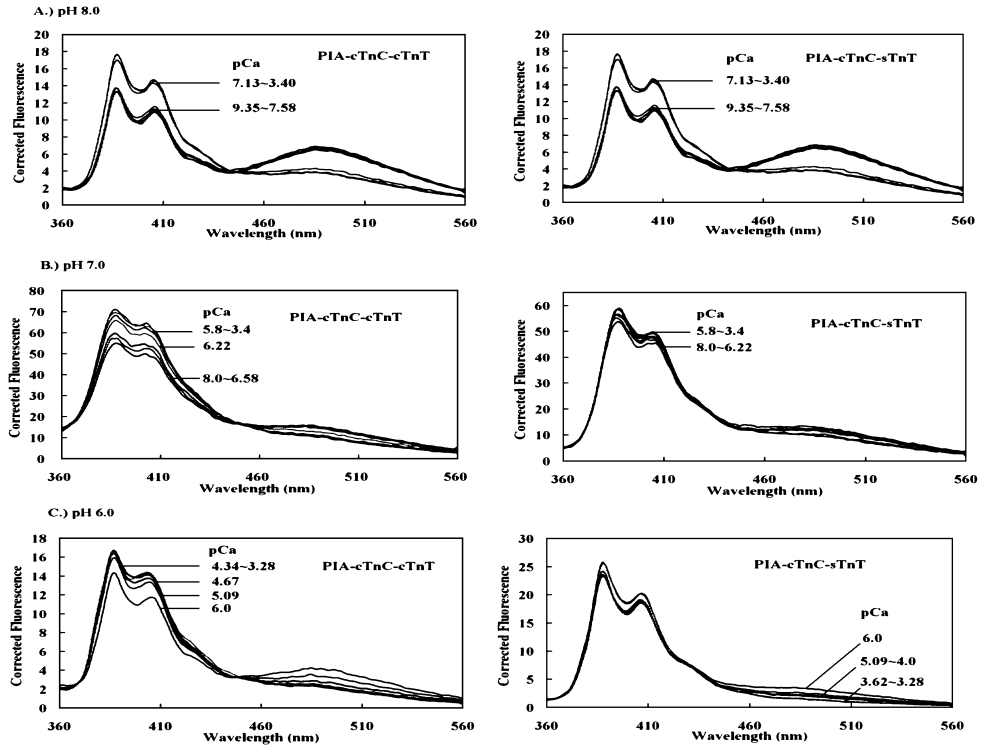


Fig. 4. **Ca^{2+}-titrated changes in fluorescence spectra of PIA-cTnC in the binary complex with cTnT (left) or sTnT (right) (A) at pH 8.0, (B) at pH 7.0, and (C) at pH 6.0.** PIA-cTnC (1 μM) complexed with 1 μM cTnT or 1 μM sTnT was dissolved in different pH buffers (100 mM MOPS, pH 7.0; 100 mM Tris-OH, pH 8.0; and 100 mM MES, pH 6.0) containing 0.1 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, and varying pCa as indicated.

are similar in both PIA-cTnC-cTnT (n: 2.17 ± 0.07; $pCa_{1/2}$: 7.34 ± 0.01) and PIA-cTnC-sTnT (n: 2.54 ± 0.07; $pCa_{1/2}$: 7.37 ± 0.01). However, at both pH 7.0 and pH 6.0, a greater cooperation of the Ca^{2+}-induced fluorescence changes was found in PIA-cTnC-cTnT (pH 7.0: n = 2.0 ± 0.2; pH 6.0: n = 1.48 ± 0.15) than in PIA-cTnC-sTnT (pH 7.0: n = 1.6 ± 0.1; pH 6.0: n = 1.00 ± 0.05). Although the $pCa_{1/2}$ values are not different between PIA-cTnC-cTnT (6.2 ± 0.1) and PIA-cTnC-sTnT (6.2 ± 0.2) at pH 7.0, a greater decrease in $pCa_{1/2}$ was observed for PIA-cTnC-cTnT (4.67 ± 0.07) than for PIA-cTnC-sTnT (5.26 ± 0.03) at pH 6.

Figures 5A and 6A show the effects of decreasing pH from 8.0 to 6.0 on the excimer fluorescence-pCa curve of PIA-cTnC in the binary complex with cardiac and skeletal TnT (Fig. 5A) or TnI (Fig. 6A). Replacement of cTnT and/or cTnI by sTnT (Fig. 5) and/or sTnI (Fig. 6) in varying from pH 8.0 to pH 7.0 has apparently little effect in altering the $pCa_{1/2}$. However, replacing cTnT with sTnT (Fig. 5) or cTnI with sTnI (Fig. 6) reduces the effect of pH decrease (7.0 to 6.0) on Ca^{2+} binding to PIA-cTnC.

When cTnI and cTnT are replaced with the skeletal isoforms, the effect of pH reduction (7.0 to 6.0) on Ca^{2+}-(PIA-cTnC) affinity is appreciably diminished (Fig. 7). The smaller effect of pH decrease on Ca^{2+} sensitivity is also seen when only cTnI or cTnT is replaced by the respective skeletal isoform. Replacing cTnT with sTnT in the ternary complex was without effect. In contrast, the replacement of cTnI with sTnI caused a greater attenuation of the pH response than replacement with both sTnI and sTnT. It seems that it is the TnI isoform which plays the dominant role in imparting pH sensitivity to the ternary complex. The data showing the differential effects of pH on the various binary and ternary complexes containing PIA-cTnC are summarized in Table 1.

DISCUSSION

Intracellular acidosis, with the resultant reduction in myofilament Ca^{2+} sensitivity, may be an important factor in the decreased contractility associated with myocardial ischemia (*38–40*). This depressed Ca^{2+} sensitivity may be ascribed to either a direct effect of pH on Ca^{2+}-cTnC affinity (*41*) or an indirect effect *via* the protein-protein interactions involved in the transmission of the Ca^{2+}-binding signal (*42*). Fluorescence resonance energy transfer measurement of the distance between sites in the N- and C-terminal domains of sTnC showed that there was a transition from an elongated conformation at pH 5.0 to a more compact conformation at pH 6.8 (*43–44*). In this study we have measured the effects of Ca^{2+} and pH on the intrinsic tyrosine fluorescence of unlabeled cTnC and the monomer and excimer fluorescence of PIA-cTnC. The data obtained indicate that proton-induced changes in Ca^{2+} binding at both the C-terminal and N-terminal domains of cTnC are associated with conformational changes in both regions (Fig. 2 and Table 1).

The structure of cTnC has been resolved by the use of multidimensional multinuclear magnetic resonance (NMR) (*45–46*) and by crystallography to 2.15 Å resolution (*47*). More recently, the core structure containing $cTnC_{1–161}$, $cTnT_{203–271}$, and $TnI_{31–163}$/$TnI_{31–210}$ from human cardiac muscle has been analyzed by X-ray crystallography by Taketa *et al.* at 2.6 and at 3.3 Å resolution (*24*). These studies indicate that the regulatory domain (N-lobe) of cTnC exists in a closed conformation in both the apoprotein (absence of metal binding) and Ca^{2+}-bound states. In contrast, the N-lobe of sTnC switches from a closed to an open conformation upon the Ca^{2+} binding to site I and II (*48–49*). The fully open conformation in the N-lobe of cTnC requires the complexation with cTnI and

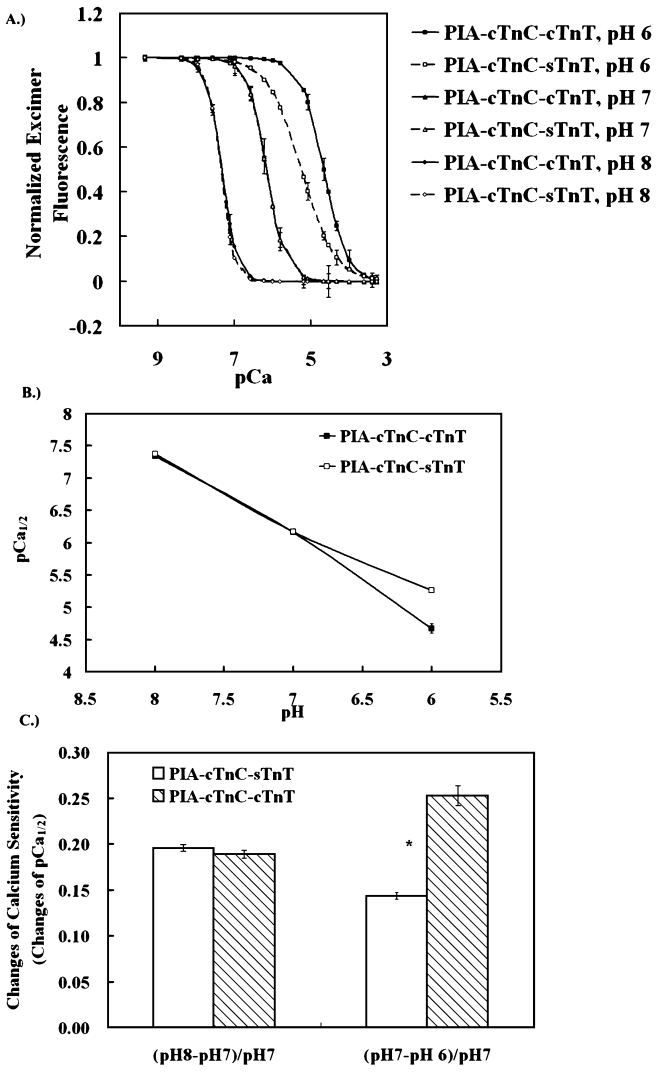


Fig. 5. **Effects of pH on Ca^{2+}-dependent excimer fluorescence changes of PIA-cTnC in the binary complex with cTnT or sTnT.** (A) The excimer fluorescence-pCa curve of PIA-cTnC in the binary complex with cTnT or sTnT at pH 8.0, 7.0, and 6.0. (B) The effect of pH on the half-maximal Ca^{2+} concentration ($pCa_{1/2}$) for excimer fluorescence changes in complex with cTnT or sTnT. (C) The effect of pH on calcium sensitivity ($pCa_{1/2}$) for PIA-cTnC-cTnT and PIA-cTnC-sTnT complexes. PIA-cTnC (1 μM) complexed with 1 μM cTnT or 1 μM sTnT was dissolved in different pH buffers (100 mM MOPS, pH 7.0; 100 mM Tris-OH, pH 8.0; and 100 mM MES, pH 6.0) containing 0.1 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, and varying Ca^{2+} additions. Relative excimer (485 nm) fluorescence at each pCa was obtained by subtracting the fluorescence measured at each pCa from that at pCa 8, and then normalizing the value to the difference at pCa 4. The curve was fitted to the data using the Hill equation as described in the Methods section. Each histogram or point is the mean ± SEM of five measurements. * indicates significant difference ($p < 0.05$).

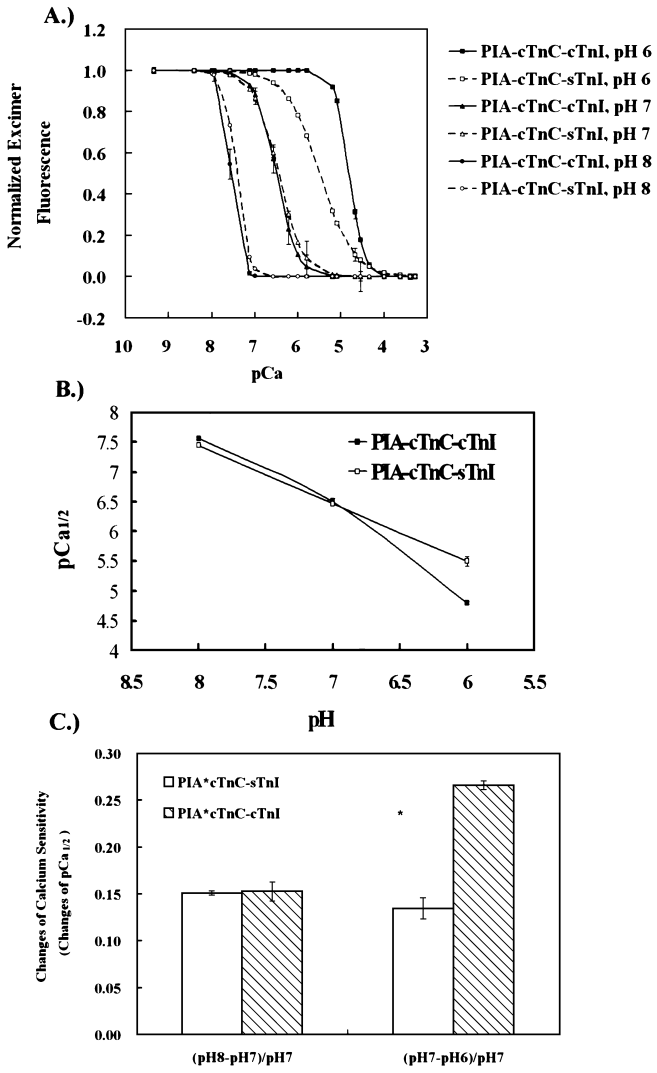


Fig. 6. **(A) The excimer fluorescence-pCa curve of PIA-cTnC in the binary complex with cTnI or sTnI at pH 8.0, 7.0, and 6.0. (B) The effect of pH on the half-maximal Ca^{2+} concentration ($pCa_{1/2}$) for excimer fluorescence changes in complex with cTnI or sTnI. (C) The effect of pH on calcium sensitivity ($pCa_{1/2}$) for PIA-cTnC-cTnI and PIA-cTnC-sTnI complexes.** PIA-cTnC (1 μM) complexed with 1 μM cTnI or 1 μM sTnI was dissolved in different pH buffers (100 mM MOPS, pH 7.0; 100 mM Tris-OH, pH 8.0; and 100 mM MES, pH 6.0) containing 0.1 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, and varying Ca^{2+} additions. Each histogram or point is the mean ± SEM of five measurements. * indicates significant difference ($p < 0.05$).

the binding of Ca^{2+} to site II (*47*). Apparently, the conformational change induced by Ca^{2+} through the TnC-TnI interaction is different in cardiac and skeletal muscle. In this study, the complexation of PIA-cTnC with cTnI produced a greater increase in monomer fluorescence and a smaller decrease in excimer fluorescence, than did the complex formed with sTnI (Fig. 3, A, B, and G). Ca^{2+} binding elicited a further increase in monomer fluorescence and a decrease in excimer fluorescence for the PIA-cTnC-cTnI complex, while there was no effect on monomer fluorescence but a decrease in excimer fluorescence for the PIA-cTnC-sTnI complex (Fig 3, A and B). The amino acid sequences of skeletal and cardiac TnI (*50*) and TnC (*8*) are different, in particular, at the binding interfaces. These differences might alter the TnC-TnI interaction and thus affect the differential response of skeletal and cardiac myofilaments to acidosis. An earlier fluorescent probe study indicated that TnC conformation was proton sensitive in cTnC-cTnI and sTnC-sTnI complexes but not in cTnC-sTnI and sTnC-cTnI complexes (*12*). This finding

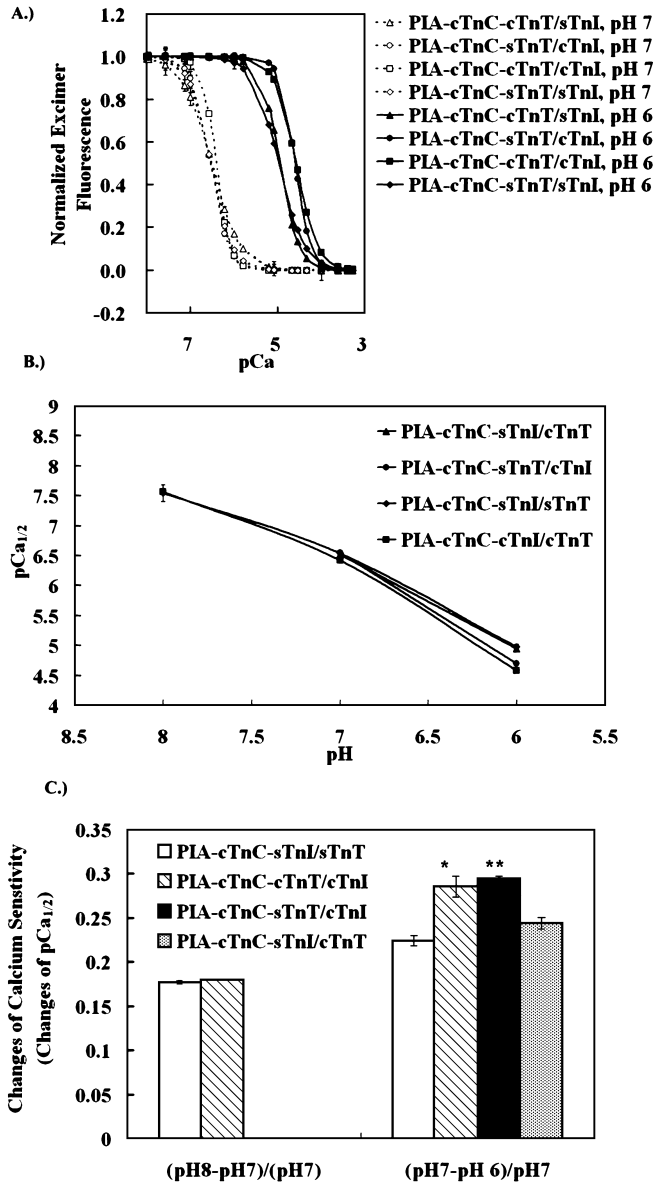


Fig. 7. **(A) The excimer fluorescence-pCa curve of PIA-cTnC in the ternary complex with the cTnT and/or cTnI replaced by sTnT and/or sTnI at pH 7.0, and 6.0. (B) The effect of pH on the half-maximal Ca^{2+} concentration ($pCa_{1/2}$) for excimer fluorescence changes in the ternary complex with the cTnT and/or cTnI replaced by sTnT and/or sTnI (C) The effect of pH on calcium sensitivity ($pCa_{1/2}$) for the ternary complexes of PIA-cTnC-cTnI/cTnT, PIA-cTnC-sTnI/sTnT, PIA-cTnC-cTnI/sTnT, and PIA-cTnC-sTnI/cTnT.** Tn ternary (1 μM) complex with a equimolar ratio of TnC, TnT and TnI was dissolved in different pH buffers (100 mM MOPS, pH 7.0; 100 mM Tris-OH, pH 8.0; and 100 mM MES, pH 6.0) containing 0.1 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, and varying Ca^{2+} additions. Each histogram or point is the mean ± SEM of five measurements. * indicates significant difference ($p < 0.05$) between PIA-cTnC-cTnI/cTnT and PIA-cTnC-sTnI/cTnT, and ** indicates significant difference between PIA-cTnC-cTnI/cTnT and PIA-cTnC-sTnI/sTnT.

indicated that Ca^{2+}-dependent, isoform specific interactions between TnI and TnC could play a role in pH responsiveness. However, for the IAANS-labeled cTnC used, the authors reported that the decrease in the Ca^{2+} affinity of site II in cTnC by acidic pH (pH 6.5) (–0.21 pCa) was potentiated when complexed with cTnI (–0.33 pCa), while replacement by sTnI (–0.20 pCa) elicited no enhancement of the pH effect (*12*). In contrast, for the PIA-cTnC used in this study, we found that the decrease in the Ca^{2+} affinity of site II in cTnC (–1.82 pCa) by acidic pH (pH 6.0) was slightly reduced when complexed with cTnI (–1.70 pCa) and was greatly reduced when complexed with sTnI (–0.96 pCa). It may be that IAANS and PIA report different information about conformational change in cTnC. Nevertheless, our data also indicate that the Ca^{2+}-induced excimer fluorescence change of the cTnC-cTnI and cTnC-cTnI-cTnT complexes is more pH-sensitive than that of the hybrid complexes (Figs. 6 and 7 and Table 1). Thus the isoform-specific interaction of TnI with cTnC may modulate the conformational state of cTnC and convey the differential effect of acidic pH on Ca^{2+} binding to the N-terminal domain of cTnC.

There are multiple interactions between TnI and TnC, involving regions located in the N-terminal hydrophobic cleft (residues 51–62), central helix (residues 89–100), and the C-terminal hydrophobic cleft (residues 127–138) of TnC and regions located in the N-terminal part ($sTnI_{1–47}$, $cTnI_{33–80}$) and the C-terminal part ($sTnI_{96–117}$, $cTnI_{129–151}$, and $sTnI_{118–129}$, $cTnI_{152–199}$) of TnI (*51–54*). Several studies have indicated that differences in charged amino acids in C-terminal regions of cTnI (residues 153–164: MMQALLGARAKE) and sTnI (residues 121–132: MLRALLGSKHKV) are responsible for the differential effects of acidic pH on the Ca^{2+} sensitivities of cardiac and skeletal myofilaments (*10–15*, *55*).

Cardiac and skeletal TnT isoforms contain conserved sequences in the C-terminal and central regions of TnT but a highly variable region in the N-terminal domain of each TnT isoform (*16–21*). The difference in the N-terminal amino acid composition would cause significant differences in the charge and isoelectric point (pI) of the protein molecules and these might further affect TnT isoform specific interactions with other troponin components during Ca^{2+} activation. Studies with transgenic mouse heart overexpressing chicken pectoral sTnT have shown that cardiac and skeletal TnT isoforms with major structural differences in the N-terminal domain could modify Ca^{2+} regulation and kinetics of cardiac muscles (*56–57*). Consistent with these published results, the data presented in this study showed an increase in monomer fluorescence but no apparent change in excimer fluorescence for the PIA-cTnC-cTnT complex, whereas there was an increase in monomer and a decrease in excimer fluorescence for the PIA-cTnC-sTnT complex (Fig. 3, A, B, and G). Apparently neither sTnT nor cTnT has any significant effect on the conformation of the regulatory domain of cTnC.

A recent study has shown that chicken pectoral sTnT isoform containing the motif with several repeating numbers of His pairs $(HEEAH)_n$ in the N-terminal hypervariable region could also bind Ca^{2+} with a binding affinity of 10^5–10^6 M^{-1} and an on-rate of 4×10^6 M^{-1} s^{-1} (*58*). In this study, with the PIA-cTnC-cTnT ($pCa_{1/2}$: 6.16 ± 0.01) and PIA-cTnC-sTnT ($pCa_{1/2}$: 6.17 ± 0.01) complexes there was no shift in the excimer fluorescence-pCa curve, as compared to PIA-cTnC ($pCa_{1/2}$: 6.11 ± 0.07). Apparently, sTnT

or cTnT has no significant effect on the Ca^{2+} sensitivity of cTnC.

It has been reported that the N-terminal charge of TnT isoform could contribute to the tolerance of the thin filament regulatory system to acidosis (*27*). More recently, a study with transgenic mouse hearts expressing normal (R92) and mutant (Q92) human troponin T suggests that TnT isoforms with minor structural variation may convey differential pH effects on myofilament Ca^{2+}-activation (*6*). As shown in this study, the Ca^{2+}-induced decrease in excimer fluorescence of the PIA-cTnC-cTnT complex is more pH-sensitive than that of the PIA-cTnC-sTnT complex (Figs. 4 and 5 and Table 1). However, this effect was appreciably diminished when cTnI or sTnI was incorporated into the PIA-cTnC-cTnT and PIA-cTnC-sTnT in the ternary complex (Fig. 7 and Table 1). This result suggested that the muscle-type–specific TnI isoform is the major determinant of the differential effect of acidic pH on myofilament Ca^{2+} sensitivity in cardiac and skeletal muscle. Future study will be needed to determine if the incorporation of sTnT into myocardium would confer on the cardiac muscle a higher tolerance to the acidic pH. In summary, the conformational change induced by Ca^{2+} through isoform specific interactions among troponin subunits appears important in the differential response of skeletal and cardiac myofilament to acidosis.

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