Ca\(^{2+}\) binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms

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Ca\(^{2+}\) binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. Am J Physiol Regulatory Integrative Comp Physiol 279: R1707–R1715, 2000.—A reduction in temperature lowers the Ca\(^{2+}\) sensitivity of skinned cardiac myofilaments but this effect is attenuated when native cardiac troponin C (cTnC) is replaced with skeletal TnC. This suggests that conformational differences between the two isoforms mediate the influence of temperature on contractility. To investigate this phenomenon, the functional characteristics of bovine cTnC (BcTnC) and that from rainbow trout, Oncorhynchus mykiss, a cold water salmonid (ScTnC), have been compared. Rainbow trout maintain cardiac function at temperatures cardioplegic to mammals. To determine whether ScTnC is more sensitive to Ca\(^{2+}\) than BcTnC, F27W mutants were used to measure changes in fluorescence with in vitro Ca\(^{2+}\) titrations of site II, the activation site. When measured under identical conditions, ScTnC was more sensitive to Ca\(^{2+}\) than BcTnC. At 21°C, pH 7.0, as indicated by \(K_{1/2} (\log[Ca])\) at half-maximal fluorescence, where [Ca] is calcium concentration, ScTnC was 2.29-fold more sensitive to Ca\(^{2+}\) than BcTnC. When pH was kept constant (7.0) and temperature was lowered from 37.0 to 21.0°C and then to 7.0°C, the \(K_{1/2}\) of BcTnC decreased by 0.13 and 0.32, respectively, whereas the \(K_{1/2}\) of ScTnC decreased by 0.76 and 0.42, respectively. Increasing pH from 7.0 to 7.3 at 21.0°C increased the \(K_{1/2}\) of both BcTnC and ScTnC by 0.14, whereas the \(K_{1/2}\) of both isoforms was increased by 1.35 when pH was raised from 7.0 to 7.6 at 7.0°C.

calcium affinity; fluorescence; contractility

CONTRACTION OF STRIATED MUSCLE is initiated by the binding of Ca\(^{2+}\) to troponin C (TnC), a troponin subunit located on the thin filament, triggering a series of structural alterations through the components of the thin filament. This cascade of reactions culminates in cross-bridge cycling between actin and myosin and force generation by the cell. Mammalian cardiac and slow skeletal muscle contain the cardiac isoform of TnC (cTnC), which consists of two homologous, globular domains each containing two possible Ca\(^{2+}\) binding sites. The NH\(_2\)-terminal, regulatory domain contains sites I and II, whereas the COOH-terminal domain contains sites III and IV. The regulatory domain of cTnC contains only a single functional Ca\(^{2+}\) binding site (site II), as the Ca\(^{2+}\) coordinating characteristics of site I have been disrupted through changes in protein sequence. Therefore the binding of Ca\(^{2+}\) to site II is believed to be solely responsible for initiating the conformational response and triggering cardiac myofilament contraction (32, 33). Sites III and IV in the COOH terminus, or high-affinity domain, bind either Ca\(^{2+}\) or Mg\(^{2+}\) and remain saturated with these divalent metals under physiological conditions.

A reduction in environmental temperature reduces the maximum Ca\(^{2+}\)-activated force (\(C_{\text{max}}\)) in cardiac muscle and reduces the sensitivity of the contractile element to calcium concentration ([Ca\(^{2+}\)]) (5, 19, 21, 40). However, replacement of native cTnC with mammalian skeletal TnC (sTnC) in rat cardiac muscle relieves the desensitizing effect of low temperature on contractility (20), suggesting that differences in the structures of cTnC and sTnC affect the impact of temperature on cardiomyocyte Ca\(^{2+}\) sensitivity.

Insight into the specific molecular mechanisms by which temperature affects TnC structure may be determined by looking at the structure/function of cTnC isoforms from ectothermic species such as the rainbow trout (Oncorhynchus mykiss), a salmonid fish that remains active at temperatures (5–21°C) that are cardioplegic to mammalian species. One adaptive feature of the salmonid heart may be its contractile element sensitivity to Ca\(^{2+}\). Over physiological temperatures, Ca\(^{2+}\) sensitivity of salmonid cardiac myofibrils is much greater than those isolated from rat (6) and other mammals.

The purpose of this study is to determine whether the differences in primary structure between BcTnC and ScTnC result in differences in the Ca\(^{2+}\) sensitivity of the two molecules. If ScTnC is more sensitive to Ca\(^{2+}\) than BcTnC, then this result would suggest that cTnC is at least partially responsible for the differences in the Ca\(^{2+}\) sensitivity between intact salmonid and mammalian skeletal TnC.
mammalian cardiac myofibrils. To accomplish this, Ca$^{2+}$ binding to site II in these two cTnC isoforms was measured in solution over a range of temperatures (7–37°C) using F27W mutants of BcTnC and ScTnC. We have previously shown that the troponin acts as a fluorescent reporter of Ca$^{2+}$ binding to site II (28). In mammals, cardiac temperature and intracellular pH are homeostatically regulated at ~37.0°C and 7.0, respectively. However, in poikilotherms, as body temperature changes so does blood and tissue pH to keep the relative alkalinity ([OH$^-$/[H$^+$]]) approximately constant. The observed relationship, called α-stat regulation, is -0.016 to -0.019 pH units/°C. To determine the effects of such pH regulation on Ca$^{2+}$ affinity, measurements were made under two different pH regimes. In the first, pH was kept at 7.0 under all temperatures measured and, in the second, pH was allowed to vary in an α-stat manner. In the α-stat experiments pH was 7.0, 7.3, and 7.6 at 37.0°C, 21.0°C, and 7.0°C, respectively.

**MATERIALS AND METHODS**

**Construction of BcTnC and ScTnC F27W mutants.** Replacement of the phenylalanine at residue 27 with a tryptophan was done in both the bovine and salmonid cTnC cDNA that was cloned into pET-23a vectors (Novagen, Mississauga, ON, Canada) using the Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). In brief, the parental wild-type plasmids containing gene inserts were used as templates for the extension of sense and antisense oligonucleotide primers. Primers contained the tryptophan point mutation were then cut out of the plasmids using the restriction enzymes 

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\text{Xba I and Bsm I (New England Biolabs, Mississauga, ON., Canada). Following digestion, the cassette and plasmid were purified by gel electrophoresis using low-melting-point agarose. The bands corresponding to the correct size of nucleotide sequence were cut from the gel and purified using ethanol/sodium acetate precipitation. From this point on, BcTnC will refer to F27WBcTnC, whereas ScTnC will refer to F27WScTnC.}
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**Expression and digestion of GST-cTnC fusion protein.** The pGex vectors containing the ScTnC and BcTnC inserts were transformed into the E. coli strain BL21 for protein expression. The fusion protein GST-cTnC was expressed and purified according to the manufacturer’s recommendations. In brief, 15 ml of bacto-tryptone and yeast extract (TY) media plus ampicillin (100 μM final concentration) were inoculated with the bacteria and allowed to grow for 4–6 h to a cell density that gave an absorbance at 600 nm ($A_{600}$) of 0.6. This culture served as an inoculum for flasks of 1.5 l of TY plus ampicillin. Once inoculated, the culture was grown overnight to an $A_{600}$ of 0.6–0.8, then isopropyl-1-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.1 mM to induce expression of the fusion protein GST-cTnC. After 6 h, the cells were precipitated from the culture supernatant by centrifugation (7,700 g for 10 min). Cell pellets were stored frozen at −20°C until needed.

Cell pellets were thawed and sonicated using a Sonics and Materials (Danbury, CT) Vibra-Cell sonicator, followed by treatment with Triton X-100 (1% final concentration) to further solubilize proteins. The cell lysate was then centrifuged at 12,000 g for 15 min to remove cellular debris, and the supernatant was collected. The supernatants isolated from four 1.5-liter samples of cell culture were pooled and frozen. Cell supernatants were then applied directly to a 1.5 × 2 cm glutathione-Sepharose 4B column (Pharmacia Biotech) equilibrated with buffer A (in mM: 140 NaCl, 2.7 KCl, 10 Na$_2$HPO$_4$, 1.8 KH$_2$PO$_4$, pH 7.3). After loading, the column was then washed with 150 ml of buffer A and then equilibrated with buffer B (in mM: 50 Tris·HCl, 150 NaCl, and 1 CaCl$_2$, pH 7.5). To separate the GST fusion protein, 0.1 mg of the serine protease factor Xa (Pharmacia Biotech) in 4 ml of buffer B was applied and allowed to remain on the column overnight at room temperature. The next day, free recombinant cTnC was collected by passing 10 ml of buffer B over the column. Column fractions were pooled, phenylmethylsulfonyl fluoride and dithiothreitol (DTT) were added to final concentrations of 0.1 and 1 mM, respectively, and the eluent was frozen. Eluents from four separate column separations (total bacterial culture = 24 l) were pooled and dialyzed against 100 volumes of buffer C (in mM: 50 Tris, 15 CaCl$_2$, 500 NaCl, and 1 DTT, pH 7.5) with three changes. The pooled and dialyzed fraction was then applied directly to a 2.6 × 4.5 cm column packed with phenyl Sepharose 6 Fast Flow, high sub (Pharmacia Biotech) equilibrated in buffer C. After loading, the column was washed with 100 ml of buffer.
C and then with 100 ml of buffer C without NaCl. Recombinant cTnC was then eluted with buffer D (in mM: 50 Tris, 15 EDTA, and 1 DTT, at pH 7.5), dialyzed twice against 100 volumes of HPLC-grade water, lyophilized, and stored frozen at −20°C until needed.

The identities of ScTnC and BcTnC were confirmed as follows. Each protein was electrophoresed on a SDS-page gel and then electroblotted onto a polyvinylidene difluoride membrane. Putative mutants of F27WcTnC were analyzed for total amino acid content, and the first 5 amino acids from the amino terminal end of the isolated protein were determined by microsequencing at the University of Victoria Protein Chemistry Center (Victoria BC). Collectively, these tests confirmed the identity of both ScTnC and BcTnC.

Solutions used in fluorescence studies. The program MaxChelator 6.5 (4) was used to estimate the pCa (−log[Ca2+]) under all conditions at which fluorescence of the cTnC isoforms was to be measured (buffer composition, temperature, pH). To calculate the initial pCa and the change in pCa of the solutions during Ca2+ titration, the apparent Ca2+ affinity constants (KCa) of EGTA were determined under all conditions at which fluorescence of the cTnC isoforms was to be measured according to Bers et al. (4), using a Ca2+ electrode. In brief, miniature Ca2+ electrodes, made according to Baudet et al. (3) using the highly specific Ca2+ ligand ETH 129, were calibrated using Ca2+ standards (Orion Research, Boston, MA) of pCa 2–5 and produced a standard curve with a Nernstian slope of ~29 mV/pCa unit. The output of the electrode was read with an Orion model EA940 Expandable IonAnalyzer pH meter. Using this standard curve and Ca2+ solutions containing EGTA, this electrode was accurate to a pCa of ~8.0. The pCa values of all of the solutions, at the conditions under which they were to be used, were measured with the electrode during Ca2+ titration and expressed in mV. These data along with the corresponding total [Ca2+] values and Ca2+ electrode calibration data were used to calculate the concentration of Ca2+ bound to EGTA and the ratio of bound Ca2+ to free Ca2+ according to the method of Bers et al. (4). Scatchard plots of these data were used to determine KCa and the actual total EGTA concentration (4). For reasons that are not apparent, electrode stability in one (7°C, pH 7.0) of the five different solutions was not acceptable. The KCa used for this solution was calculated as 0.94 × 106 M−1 by extrapolation from that derived in solutions at higher temperatures but otherwise under identical conditions (e.g., pH, buffer, and ionic strength). The temperature coefficient that we determined was 0.048 × 106 M−1°C, and this value compares favorably with that determined by Bers et al. (4).

The electrode-derived KCa values (Table 1) and actual EGTA concentrations were then used to determine the free [Ca2+] of the solutions under all experimental conditions. We wish to emphasize here that great care was taken in the determination of these values, as the interpretation of the data is critically dependent upon the pCa of the solutions during Ca2+ titration. It was assumed that the pCa would not differ significantly when cTnC was added, as EGTA was in a 400-fold molar excess.

The pH buffer MOPS was used in the solutions when pH was maintained at 7.0, whereas TES was used when the pH of the solutions was varied according to α-stat. For the measurement of Ca2+ binding in the MOPS buffers (pH 7.0), three different recipes were formulated, and then pH was adjusted to 7.0 at 37.0°C (buffer E), 21.0°C (buffer F), and 7.0°C (buffer G). One TES buffer was formulated, and then pH was adjusted to 7.0 at 37.0°C (buffer H). The dpK/dpH of TES (−0.2 pH units°C−1) is similar to α-stat, so the pH of the TES buffer adjusted to pH 7.0 at 37.0°C increased accordingly as the temperature of the buffer was decreased.

Buffers E–H all contained (in mM) 1.0 EGTA, 0.03 CaCl2, 112.0 KCl and 50 of the appropriate buffer at the appropriate pH (final ionic strength = 150 mM). For buffers E–G, the concentration of MOPS free acid and its sodium salt required to equal 50 mM varied according to the temperature at which the buffer was to be used. The basal pCa values of the solutions at their respective temperatures were: buffer E, 7.97; buffer F, 7.66; buffer G, 7.36; and buffer H at 21.0°C was 8.08 and buffer H at 7.0°C was 8.90, reflecting the effects of pH and temperature on the KCa of EGTA.

Preparation of F27WcTnC for fluorescence studies. Each protein was dissolved in 6 M urea to a final concentration of 10 mg of lyophilized protein/ml. Samples were immediately diluted with an equal volume of buffer E. This volume was then aliquoted into four fractions, and individual fractions were dialyzed against 500 volumes of buffers E–H as appropriate with two changes. The pair of ScTnC and BcTnC isoforms to be measured under the same conditions was dialyzed simultaneously in the same vessel/buffer. After dialysis, the proteins were removed from the dialysis bags and stored frozen at −80°C until required. All plasticware used for the preparation of proteins and buffers was rinsed with 6 N HCl prior to use.

Fluorescence studies. The cTnC samples were diluted to 2.5 μM with the required buffer in a quartz cuvette (1 cm2). Fluorescence was measured using an SLM Instruments (Urbana, IL) model 4800C spectrofluorometer, which had a NesLab (Portsmouth, NJ) water bath attached to maintain the cuvette at the desired temperature. Compressed air was used to keep the reaction chamber free of moisture to avoid condensation on the cuvette at 7.0°C.

The protein samples were titrated by pipetting Ca2+ stock (0.101 M or 0.051 M) in 1-μl increments into the cuvette containing an active stir bar. After 60 s of mixing, fluorescence was measured over a 10-s period. Pilot studies indicated that the fluorescence emission was stable after 60 s at each pCa. The cuvette was only exposed to the excitation beam for 10 s at each pCa. The maximum number of fluorescence measurements per titration experiment was 16. Fluorescence was measured during Ca2+ titration of the F27WcTnC isoforms using an excitation wavelength of 276 nm and an emission wavelength of 330 nm.

The relative fluorescence was calculated as the ratio of the fluorescence of the protein and an internal rhodamine standard. Before the beginning of fluorescence measurements, the current gain setting of each photomultiplier tube of the spectrofluorometer was adjusted so that the relative fluorescence of the Ca2+-saturated cTnC mutants was equal to unity for each isoform under each condition measured (temperature, pH). The resultant spectra of ScTnC at basal and

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Table 1. Apparent affinity constants for Ca2+
saturating [Ca\(^{2+}\)] at 21.0°C pH 7.0 were similar to that published by Moye et al. (28). The relative fluorescence of ScTnC (21.0°C, pH 7.0) at basal [Ca\(^{2+}\)] was 0.83 ± 0.01 (n = 9) and when saturated with [Ca\(^{2+}\)] was 1.00 ± 0.01 (n = 9). For BcTnC these values were 0.75 ± 0.01 (n = 10) and 1.01 ± 0.01 (n = 10), respectively. These data demonstrate that the fluorescence of the mutant proteins was stable and that the cTnC was not affected by the brief exposure to the ultraviolet excitation during the titration.

Data manipulation and statistical analysis. The Ca\(^{2+}\)-dependent component of the fluorescence measurements from each titration was determined by subtracting the fluorescence at basal [Ca\(^{2+}\)] from all measurements and then expressing the resultant values as a percentage of the maximum fluorescence. Each data set was fitted using a Hill equation with the program Origin 6.0. (Microcal Software, Northampton, MA)

$$Y = \frac{F_{\text{max}}[(x^n)/(K_{1/2} + x^n)]}{n}$$

where Y is the relative fluorescence, F\(_{\text{max}}\) is the maximum Ca\(^{2+}\)-dependent fluorescence equal to 1, x is the free Ca\(^{2+}\) (M), n is the Hill coefficient, and K\(_{1/2}\) is the Ca\(^{2+}\) concentration at half-maximum Ca\(^{2+}\)-dependent fluorescence. The \(x^n\), which was used as a goodness of fit index of the Hill equation to our data, ranged from 0.0042 ± 0.0005 (ScTnC 7.0°C, pH 7.6) to 0.0002 ± 0.0001 (BcTnC 21.0°C, pH 7.0). Because some of the curves showed deviations from Hill equation predictions, the data were also fitted with a multi-function two-site binding nonlinear equation and by interpolation of pCa values at 50% fluorescence from the spline curve fits (data not shown). The K\(_{1/2}\) values derived from these two alternative procedures were never more than 0.8% different from those calculated using the Hill equation. The effects of temperature, pH, and isoform on K\(_{1/2}\) values as determined by the Hill equation curve fitting were analyzed statistically using a one-way repeated measures ANOVA followed by Bonferroni post hoc tests using the statistical software package SigmaStat. The values reported for K\(_{1/2}\) are expressed as means ± SE in pCa units. Two means were considered to be significantly different when the P value was less than 0.05.

RESULTS

Effect of temperature on Ca\(^{2+}\) binding in BcTnC and ScTnC. When pH was kept constant (7.0), a reduction in temperature lowered the Ca\(^{2+}\) sensitivity of both isoforms. The K\(_{1/2}\) of BcTnC was reduced by 0.13 when temperature was lowered from 37°C to 21°C and then by 0.32 when temperature was reduced from 21.0 to 7.0°C (Fig. 1A, Table 2). The K\(_{1/2}\) of ScTnC for Ca\(^{2+}\) was reduced by 0.76 when temperature was lowered from 37.0 to 21.0°C and then by 0.42 when temperature was lowered from 21.0 to 7.0°C (Fig. 1B, Table 2). Visual comparison of the titration curves obtained for ScTnC at 7.0°C, pH 7.0; 21.0°C, pH 7.0; and 37.0°C, pH 7.0, reveals that the curve obtained at 37.0°C is aberrant (Fig. 1B). The fluorescence of ScTnC at 37.0°C did not reach an asymptote even at pCa 3.0; however, it was arbitrarily taken as maximal.

Effect of pH on Ca\(^{2+}\) binding in BcTnC and ScTnC. When temperature was kept constant at either 21.0°C or 7.0°C, an increase in pH increased the Ca\(^{2+}\) sensitivity of both BcTnC (Fig. 1C, Table 2) and ScTnC (Fig. 1D, Table 2). At 21.0°C the increase in pH from 7.0 to 7.3 increased the K\(_{1/2}\) of Ca\(^{2+}\) binding by 0.14 for both BcTnC and ScTnC (Table 2). At 7.0°C, the increase in pH from 7.0 to 7.6 increased the K\(_{1/2}\) of both BcTnC and ScTnC by 1.35 (Fig. 1D, Table 2). When the titration data were fit with the Hill equation, some of the data were not adequately described by the resultant curves, particularly at the higher pCa values. More specifically, the fluorescence of ScTnC appears to be non-Hillian as Ca\(^{2+}\) increases from pCa 8 to ~6.5 at 21.0°C, pH 7.3, and 7.0°C, pH 7.6 (Fig. 1F).

Effect of pH and temperature on Ca\(^{2+}\) binding in BcTnC and ScTnC. When temperature was decreased from 37.0 to 21.0°C and pH was increased according to α-stat regulation (pH 7.0 → 7.3), the affinity of BcTnC for Ca\(^{2+}\) did not change (Fig. 1E, Table 2). However, the K\(_{1/2}\) of BcTnC was increased by 0.89 when temperature was decreased from 21.0 to 7.0°C and pH was increased from 7.3 to 7.6 (Table 2). The K\(_{1/2}\) of ScTnC was decreased by 0.62 when temperature was reduced from 37.0°C to 21.0°C and pH was increased from 7.0 to 7.3 (Table 2). When temperature was further decreased to 7.0°C, pH 7.6, the K\(_{1/2}\) increased by 0.79 (Fig. 1F, Table 2).

Difference in Ca\(^{2+}\) sensitivity between isoforms. When measured under identical experimental conditions (temperature, pH), ScTnC was more sensitive to Ca\(^{2+}\) than BcTnC (Fig. 2A). When Ca\(^{2+}\) binding was measured at 37.0°C pH 7.0, the K\(_{1/2}\) of ScTnC was 0.99 greater than BcTnC. At 21.0°C, pH values of 7.0 and 7.3, the difference between isoforms was 0.36 in both cases (Table 2). When measured at 7.0°C and pH values of 7.0 and 7.6, the difference in K\(_{1/2}\) between isoforms was 0.26 for both pH values (Table 2). Comparison of the isoforms under the experimental conditions closest to their respective physiological conditions (ScTnC, 7.0°C, pH 7.6; BcTnC, 37.0°C, pH 7.0) reveals that ScTnC was significantly more sensitive to Ca\(^{2+}\) than BcTnC (Fig. 2B). The K\(_{1/2}\) of ScTnC under these conditions was 1.17 more (14.8-fold) than BcTnC (Table 2). A consistent finding when comparing the shape of the titration curves of the two isoforms of cTnC under identical conditions is that as ScTnC begins loading Ca\(^{2+}\) at lower [Ca\(^{2+}\)] than BcTnC.

DISCUSSION

The engineering of fluorescent probes into TnC through phenylalanine-to-tryptophan mutation has been used previously to study the Ca\(^{2+}\) binding dynamics of this molecule (12, 24, 25, 28, 31, 34, 41). The effectiveness of tryptophan at residue 27 in reporting Ca\(^{2+}\) binding to site II in BcTnC and ScTnC without significantly affecting the tertiary structure of the molecule has been previously established (28). In this study, we demonstrated that the far ultraviolet circular dichroism of wild-type BcTnC and ScTnC were nearly identical to the F27W mutants under both Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturating conditions (28). These results suggest that the F27W mutation does not have a significant effect on the α-helical structure of the proteins or how the degree of α-helicity changes with
Ca$^{2+}$ binding. In the present study, the $K_{1/2}$ values of Ca$^{2+}$ binding to the two mutant proteins are also within the range previously reported for that of Ca$^{2+}$-triggered tension generation in cardiac myocytes under similar conditions (21, 23, 26, 27, 29, 44). Pearlstone et al. (31) using a series of tryptophan mutants of sTnC, have also demonstrated that Ca$^{2+}$ affinity measured with fluorescence is similar to when measured directly by Ca$^{2+}$ binding (7). For these reasons, we believe that the F27W mutation is accurately reporting on the Ca$^{2+}$-induced conformational response of BcTnC and ScTnC without significantly altering the functional characteristics of the molecule. As the recombinant cTnCs were studied in isolation from troponin I and troponin T, the modulatory effect of these proteins on Ca$^{2+}$ binding has been removed. Hence, a direct comparison of Ca$^{2+}$ binding by the two isoforms is possible under very specific conditions.

Fig. 1. The effect of temperature and pH on Ca$^{2+}$ sensitivity of bovine cardiac troponin C (BcTnC, left) and cardiac troponin C from the cold water salmonid, Oncorhynchus mykiss (ScTnC, right). Data are normalized with respect to the maximal fluorescence of each Ca$^{2+}$ titration and presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added for comparison against the data points. A: titration of fluorescence of BcTnC at 37.0°C ($n=8$), 21.0°C ($n=10$), and 7.0°C ($n=8$) with pH constant at 7.0. B: titration of fluorescence of ScTnC at 37.0°C ($n=8$), 21.0°C ($n=9$), and 7.0°C ($n=11$) with pH constant at 7.0. C: titration of fluorescence of BcTnC with temperature constant at 7.0°C and pH at 7.0 ($n=10$) and 7.6 ($n=9$). D: titration of fluorescence of ScTnC with temperature constant at 7.0°C and pH at either 7.0 ($n=9$) or 7.6 ($n=10$). E: titration of fluorescence of BcTnC at 37.0°C ($n=8$), 21.0°C ($n=9$), and 7.0°C ($n=6$) while pH is allowed to vary in an α-stat manner, 7.0, 7.3, and 7.6, respectively. F: titration of fluorescence of ScTnC at 37.0°C ($n=8$), 21.0°C ($n=10$), and 7.0°C ($n=8$) while pH is allowed to vary in an α-stat manner, 7.0, 7.3, and 7.6, respectively. In A–D and F, the pCa at half-maximal fluorescence, $K_{1/2}$, of each curve is significantly different from the $K_{1/2}$ values of the other curves on the same plot ($P<0.05$). In E, the $K_{1/2}$ of BcTnC at 7.0°C, pH 7.6, is significantly greater than the $K_{1/2}$ values determined at 37.0°C and 21.0°C, although these two $K_{1/2}$ values are not significantly different from each other ($P<0.05$).
The effect of temperature on Ca$^{2+}$ sensitivity. It has been clearly demonstrated that a reduction in environmental temperature reduces the sensitivity of the contractile element to [Ca$^{2+}$] in cardiac myofibrils isolated from mammals, frogs, and salmonids (5, 6, 19, 21, 40). This effect of lowered temperature is exploited as a cardioprotectant during surgery, as in addition to inhibiting electrical activity, it also acts to desensitize the cells to Ca$^{2+}$, thereby reducing the possibility of contracture when intracellular [Ca$^{2+}$] rises during the imposed ischemia. The results of the present study suggest that it is the effect of temperature on the Ca$^{2+}$ binding characteristics of cTnC that is at least partly responsible for this effect. The decrease in the $K_{1/2}$ of both ScTnC and BcTnC as temperature was decreased from 37.0 to 21.0°C and from 21.0 to 7.0°C (at constant pH) indicates that the molecule was becoming desensitized to Ca$^{2+}$ in the absence of other thin filament proteins. This decrease was equal to 0.048 pCa units/°C for ScTnC and 0.008 pCa units/°C for BcTnC when temperature was decreased from 37 to 21°C. When temperature was decreased from 21.0 to 7.0°C, this decrease in $K_{1/2}$ was equal to 0.030 pCa units/°C for ScTnC and 0.022 pCa units/°C for BcTnC.

The shape of the curve produced by the titration of ScTnC at 37.0°C is anomalous. In all previous studies that describe the change in fluorescence as Ca$^{2+}$ binds to TnC or of Ca$^{2+}$ triggering tension generation achieve asymptotic levels at pCa values of 3.5 or higher. The change in the Ca$^{2+}$ binding properties of ScTnC at 37°C likely indicates loss of tertiary structure and perhaps denaturation. On the one hand, this is not surprising, as temperatures above 25°C are lethal to salmonids. Previous studies have demonstrated that the function of various enzymes from cold-adapted organisms may degrade as temperature increases significantly above that normally experienced (8, 11, 14, 35, 37, 43). On the other hand, this finding is somewhat surprising, given how highly conserved cTnC is (13 differences out of 161 amino acids) between the two species. This therefore suggests that the sequence differences between the two isoforms have significant effects on the thermal stability of the molecule.

This is the first known study that has examined the effects of temperature on Ca$^{2+}$ binding in cTnC. The effect of low temperature on avian sTnC has been studied using an N-domain mutant (residues 1–90) containing an F29W mutation. In this study the authors showed that Ca$^{2+}$ sensitivity increased 4.4-fold when temperature was decreased from 20°C, pH 7.0, to 1.0°C, pH 7.0, as reflected in $K_{1/2}$ values of 5.90 and 6.54, respectively (12). This increase in $K_{1/2}$ is equal to 0.03 pCa units/°C.

How temperature effects the interaction of cTnC with the other components of the thin filament is not known. The binding of Ca$^{2+}$ to the low-affinity binding sites of TnC leads to the exposure of a hydrophobic surface in the NH2-terminal domain as the molecule “opens” (42). The hydrophobic patch is believed to interact with troponin I to remove the inhibitory effect on troponin I on cross-bridge formation (13). However, there is a comparatively smaller conformational re-

### Table 2. pCa at half-maximal fluorescence of Ca$^{2+}$ binding to BcTnC and ScTnC

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<th>pH 7.0</th>
<th>pH 7.6</th>
<th>pH 7.0</th>
<th>pH 7.3</th>
<th>37.0°C at pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BcTnC</td>
<td>4.97 ± 0.04 (8)</td>
<td>6.32 ± 0.08 (6)</td>
<td>5.29 ± 0.04 (10)</td>
<td>5.43 ± 0.03 (9)</td>
<td>5.42 ± 0.02 (8)</td>
</tr>
<tr>
<td>ScTnC</td>
<td>5.23 ± 0.03 (11)</td>
<td>6.58 ± 0.03 (8)</td>
<td>5.65 ± 0.04 (9)</td>
<td>5.79 ± 0.05 (10)</td>
<td>6.41 ± 0.02 (8)</td>
</tr>
</tbody>
</table>

Values are means ± SE; numbers of experiments are in parentheses. All values for pCa at half-maximal fluorescence ($K_{1/2}$) were significantly different from each other. BcTnC, bovine cardiac troponin C; ScTnC, salmonid cardiac TnC.

Fig. 2. Difference in Ca$^{2+}$ sensitivity between BcTnC and ScTnC. The curves generated by fitting the data with the Hill equation have been added for comparison against the data points. A: titration of fluorescence of BcTnC (n = 10) and ScTnC (n = 9) at 21.0°C, pH 7.0. B: titration of fluorescence of ScTnC (n = 9) at 7.0°C, pH 7.6, and BcTnC (n = 8) at 37.0°C, pH 7.0. Data are normalized with respect to the maximal fluorescence of each Ca$^{2+}$ titration and presented as means ± SE. In both plots, the $K_{1/2}$ of each curve is significantly different from the $K_{1/2}$ values of the other curve on the same plot (P < 0.05).
The purpose of measuring Ca\(^{2+}\) temperature on Ca\(^{2+}\)decreases acts to compensate for the effect of lowered temperature on the structure of TnC was done using a NH\(_2\)-terminal mutant of sTnC (42). These authors used nuclear magnetic resonance to examine the solution structure of the NH\(_2\)-terminal domain of BsTnC and demonstrated that low temperature (4.0°C) imparts a structural change on the molecule, causing the apo-NTnC domain to contract. It is suggested that this low-temperature effect could increase the activation energy required to trigger the conformational response (42). Full-length mammalian sTnC is 66.7% identical in amino acid sequence to mammalian cTnC, and the two isoforms display distinct functional differences (1, 17, 18, 20, 39) and differ in the number of functional Ca\(^{2+}\) binding sites. For these reasons, prediction of how lowered environmental temperature would affect the structure of mammalian cTnC from data on sTnC is tenuous.

The Hill coefficient is typically used to describe the cooperativity of Ca\(^{2+}\) binding to a single molecule with multiple binding sites (sTnC) or of Ca\(^{2+}\)-activated tension in a functioning myofibril. However, as this study examined Ca\(^{2+}\) binding of cTnC, which has a single activation site, cooperativity is highly unlikely. For this reason, the Hill coefficient is used only as a parameter of curve fitting, and a physiological interpretation of these differences has been avoided (Table 3).

Effect of pH on Ca\(^{2+}\) sensitivity in BcTnC and ScTnC. The purpose of measuring Ca\(^{2+}\) sensitivity while keeping temperature constant but altering pH was to determine the physiological role of \(\alpha\)-stat regulation in maintaining cTnC function in salmonid myocytes. It has been suggested that the rise in pH that occurs when the body temperature of a poikilotherm decreases acts to compensate for the effect of lowered temperature on Ca\(^{2+}\) sensitivity (6). A similar compensatory effect of pH on the \(K_m\) of M\(_2\)-lactate dehydrogenase from different fish species and rabbits has been demonstrated in response to temperature reduction (38). The increase in Ca\(^{2+}\) sensitivity of BcTnC and ScTnC when pH was increased at both 21.0°C and 7.0°C is supported by previous studies looking at the effects of pH on Ca\(^{2+}\) sensitivity of ventricular myofibrillar ATPase activity isolated from rat and rainbow trout ventricles (6). In this previous study, an increase in pH increased the Ca\(^{2+}\) sensitivity of both mammalian and salmonid ventricular myofibrillar ATPase. The effect of pH on the Ca\(^{2+}\) sensitivity of cardiac myocytes is well established (10, 15, 22, 36), and TnC has been demonstrated to be at least partly responsible (2, 9, 26, 27, 29, 30). Through manipulation of the first 41 residues of BcTnC, Ding et al. (9) have demonstrated that this region contains a pH “sensor” capable of altering the effect of pH on myocyte contractility.

In the present study, the sensitivity of both BcTnC and ScTnC was affected to the same degree by an increase in pH with the effect being greatest at 7.0°C. At 21.0°C (pH 7.0 → 7.3), the increase in \(K_{1/2}\) was equal to 0.047 pCa units/pH unit for both isoforms, whereas at 7.0°C (pH 7.0 → 7.6), the increase in \(K_{1/2}\) was equal to 0.23 pCa units/pH unit for both isoforms. It is not known why the effect of pH was greater at 7.0°C. It is clear, however, that an increase in pH, as would occur during \(\alpha\)-stat regulation, sensitizes the cTnC molecule to Ca\(^{2+}\), and this effect could help maintain contractility in the salmonid myocyte at low temperatures.

The unexpected non-Hillian increase in fluorescence of ScTnC at pH values greater than 7.0 as Ca\(^{2+}\) increases in the range of pCa 8 to ~6.5 suggests that an increase in pH alters the nature of Ca\(^{2+}\) binding to ScTnC. The mechanism of this effect is not known at this time but is clearly species dependent and deserves further investigation.

Differences between isoforms. The differences in amino acid sequence between ScTnC and BcTnC appear to have a significant effect on their ability to bind Ca\(^{2+}\). The higher Ca\(^{2+}\) sensitivity of tension generation of salmonid ventricular fibers compared with those of a rat (6) seems to be due, at least in part, to the enhanced Ca\(^{2+}\) sensitivity of ScTnC. The differences in Ca\(^{2+}\) sensitivity of ScTnC and BcTnC under their respective physiologically relevant conditions is 14.8-fold. As there is complete sequence identity of site II between the two isoforms, the variation in Ca\(^{2+}\) sensitivity must be as a result of differences elsewhere in the protein, for example, the region of cTnC that encompasses the nonfunctional Ca\(^{2+}\) binding site I (the first 41 residues of the protein). Gulati et al. (16) have demonstrated that this region of cTnC, while unable to bind Ca\(^{2+}\), is critical to the normal function of the protein. Manipulation of the protein sequence in this region has a significant effect on the tension-generating abilities of skinned cardiac trabeculae (16).

In summary, although the sensitivity of both isoforms was reduced when temperature was lowered and pH was maintained at 7.0, ScTnC was always more sensitive to Ca\(^{2+}\) than BcTnC when measured under identical conditions. An increase in pH at either 21.0°C or 7.0°C increases the Ca\(^{2+}\) affinity of both isoforms to a similar degree.

Table 3. Hill coefficients of Ca\(^{2+}\) binding to BcTnC and ScTnC

<table>
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<tr>
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<th>pH 7.0</th>
<th>pH 7.6</th>
<th>pH 7.0</th>
<th>pH 7.3</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BcTnC</td>
<td>1.31 ± 0.03(8)</td>
<td>3.50 ± 0.54(6)</td>
<td>1.47 ± 0.06(10)</td>
<td>1.99 ± 0.08(9)</td>
<td>1.18 ± 0.06(8)</td>
</tr>
<tr>
<td>ScTnC</td>
<td>0.81 ± 0.02(11)</td>
<td>0.96 ± 0.03(8)</td>
<td>1.27 ± 0.06(9)</td>
<td>0.79 ± 0.07(10)</td>
<td>0.59 ± 0.03(8)</td>
</tr>
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</table>

Values are means ± SE; number of experiments is in parentheses.
The results of this study suggest that ScTnC is at least partially responsible for the higher Ca\(^{2+}\) sensitivity of the intact salmonid cardiac myofibril, as it was clearly shown that this isoform is significantly more sensitive to Ca\(^{2+}\) than BcTnC. Visual comparison of the shape of ScTnC and BcTnC Ca\(^{2+}\) titration curves under identical conditions reveals that ScTnC begins to load Ca\(^{2+}\) at a higher pCa than BcTnC; as a result, the curve has a "shoulder" at low Ca\(^{2+}\) concentrations and therefore a distinctive shape. One possibility is that, at higher pH values, a second Ca\(^{2+}\) binding site is observed in the ScTnC isoform, but clearly, further experiments are required to test this hypothesis. While extreme caution must be taken when extrapolating the Ca\(^{2+}\) sensitivity of isolated cTnC to the Ca\(^{2+}\) sensitivity of intact cardiac myofibrils, previous studies have demonstrated that the contractility of chemically skinned cardiac myocytes can be manipulated by cTnC replacement (20).

Comparing the NH\(_2\)-terminal region (residues 1–41) of BcTnC and ScTnC reveals that there are a total of five amino acid differences. Of these, three have the potential of having functional consequences either individually or in concert. The first of these is at residue 2 in ScTnC, where asparagine has replaced aspartate; the second is at residue 29, in which glutamine has replaced leucine; and the third is at residue 30, in which aspartate has replaced glycine (28). The location of a negatively charged side chain, aspartate, could have significant effects on the tertiary structure of the molecule, as could the removal of an additional hydrophobic side chain, leucine. One possibility is that site 2 is functional in ScTnC due to the insertion of aspartate into this region of the protein; however, further study is required.

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REFERENCES


