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Increasing mammalian cardiomyocyte contractility with residues identified in trout troponin C

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Gillis, Todd E., Bo Liang, Franca Chung, and Glen F. Tibbits. Increasing mammalian cardiomyocyte contractility with residues identified in trout troponin C. *Physiol Genomics* 22: 1–7, 2005. First published March 22, 2005; 10.1152/physiolgenomics.00007.2005.—The Ca^{2+} sensitivity of force generation in trout cardiac myocytes is significantly greater than that from mammalian hearts. One mechanism that we have suggested to be responsible, at least in part, for this high Ca^{2+} sensitivity is the isoform of cardiac troponin C (cTnC) found in trout hearts (ScTnC), which has greater than twice the Ca^{2+} affinity of mammalian cTnC (McTnC). Here, through a series of mutations, the residues in ScTnC responsible for its high Ca^{2+} affinity have been identified as being Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30}. When these residues in McTnC were mutated to the trout-equivalent amino acid, the Ca^{2+} affinity of the molecule, determined by monitoring the fluorescence of a Trp inserted for a Phe at residue 27, is comparable to that of ScTnC. To determine how a McTnC mutant containing Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30} (NIQD McTnC) affects the Ca^{2+} sensitivity of force generation, endogenous cTnC in single, chemically skinned rabbit cardiomyocytes was replaced with either wild-type McTnC or NIQD McTnC. Our results demonstrate that the cardiomyocytes containing NIQD McTnC were approximately twice as sensitive to Ca^{2+}, illustrating that a McTnC mutant with similar Ca^{2+} affinity as ScTnC can be used to sensitize mammalian cardiac myocytes to Ca^{2+}.

temperature; cardiac contractility; cold adaptation of proteins; Ca^{2+} affinity

A REDUCTION IN TEMPERATURE decreases the Ca^{2+} sensitivity of the contractile element in the vertebrate heart (2, 19). Because Ca^{2+} is used to trigger and regulate cardiac contraction, this effect of temperature represents a significant challenge for ectothermic species living in cold environments. Comparison of the ability of cardiac myofibrils isolated from the trout (a salmonid fish), frog, rat, and rabbit to generate force over a range of Ca^{2+} concentrations ($[Ca^{2+}]$) reveals that trout cardiac myofibrils are significantly more sensitive to Ca^{2+} than those from the other species when compared at the same temperature and pH (2, 18). It is thought that this increased Ca^{2+} sensitivity of force generation in the trout heart helps offset the desensitizing effect of low temperature (2).

One mitigating factor that has to be taken into account when considering physiological processes at low temperature is the fact that the pH of both blood (pH_o) and tissue cytosol (pH_i) increases as temperature decreases, keeping the relative alkalinity ($[OH^-]/[H^+]$) approximately constant. This relationship, which has a slope of −0.016 to −0.019 pH units/°C, has been termed α-stat regulation. The pH_i of trout cardiomyocytes is likely higher at 15°C than for mammals at 37°C (26). Because an increase in pH_i is known to increase the Ca^{2+} sensitivity of the cardiac contractile element (2, 5, 16, 21, 34), it is likely that the higher pH_i of the trout heart also helps to offset the effect of low temperature on cardiac function (2, 12, 14).

Cardiac troponin C (cTnC) is a small (161 amino acids) acidic protein with two Ca^{2+}-binding domains separated by an α-helical linker. The low-affinity domain, or NH_2 domain, contains Ca^{2+}-binding sites I and II. Site I is nonfunctional in cTnC due to sequence alterations that have disrupted its ability to bind Ca^{2+}. The high-affinity domain, or COOH domain of cTnC, contains-binding sites III and IV. These sites remain saturated with either Ca^{2+} or Mg^{2+} under all physiological conditions. It is, therefore, Ca^{2+} binding to site II that initiates contraction of the mammalian cardiac myocyte. As a result, the Ca^{2+} affinity of site II controls the $[Ca^{2+}]$ at which the contractile reaction is initiated and thus plays a significant role in determining cardiac contractility.

The activation of site II by Ca^{2+} triggers a conformational change in cTnC resulting in the "opening" of the NH_2 terminus and the exposure of a hydrophobic patch (24). This hydrophobic patch acts to pull cardiac troponin I (cTnI) away from its interaction with the actin filament (24), and, as a result, tropomyosin is released to move across the surface of actin-exposing myosin-binding sites. This conformational change allows for the formation of strong cross-bridges and the generation of contractile force (15).

The sequence of human cTnC is identical to that of either the bovine or porcine heart. For this reason, we refer to this isoform of cTnC as mammalian cTnC (McTnC). Using a fluorescent reporter that monitors Ca^{2+} binding to site II, we have previously demonstrated that site II of cTnC cloned from the trout (ScTnC) has approximately twice the Ca^{2+} affinity as that of McTnC when compared at the same temperature (12). The reporter is a Trp inserted for Phe at residue 27 (F27W). The above result suggests that the high Ca^{2+} sensitivity of trout cardiac myocytes is due, at least in part, to the high Ca^{2+} affinity of the activation site of ScTnC.

There are 13 sequence differences between ScTnC and McTnC (92% identity) (29) (Fig. 1). By removing the COOH terminus from F27W ScTnC and F27W McTnC (amino acids 90–161) and then measuring the ability of these NH_2-terminal

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                                              EF-hand site I
                                             X Y Z-Y-X -Z
ScTnC:       1    MNDIYKAAVEQLTDEQKNEFKAAFDIFIQDAEDGCISTKELGKVMRMLGQ  50
McTnC:       1    MDDIYKAAVEQLTEEQKNEFKAAFDIFVLGAEDGCISTKELGKVMRMLGQ  50
NIQD McTnC:  1    MNDIYKAAVEQLTEEQKNEFKAAFDIFIQDAEDGCISTKELGKVMRMLGQ  50
                    helix N       helix A                  helix B
                             EF-hand site II
                            X Y Z-Y-X -Z
ScTnC:       51   NPTPEELQEMIDEVDEDGSGTVDFDEFLVMMVRCMKDDSK  90
McTnC:       51   NPTPEELQEMIDEVDEDGSGTVDFDEFLVMMVRCMKDDSK  90
NIQD McTnC:  51   NPTPEELQEMIDEVDEDGSGTVDFDEFLVMMVRCMKDDSK  90
                      helix C               helix D

                            EF-hand site III
                             X Y Z-Y-X -Z
ScTnC:       91   GKTEEELADLFCMFDKNADGYIDLQELKVMLEATGEA  127
McTnC:       91   GKSEEELSDLFRMFDKNADGYIDLEELKIMLQATGET  127
NIQD McTnC:  91   GKSEEELSDLFRMFDKNADGYIDLEELKIMLQATGET  127
                     helix E                 helix F

                         EF-hand site IV
                        X Y Z-Y-X -Z
ScTnC:       128  ITEDDIEELMKDGDKNNDGKIDYDEFLEFMKGVE  161
McTnC:       128  ITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE  161
NIQD McTnC:  128  ITEDDIEELMKDGDKNNDGRIDYDEFLEFMKGVE  161
                     helix G               helix H
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Fig. 1. Alignment of ScTnC, McTnC, and NIQD McTnC. The differences between sequences are shown in bold. The Ca^{2+}-coordinating positions in each EF-hand site are shown above the sequences. ScTnC, isoform of cardiac troponin C (cTnC) found in trout hearts; McTnC, mammalian cTnC; NIQD McTnC, McTnC mutant containing Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30}.

mutants (NTnC) to be activated by Ca^{2+}, we have demonstrated that the difference in site II Ca^{2+} affinity, exhibited in the full-length proteins, is retained. It is therefore the sequence differences in the low affinity domain, of which there are five, that are responsible for the functional difference between F27W ScTnC and F27W McTnC (13).

The purpose of the present study is to identify the specific residues responsible for the high Ca^{2+} affinity of ScTnC in vitro and then determine whether these effects are observed in situ by introducing an McTnC mutant, containing the identified residues, into single, skinned, mammalian cardiac myocytes. The single myocyte preparation was utilized, as it allows for significant control over *1*) sarcomere length, which affects Ca^{2+} sensitivity and *2*) intracellular [Ca^{2+}], by minimizing diffusion distances. Our results demonstrate that *1*) the residues responsible for the high Ca^{2+} affinity of F27W ScTnC are Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30} and *2*) the insertion of D2N/V28I/L29Q/G30D McTnC (NIQD McTnC) into the cardiomyocytes increases the Ca^{2+} sensitivity of force generation by approximately twofold relative to myocytes containing either endogenous rabbit cTnC (RcTnC) or recombinant wild-type (WT) McTnC.

MATERIALS AND METHODS

Molecular biology. The following cDNA constructs were synthesized by polymerase chain reaction according to methods similar to those previously described (13): F27W/V28I/L29Q/G30D McNTnC (F27W IQD McNTnC), D2N/F27W/V28I/L29Q/G30D McNTnC (F27W NIQD McNTnC), F27W/V28I/L29Q/G30D McTnC (F27W IQD McTnC), D2N/F27W/V28I/L29Q/G30D McTnC (F27W NIQD McTnC) and NIQD McTnC. All mutations were made with the use of the Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and all nucleotide sequences were confirmed by sequencing.

Protein expression and purification. F27W IQD McNTnC, F27W NIQD McNTnC, F27W IQD McTnC, and F27W NIQD McTnC were expressed as glutathione *S*-transferase fusion proteins that were then digested and purified as described previously (13). WT McTnC and NIQD McTnC were expressed and purified according to Dong et al. (4). The identities of all cTnC mutants were confirmed by NH_2-terminal microsequencing and amino acid analysis, completed at the Nucleic Acid Protein Service Unit, the University of British Columbia (UBC). The purities of the isolated proteins as well as their atomic masses were confirmed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry completed at the UBC Mass Spectrometry Center. Collectively, these tests confirmed the identity and purity of all cTnC mutants.

Fluorescence studies. The fluorescence studies of all cTnCs were carried out as previously described (13) using a PerkinElmer (Wellesley, MA) LS50B spectrofluorometer attached to a NesLab (Portsmouth, NJ) water bath to maintain the cuvette at 21.0 ± 0.1°C. Briefly, fluorescence was measured during Ca^{2+} titration by use of an excitation wavelength of 276 nm and an emission wavelength of 330 nm. 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 percentages of the maximum fluorescence. Solutions used in these studies were identical to those previously described (13). The concentration of MOPS free acid and its sodium salt required to equal 50 mM and achieve a pH of 7.0 at 21°C was calculated, and then actual pH was measured and adjusted to 7.0 at 21°C.

Cardiac myocyte preparation. Ventricular myocytes were obtained by enzymatic digestion of hearts from adult New Zealand White rabbits (weighing 1.5–2 kg). The cell isolation method was modified from Osaka et al. (30) as described by Huang et al. (22). The myocytes were subsequently skinned, using the method of Fan et al. (6).

Experimental apparatus. An experimental chamber was constructed for attaching single cardiac myocytes to a force transducer and a servomotor for the activation and relaxation of the attached cell. Cells were attached using the method described by Fitzsimons and Moss (8). The experimental chamber was positioned on the stage of a Nikon (Mississauga, ON, Canada) Diaphot inverted microscope. The

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temperature of the experimental chamber was controlled to 15 ± 0.5°C with the use of a Julabo (Allentown, PA) F25 water bath. The inverted microscope and experimental chamber were mounted on a Melles Griot (Ottawa, ON, Canada) Science Desk anti-vibration table. The single cardiac myocytes were attached to the experimental apparatus by gluing the two ends of the myocyte to steel pins, with an outer diameter of ≤10 μm, using Dow Corning (Midland, MI) Aquarium Sealant. One of these steel pins was attached with paraffin to an Aurora Scientific (Aurora, ON, Canada) 308B servomotor, while the other was attached to a Cambridge Technology (Cambridge, MA) 406 force transducer. The force transducer and servomotor were mounted on micromanipulators for precise three-way positioning. Myocytes were imaged using a Pulnix (Sunnyvale, CA) 6710 CCD camera, and the sarcomere length was measured by fast Fourier transform analysis of the digitized striated images of the attached cell. The output signals from the force transducer and the Pulnix CCD camera were digitized using a National Instruments (Austin, TX) PCI-MIO-16E-1 16-bit A/D converter and a National Instruments 1424 frame grabber, respectively, and displayed and stored on a computer using custom-written software in LabView 6i for Windows (National Instruments). A single myocyte was gravity superperfused using a three-barrel pipette attached to a Warner Instruments (Hamden, CT) SF-77B fast-step switcher. Each of the barrels was made of square glass tubing, 700-μm wide. The pipette and stepper motor were attached to a micromanipulator to allow precise positioning of the pipette opening over the myocyte. The stepper motor could rapidly switch the adjacent barrel over the myocyte within 20 ms, effectively switching the solution bathing the myocyte.

Force-pCa relationship. The method and solutions described in Fan et al. (6) were used to determine the Ca^{2+} sensitivity of isometric force generation before RcTnC was extracted or after the cells were reconstituted with either recombinant McTnC or NIQD McTnC. The pH of all solutions was adjusted to 7.0 at 15°C. In brief, single myocytes were exposed to a series of solutions of increasing Ca^{2+} concentration (pCa 6.0–4.5), and isometric force generated at each pCa was measured using the force transducer. Each activation was allowed to continue for at least 6 s, enabling force generation to reach steady state, after which the solution bathing the myocyte was changed. Sarcomere length was maintained at 2.1 μm during each experiment. Any myocyte found to lose its sarcomere pattern after the extraction/reconstitution protocol or during the force-pCa measurements was discarded. To determine any decline in force-generating capability, the myocytes were maximally activated at the start and at the end of each protocol. Myocytes not achieving 80% of original maximal Ca^{2+}-activated force (F_{max}) at pCa 4.5 were rejected. Ca^{2+}-activated force (F) was measured at submaximal pCa values, and the data were expressed relative to F produced during that experiment at pCa 4.5 ($F_{4.5}$). Each force-pCa curve was fitted with the Hill equation: $F/F_{4.5} = [Ca^{2+}]^{n_H}/(k^{n_H} + [Ca^{2+}]^{n_H})$, where n_H is the Hill coefficient and k corresponds to the $[Ca^{2+}]$ required for pCa at half-maximal force ($K_{F½}$) using Origin 6.0 (Microcal Software, Northhampton, MA). Each of these values was then averaged.

cTnC extraction and reconstitution. Endogenous RcTnC was extracted from the myocytes, using a modification of the method described by Gulati et al. (17), by exposing the skinned cells to an extraction solution that contained (in mM) 5 K_2-EDTA, 20 Tris, pH 7.2, at 8°C for 5 min, after which the temperature was raised to 15°C for 20 min. Before extraction F_{max} was determined and then used in subsequent calculations, including the determination of force loss and recovery due to cTnC extraction and reconstitution, respectively. Using the method described by Martyn and Gordon (25) for cardiac trabeculae, we then reconstituted the skinned myocytes by incubation for 30 min in a relaxing solution containing 10 μM recombinant WT McTnC or NIQD McTnC.

Data and statistical analysis. The Ca^{2+}-dependent component of the fluorescence measurements from each titration was determined as previously described (12). Each data set was fit using the Hill equation with the program Origin 6.0. The effect of the mutations on the half-maximal fluorescence ($K_{f½}$) values determined by the Hill equation curve fitting were statistically analyzed as previously described (12). The effect of the different cTnC isoforms (RcTnC, WT McTnC, and NIQD McTnC) on the $K_{f½}$ values determined by the Hill equation curve fitting were analyzed using a one-way repeated measures analysis of variance followed by Bonferroni post hoc tests, as previously described for the $K_{f½}$ values (12).

RESULTS

Ca^{2+} affinity measurements. The five differences in sequence between ScNTnC and McNTnC include the presence of Asn, Asp, Ile, Gln, and Asp at residues 2, 14, 28, 29, and 30, respectively, in ScNTnC, instead of Asp, Glu, Val, Leu, and Gly, respectively (Fig. 1). The results of a previous study (13) suggest that Gln^{29} and Asp^{30} are required for the high Ca^{2+} affinity of ScNTnC, but other residues in place of, or in addition to, Asn^2 are also required. We have previously demonstrated that Gln^{29} and Asp^{30} are required for the high Ca^{2+} affinity of ScNTnC, but the data indicated that other residues, possibly Asn^2, are also involved (13). On the basis of these findings, the first NH_2-domain mutant characterized in the present study was F27W IQD McNTnC. The Ca^{2+} titration curve of this mutant is shifted to the left of F27W McNTnC. Using the $K_{f½}$ (pCa at half-maximal fluorescence) as a measure of affinity indicates that the Ca^{2+} affinity of this mutant is 0.89-fold that of F27W ScNTnC ($P < 0.01$) (Fig. 2*A*). Thus the Ca^{2+} affinity of this mutant is greater than that of F27W McNTnC but is not equal to that of F27W ScNTnC. When F27W IQD McNTnC was mutated to contain Asn^2, the Ca^{2+} affinity of this mutant, F27W NIQD McNTnC, was increased to ~1.2-fold that of F27W ScNTnC ($P < 0.0003$) (Fig. 2*A*). Replication of these experiments using full-length McTnC mutants demonstrated that F27W IQD McTnC has greater Ca^{2+} affinity than F27W McTnC but less than F27W ScTnC, while the Ca^{2+} affinity of F27W NIQD McTnC is equal to that of F27W ScTnC ($P = 0.53$) (Fig. 2*B*). The Ca^{2+} titration curve of F27W NIQD McTnC was also virtually superimposable with that of F27W ScTnC (Fig. 2*B*). These data demonstrate that Asn^2, Ile^{28}, Gln^{29}, and Asp^{30} are responsible for the high Ca^{2+} affinity of F27W ScTnC as determined in vitro.

The Ca^{2+} affinity of the full-length F27W McTnC mutants was found to increase, relative to F27W ScTnC, in a manner quantitatively similar to that of the NH_2-terminal mutants (Fig. 3, *A* and *B*). This result suggests that it is only sequence differences in the NH_2-terminal domain, and not those in the COOH terminus, that are involved in conferring the high Ca^{2+} affinity to site II of F27W ScTnC. As previously demonstrated, the removal of the COOH terminus does affect the Ca^{2+}-binding properties of the protein, as exhibited by the differences in $K_{f½}$ values, but this affect appears to be uniform with all mutants (13).

Force-pCa measurements in single cardiac myocytes. Before RcTnC was extracted, the average F_{max} generated by the single myocytes was 1.21 ± 0.12 mg, and this value was taken as the maximum force-generating capacity of the myocytes. After extraction, $F_{4.5}$ was 11.2 ± 2.0% of F_{max}, indicating that the extraction protocol was effective (Fig. 4). On reconstitution with WT McTnC and NIQD McTnC, $F_{4.5}$ returned to 82.7 ± 2.4 and 80.8 ± 2.0%, respectively, of F_{max}. Although the amount of force produced by the reconstituted myocytes is

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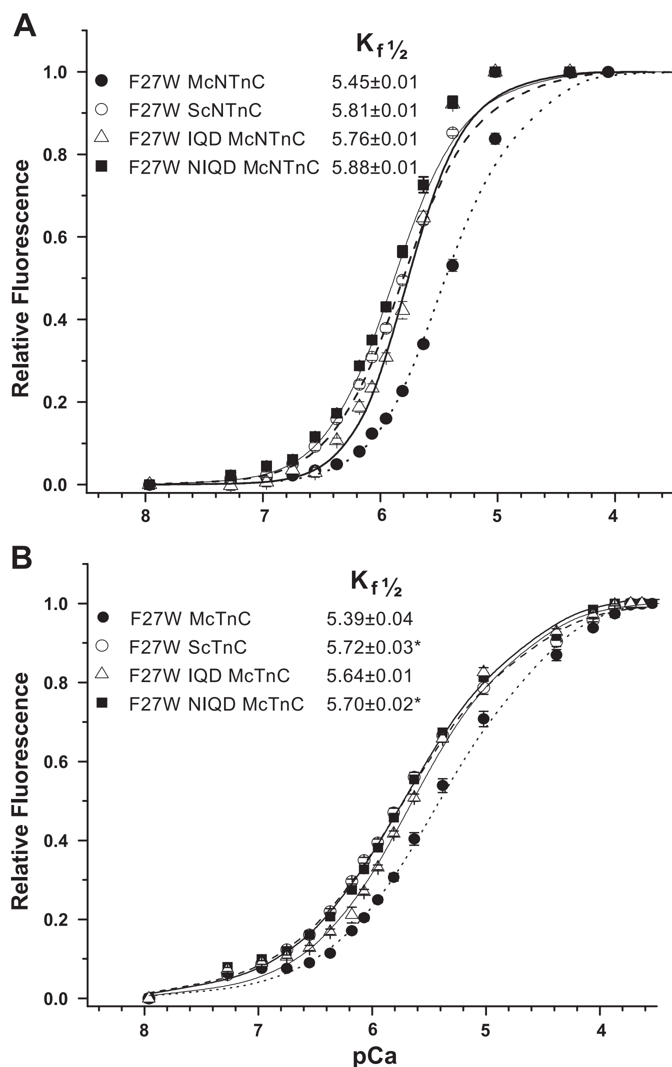

Fig. 2. Comparison of the steady-state Ca^{2+} binding of F27W McNTnC, F27W ScNTnC, F27W IQD McNTnC, F27W NIQD McNTnC, F27W McTnC, F27W ScTnC, F27W IQD McTnC, and F27W NIQD McTnC at 21°C, pH 7.0. Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added to the figures for comparison against the data points. *A*: titration of fluorescence of F27W McNTnC (n = 7), F27W ScNTnC (n = 9), F27W IQD McNTnC (n = 7), and F27W NIQD McNTnC (n = 9). *B*: titration of fluorescence of F27W McTnC (n = 10), F27W ScTnC (n = 10) and F27W IQD McTnC (n = 10), and F27W NIQD McTnC (n = 10). pCa values at half-maximal fluorescence ($K_{f1/2}$) are shown as means ± SE. *Not significantly different from each other (i.e., $P > 0.05$).

statistically less ($P < 0.05$) than that produced by the preextracted myocytes, this amount of force recovery suggests that the myocytes were successfully reconstituted with the McTnC isoforms. This result also suggests that there were no differences in how WT McTnC and NIQD McTnC were incorporated into the myocytes. Because the data from each experiment were normalized to unity (with 1 being F produced at pCa 4.5) before fitting the data with the Hill equation, it is possible to make relative force-pCa comparisons between treatment groups.

Previous studies using these methods report similar efficiencies of cTnC extraction and reconstitution (17, 25). The protocol used is effective in replacing native cTnC in cardiac preparations, as after extraction of native cTnC the myocytes become relatively insensitive to Ca^{2+}. However, Ca^{2+} sensitivity of the preparation is restored after incubation in the cTnC solution, illustrating that the loss of Ca^{2+} sensitivity is due to the loss of functional cTnC. Additionally, Regnier et al. (32, 33) have shown that when cardiac and skeletal myocyte preparations are reconstituted with mutant TnCs that are unable to be activated by Ca^{2+}, the preparations remain insensitive to

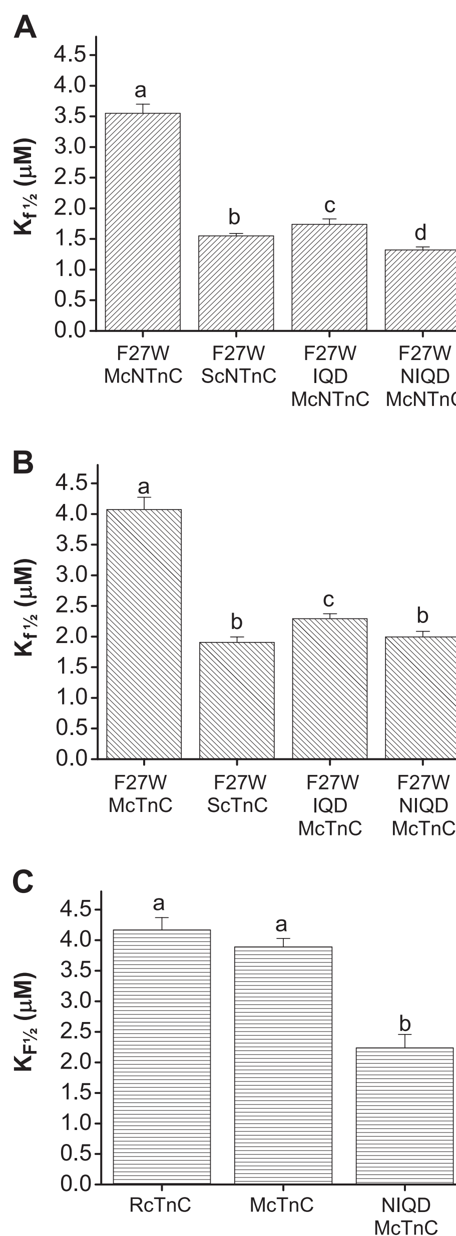

Fig. 3. Comparison of the Ca^{2+} affinity of the cNTnC and cTnC mutants and the Ca^{2+} sensitivity of single rabbit cardiac myocytes containing either endogenous rabbit cTnC (RcTnC), wild-type (WT) McTnC, or NIQD McTnC. *A*: $K_{f1/2}$ values of F27W McNTnC (n = 7), F27W ScNTnC (n = 9), F27W IQD McNTnC (n = 7), and F27W NIQD McNTnC (n = 9). *B*: $K_{f1/2}$ values of F27W McTnC (n = 10), F27W ScTnC (n = 10), F27W IQD McTnC (n = 10), and F27W NIQD McTnC (n = 10). *C*: $K_{F1/2}$ values of cardiac myocytes containing either RcTnC (n = 19), WT McTnC (n = 12), or NIQD McTnC (n = 7). All values are shown as means ± SE. Bars on the same panel with the same letter are not significantly different from each other ($P > 0.05$).

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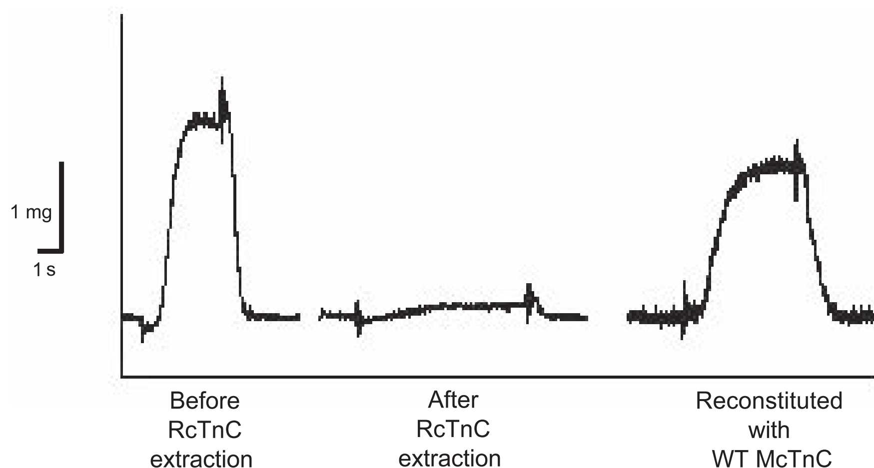

Fig. 4. Representative time-based digital recordings of force generated by cardiac myocytes before endogenous RcTnC extraction, after extraction, and after reconstitution with WT McTnC. First force break in recording represents incubation in extraction solution, and second break represents incubation in a relaxing solution containing WT McTnC.

Ca^{2+}. This demonstrates that the myocyte preparations only regain their ability to be activated by Ca^{2+} when they are reconstituted with a functional TnC.

Attempts were made to reconstitute rabbit myocytes with recombinant ScTnC; however, there was no force recovery, suggesting that the protein did not incorporate into the sarcomeres. The sequence differences between McTnC and ScTnC in the COOH terminus may, therefore, affect how these proteins incorporate into the thin filament. Efforts were also made to replace endogenous ScTnC with McTnC in trout cardiac myocytes, but it was not possible to effectively monitor sarcomere length in these preparations.

Exposure of the myocytes containing either endogenous RcTnC, WT McTnC, or NIQD McTnC to increasing [Ca^{2+}] caused an increase in the amount of force generated that reached a maximum at pCa 4.5 (Fig. 5). This result shows that

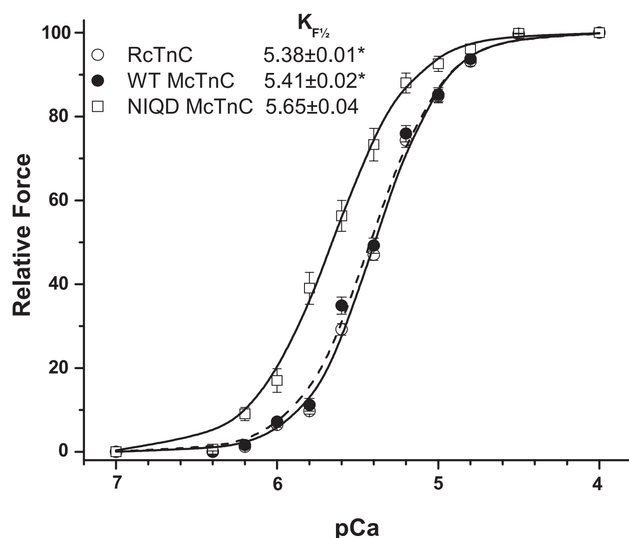

Fig. 5. Ca^{2+} titration of steady-state force generation in single, skinned, rabbit cardiac myocytes containing either endogenous (preextracted) RcTnC (n = 19), WT McTnC (n = 12), or NIQD McTnC (n = 7) at 15°C, pH 7.0. Data are normalized with respect to the maximal force generated during each Ca^{2+} titration and are presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added to the figures for comparison against the data points. $K_{F½}$ values are shown as means ± SE. Means indicated with the same superscript are not significantly different from each other (P > 0.05).

force generation in these myocytes is Ca^{2+} sensitive. The force-pCa curves of the myocytes containing either endogenous RcTnC or recombinant WT McTnC were very similar, and there was no significant difference in the $K_{F½}$ (Fig. 5). This result demonstrates that *1*) the protocol used to replace endogenous RcTnC with WT McTnC had no affect on the Ca^{2+} sensitivity of force generation and *2*) the sequence difference between RcTnC and WT McTnC did not substantively affect the Ca^{2+} sensitivity of force generation.

The force-pCa curves generated by the myocytes containing NIQD McTnC were shifted to the left of those for the myocytes containing either endogenous RcTnC or WT McTnC, and the $K_{F½}$ values calculated from this curve were 0.27 and 0.24 pCa units greater than those for cells containing endogenous RcTnC and WT McTnC, respectively (Fig. 5). Converting the $K_{F½}$ values into molar concentrations demonstrated that the cardiac myocytes containing NIQD McTnC were ~1.95-fold more sensitive to Ca^{2+} than those containing either endogenous RcTnC or WT McTnC.

DISCUSSION

Because Asn^2, Ile^{28}, Gln^{29}, and Asp^{30} are located outside of site II, it is therefore through an allosteric effect that the ability of ScTnC to be activated by Ca^{2+} is enhanced. Nuclear magnetic resonance (NMR) solution studies have provided evidence that sequence differences in site I can affect the structure of site II. Spyracopoulos et al. (36), through interpretation of $^3J_{HNH\alpha}$ coupling constants and backbone ^{15}N-relaxation measurements of McNTnC, suggest that sites I and II are structurally linked. It is not surprising, therefore, that the sequence differences in site I between ScNTnC and McNTnC affect the relative position of Ca^{2+}-binding sites I and II within the proteins when both structures were solved at 30°C (1). This illustrates that the sequence differences between ScNTnC and McNTnC affect the structural organization of site II and could therefore alter its ability to bind Ca^{2+}.

During Ca^{2+} activation, the residues comprising site I shift position within the protein in a manner similar to those of site II in both McNTnC (23) and ScNTnC (11). Modification to site I, through sequence manipulation at residues 28, 29, and 30, could therefore affect the ability of the protein to change conformation during activation. Such a change could affect the free-energy landscape associated with a conformational change

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in cTnC. For example, a reduction in the energy barrier (ΔG) needed to be overcome for a conformational change to occur would make it easier for ScTnC to be activated once Ca^{2+} binds site II. One approach to decrease ΔG is to increase the molecular flexibility at the region of the protein that undergoes the conformational change. Cold-adapted enzymes have been shown to compensate for the lower thermal energy at low temperatures by employing this strategy (3, 7, 9, 10).

The presence of the polar, hydrophilic Gln^{29} in ScTnC instead of the hydrophobic Leu has the potential to increase the molecular flexibility of ScTnC. A recent study by Tikunova and Davis (37) demonstrates that the replacement of hydrophobic residues at positions 20, 44, 45, 48, and 81 with Gln in McTnC increased the Ca^{2+} affinity of all mutants. These authors suggest that the insertion of Gln decreased internal hydrophobic interactions, thereby facilitating the opening of the molecule during Ca^{2+} activation. However, examination of the three-dimensional structure of McNTnC reveals that, as in ScNTnC, residue 29 is on the surface of the molecule (1). The loss of the hydrophobic residue should not, therefore, affect the stability of the core of the molecule. It is possible, however, that the L29Q substitution could increase the interaction between the protein and its solvent, thereby decreasing local protein stability. Functional evidence suggests that F27W ScTnC is less stable than F27W McTnC, as the former lost functionality at 37°C (12). We interpreted this result as a loss of tertiary structure, indicative of F27W ScTnC having greater molecular flexibility than F27W McTnC (12). Similar thermal denaturation has been noted by others (9, 35) studying enzymes from cold-adapted organisms. It is possible, therefore, that the residues responsible for the high Ca^{2+} affinity of ScTnC increase molecular flexibility, facilitating the protein to be activated by Ca^{2+}.

Comparison of the NMR structures of McNTnC and ScNTnC, both solved at 30°C, reveals that there are differences in the fold of the proteins. However, when ScNTnC solved at 7°C is compared with McNTnC solved at 30°C, these differences significantly decrease (1). Because 30°C and 7°C are close to the physiological temperatures of mammalian and trout hearts, respectively, these data suggest that McNTnC and ScNTnC have similar conformations at their respective physiological temperatures (1). F27W ScTnC and F27W McTnC also have similar function at their respective physiological temperatures. The $K_{f½}$ of F27W ScTnC at 7°C, pH 7.0, was 5.23 ± 0.03, and that for F27W McTnC at 37°C, pH 7.0, was 5.42 ± 0.02 (12). Although 1.5 times as much Ca^{2+} is required to half-saturate F27W ScTnC, these values are not appreciably different considering that the temperatures at which they were determined differ by 30°C. Additionally, if pH was allowed to vary with temperature according to α-stat, the pH of the F27W ScTnC at 7°C would be higher than 7.0, and, as a result, its affinity for Ca^{2+} would undoubtedly be greater. The difference in Ca^{2+} affinity between F27W ScTnC and F27W McTnC when compared under physiological conditions is likely even less. Similar conservation of protein function at physiological conditions has been previously reported for isoforms of lactate dehydrogenase (A_4-LDH) purified from different fish species that live at different temperatures (7). Together, these results suggest that, when compared under their respective physiological conditions, the structure and function of the NH_2 domain of McTnC and ScTnC are similar.

Gln^{29} has been found as a lone mutation (L29Q) in McTnC cloned from a patient with familial hypertrophic cardiomyopathy (FHC) (20). FHC, one of the most frequently inherited cardiac disorders, is caused by mutations in a number of the sarcomeric proteins (31). A common etiology of this disease is an alteration in the dynamics of Ca^{2+} activation (31). Although the functional significance of L29Q in McTnC is currently unknown, it is possible that it could affect the Ca^{2+} affinity of site II as the opposite mutation, Q29L, decreased the Ca^{2+} affinity of site II in ScNTnC (13).

The difference in Ca^{2+} affinity between F27W McTnC and F27W NIQD McTnC is almost identical to the difference in Ca^{2+} sensitivity between myocytes containing WT McTnC and NIQD McTnC (Fig. 3, *B* and *C*). In addition to confirming that Asn^2, Ile^{28}, Gln^{29}, and Asp^{30} are responsible for the high Ca^{2+} affinity of F27W ScTnC, this result demonstrates that ScTnC is at least partially responsible for the comparatively high Ca^{2+} sensitivity of trout cardiac myocytes.

To characterize the Ca^{2+} affinity of the different cTnC mutants, a Trp was inserted at residue 27, as this mutation (F27W) introduces the fluorescent reporter. It has been assumed that any affect of this mutation on cTnC function would be negated, as all proteins characterized contained the mutation (11–13). The results of the cTnC replacement studies confirmed this assumption, as none of the proteins integrated into the myocytes contained the F27W mutation, but the difference in Ca^{2+} sensitivity between the myocytes containing WT McTnC and NIQD McTnC was almost identical to the difference in Ca^{2+} affinity determined using the F27W mutation in the fluorescence studies.

The cTnC replacement study was completed at 15°C, pH 7.0, whereas the fluorescent measurements were completed at 21°C, pH 7.0. A temperature of 15°C was used, as the myocytes maintained functional integrity at this temperature longer. The difference in temperature at which these two studies were completed is not expected to affect the relative difference in Ca^{2+} affinity between cTnC isoforms, as we have demonstrated that the difference in Ca^{2+} affinity between F27W ScTnC and F27W McTnC is maintained over a range of temperatures and pHs (12). As both temperature and pH were held constant in each study, the only factors responsible for any differences in Ca^{2+} affinity or Ca^{2+} sensitivity are the differences in protein sequence.

Numerous studies have demonstrated that the Ca^{2+} sensitivity of both cardiac and skeletal myocyte preparations can be altered by replacing endogenous cTnC or skeletal TnC with other TnC isoforms or mutants (17, 19, 25, 27, 28). However, this is the first known study in which the Ca^{2+} sensitivity of force generation in cardiac myocytes has been substantively increased by replacing endogenous cTnC with a mutant McTnC isoform. Because of its sensitizing capabilities, NIQD McTnC may therefore represent a new tool in developing treatment modalities for pathologies such as heart failure to increase inotropism and stroke volume.

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