

**Ca²⁺ BINDING TO CARDIAC TROPONIN C:
EFFECTS OF TEMPERATURE AND PH
ON CLONED MAMMALIAN AND SALMONID ISOFORMS**

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Abstract

A reduction in temperature lowers the Ca²⁺ 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 if ScTnC is more sensitive to Ca²⁺ 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), 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, while 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 while 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.

Introduction

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) that consists of two homologous, globular domains each containing two possible Ca^{2+} binding sites. The N-terminal, regulatory domain contains sites I and II while the C 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. Sites III and IV in the C-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_{\max}) in cardiac muscle and reduces the sensitivity of the contractile element to $[\text{Ca}^{2+}]$ [Harrison and Bers 1990]. However, replacement of native cTnC with mammalian skeletal TnC (sTnC) in rat cardiac muscle relieves the desensitizing effect of low temperature on contractility [Harrison and Bers 1990] 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 cTnC 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 which 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 Ca²⁺. Over physiological temperatures, Ca²⁺ sensitivity of salmonid cardiac myofibrils is much greater than those isolated from rat [Churcott et al. 1994] and other mammals.

The purpose of this study is to determine if the differences in primary structure between bovine cTnC (BcTnC) and salmonid cTnC (ScTnC) result in differences in the Ca²⁺ sensitivity of the two molecules. To accomplish this Ca²⁺ binding to site II in these two cTnC isoforms was measured in solution over a range of temperatures using F27W mutants of BcTnC and ScTnC. We have previously shown that the tryptophan engineered into this position of the molecule acts as a fluorescent reporter of Ca²⁺ binding to site II [Moyes et al 1996].

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 using the Stratagene Quick Change Site Directed Mutagenesis Kit. The nucleotide sequences of the two newly mutated plasmids were confirmed by sequencing and subcloned into pGex expression vectors. The fusion protein GST-cTnC was expressed, purified, cleaved with Factor Xa and purified by column chromatography with phenyl sepharose. The identities of ScTnC and BcTnC were confirmed by sequencing the first 5 amino acids from the amino terminal end of the isolated protein.

Fluorescence Studies.

The cTnC samples were diluted to 2.5 µM and fluorescence was measured using an SLM 4800C spectrofluorometer. The protein samples were titrated by pipeting Ca²⁺ stock in 1 µl increments and fluorescence was measured over a

10 s period for each $[Ca^{2+}]$ using an excitation wavelength of 276 nm and an emission wavelength of 330 nm.

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. The effects of temperature, pH, and isoform on $K_{1/2}$ values (pCa required to give half-maximal fluorescence) as determined by the Hill equation curve fitting were analyzed statistically using a one-way repeated measures analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test using the statistical software package SigmaStat. The values reported for $K_{1/2}$ are expressed as mean \pm SEM in pCa units.

Results

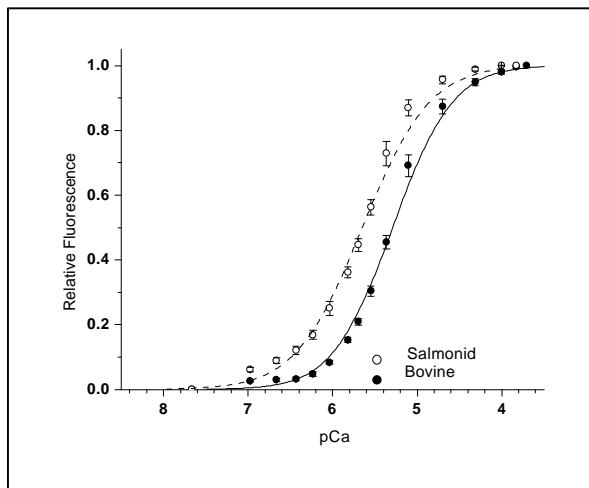


Figure 1. Shown on the left is the difference in Ca^{2+} sensitivity between bovine cTnC and salmonid cTnC under identical conditions of 21.0°C, pH 7.0. Data are normalized by the max. fluorescence of each ex-periment and presented as mean \pm SEM. The curves were generated by fitting the data with the Hill equation. The derived $K_{1/2}$ values, the pCa at

half-maximal fluorescence, are significantly ($p < 0.05$) different between the

two species. These data indicate that the Ca^{2+} sensitivity is 2.3 fold greater in salmonid compared to bovine cTnC.

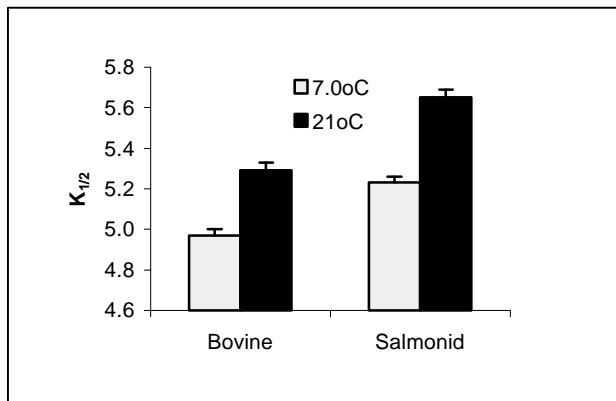


Figure 2. Shown on the left is the effect of lowering temperature from 21 to 7°C on bovine and salmonid cTnC Ca²⁺ sensitivity while keeping pH constant at 7. The K_{1/2} was reduced by 0.32 and 0.42 in bovine and salmonid cTnC isoforms, respectively. While the

degree of attenuation with hypothermia is significant (p< 0.05) and similar between the isoforms, the salmonid has significantly greater sensitivity for Ca²⁺ at both temperatures.

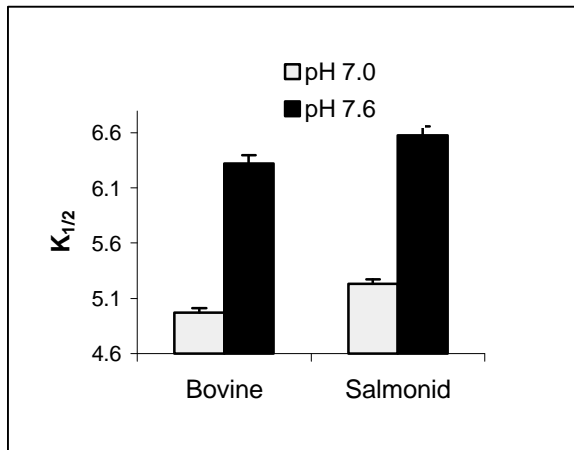


Figure 3. The effect of pH on bovine and salmonid cTnC Ca²⁺ sensitivity while keeping temperature constant at 7°C. In-creasing the pH from 7.0 to 7.6 increased the sensitivity by 1.35 pCa units (or 22 fold) for both isoforms. These data are consistent with previous on the effect of pH on cardiac myofilament Ca²⁺ sensitivity (Palmer and

Kentish 1994).

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) is shown in Figure 4 below. This figure demonstrates that salmonid cTnC is 1.17 pCa units (14.8 fold) more sensitive to Ca^{2+} than the bovine isoform despite the attenuating effect of hypothermia on $K_{1/2}$.

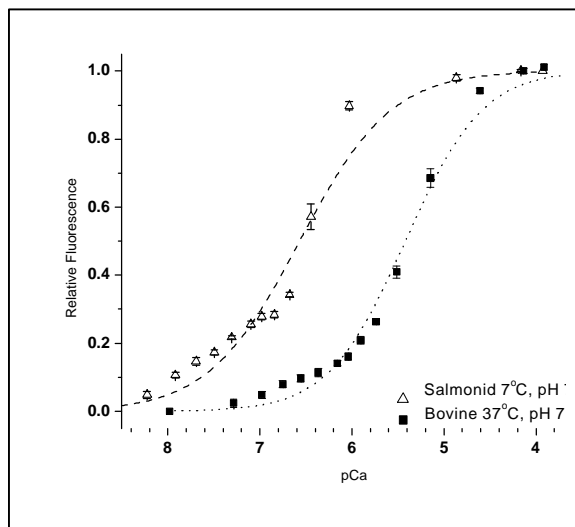


Figure 4. Ca^{2+} sensitivity of TnC isoforms under the “normal” physiological conditions of each organism.

Discussion

It has been clearly demonstrated that a reduction in environmental temperature reduces the sensitivity of the contractile element to $[\text{Ca}^{2+}]$ in cardiac myofibrils isolated from mammals, frogs and salmonids [Churcott et al 1994; Harrison and Bers 1990]. The results of the present study suggest that it is the effect of temperature on the Ca^{2+} binding characteristics of cTnC, which is at least partly responsible for this effect. Both isoforms exhibited similar degrees of reduction in Ca^{2+} sensitivity with a decrease in temperature.

The purpose of measuring Ca^{2+} sensitivity while keeping temperature constant but altering pH was to determine the physiological role of α -stat regulation in maintaining function in salmonid hearts. 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 Ca²⁺ sensitivity [Churcott et al 1994]. In the present study the sensitivity of both BcTnC and ScTnC were affected to the same degree by an increase in pH. It is clear that an increase in pH, as would occur during α -stat regulation, sensitizes the cTnC molecule to Ca²⁺ and this effect could help maintain contractility in the salmonid myocyte at low temperatures. The effect of pH on

The relative small differences in amino acid sequence between the salmonid and bovine cTnC appears to have a significant effect on their ability to bind Ca²⁺. The higher Ca²⁺ sensitivity of tension generation of salmonid ventricular fibres compared to those of a mammal is due, at least in part, to the enhanced Ca²⁺ sensitivity of ScTnC. As there is complete sequence identity of site II between the two isoforms, the variation in Ca²⁺ sensitivity must be as a result of differences elsewhere in the protein.

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References

- Churcott, C. S., C. D. Moyes, B. H. Bressler, K. M. Baldwin, and G. F. Tibbits. (1994) Temperature and pH effects on Ca²⁺ sensitivity of cardiac myofibrils: a comparison of trout with mammals. *Am. J. Physiol.* 267: R62-70.
- Harrison, S. M., and D. M. Bers. (1990) Modification of temperature dependence of myofilament Ca sensitivity by troponin C replacement. *Am. J. Physiol.* 258: C282-8, 1990.
- Moyes, C. D., T. Borgford, L. LeBlanc, and G. F. Tibbits. (1996) Cloning and expression of salmon cardiac troponin C: titration of the low-affinity

Ca²⁺-binding site using a tryptophan mutant. *Biochemistry* 35: 11756-62.

Palmer, S., and J. C. Kentish. (1994) The role of troponin C in modulating the Ca²⁺ sensitivity of mammalian skinned cardiac and skeletal muscle fibers. *J. Physiol. Lond.* 480: 45-60.

Spyracopoulos, L., M. X. Li, S. K. Sia, S. M. Gagne, M. Chandra, R. J. Solaro, and B. D. Sykes. (1997) Calcium-induced structural transition in the regulatory domain of human cardiac troponin C. *Biochemistry* 36: 12138-46.

