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RESEARCH ARTICLE

The influence of trout cardiac troponin I and PKA phosphorylation on the Ca^{2+} affinity of the cardiac troponin complex

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SUMMARY

The trout heart is 10-fold more sensitive to Ca^{2+} than the mammalian heart. This difference is due, in part, to cardiac troponin C (cTnC) from trout having a greater Ca^{2+} affinity than human cTnC. To determine what other proteins are involved, we cloned cardiac troponin I (cTnI) from the trout heart and determined how it alters the Ca^{2+} affinity of a cTn complex containing all mammalian components (mammalian cTn). Ca^{2+} activation of the complex was characterized using a human cTnC mutant that contains anilinonapthalenesulfote iodoacetamide attached to Cys53. When the cTn complex containing labeled human cTnC was titrated with Ca^{2+}, its fluorescence changed, reaching an asymptote upon saturation. Our results reveal that trout cTnI lacks the N-terminal extension found in cTnI from all other vertebrate groups. This protein domain contains two targets (Ser23 and Ser24) for protein kinase A (PKA) and protein kinase C. When these are phosphorylated, the rate of cardiomyocyte relaxation increases. When rat cTnI in the mammalian cTn complex was replaced with trout cTnI, the Ca^{2+} affinity was increased ~1.8-fold. This suggests that trout cTnI contributes to the high Ca^{2+} sensitivity of the trout heart. Treatment of the two cTn complexes with PKA decreased the Ca^{2+} affinity of both complexes. However, the change for the complex containing rat cTnI was 2.2-fold that of the complex containing trout cTnI. This suggests that the phosphorylation of trout cTnI does not play as significant a role in regulating cTn function in trout.

Key words: adrenergic stimulation, calcium activation, cardiac thin filament proteins.

INTRODUCTION

When the temperature of the vertebrate heart is reduced, its ability to contract and generate force is impaired. This effect of temperature change is due to a reduction in the activity of Ca^{2+} handling proteins, such as the sarcolemmal Na^{+}/Ca^{2+} exchanger (Elias et al., 2001), and to the contractile element losing its ability to respond to changes in intracellular Ca^{2+} (Churcott et al., 1994; Gillis et al., 2000; Harrison and Bers, 1989). Ca^{2+} is the intracellular signal that initiates contraction by binding to the thin filament *via* cardiac troponin C (cTnC). The Ca^{2+} activation of cTnC initiates a series of conformational changes through the components of the thin filament, resulting in the formation of force-generating cross-bridges between actin and myosin.

For ectothermic species, such as rainbow trout, the desensitizing effect of temperature on contractile function represents a potential challenge. Trout live, and remain active, in waters where temperatures range seasonally between 4 and 20°C (Black, 1950). Such temperatures would cause cardioplegia in the mammalian heart (Harrison and Bers, 1989; Churcott et al., 1994). One characteristic of the trout heart that enables it to function at these low temperatures is that it is 10 times more sensitive to Ca^{2+} than mammalian hearts when measured at the same temperature (Churcott et al., 1994). This helps to compensate for the desensitizing effect of low temperature, as the Ca^{2+} sensitivity of the trout heart and mammalian heart are similar at their respective physiological temperatures (Churcott et al., 1994; Gillis and Tibbits, 2002). The comparatively high Ca^{2+} sensitivity of the trout heart is due in part to trout cTnC having 2.3-fold the Ca^{2+} affinity of human cTnC (Gillis et al., 2000). The residues in trout cTnC responsible for its comparatively high Ca^{2+} affinity are Asn2, Ile28, Gln29 and Asp30 (Gillis et al., 2005). When human cTnC was mutated to contain these residues and the mutant protein was incorporated into rabbit cardiac myocytes, the Ca^{2+} sensitivity of force generation was increased 2-fold (Gillis et al., 2005). This clearly shows that trout cTnC can increase the Ca^{2+} sensitivity of force generation but indicates that other mechanisms are involved in the comparatively high Ca^{2+} sensitivity of the trout heart.

A second candidate for increasing the Ca^{2+} sensitivity of the trout heart is cardiac troponin I (cTnI). This regulatory protein, along with cTnC and cardiac troponin T (cTnT), form the cardiac troponin (cTn) complex. When the heart is relaxed, the inhibitory peptide of cTnI (residues 128–147) interacts with actin and prevents the movement of tropomyosin (Tm) across the surface of the thin filament. In this state Tm is thought to sterically block the formation of force-producing cross-bridges between actin and myosin (Kentish et al., 2001; Li et al., 2004; Rarick et al., 1999; Solaro and Rarick, 1998). Following the release of Ca^{2+} from the sarcoplasmic reticulum, Ca^{2+} binds to the regulatory domain of cTnC (site II). The resulting conformational change triggers the movement of the switch peptide of cTnI (residues 147–163) towards a hydrophobic cleft that has been exposed within the N-terminus of cTnC (Kentish et al., 2001; Li et al., 2004; Rarick et al., 1999; Solaro and Rarick, 1998). This movement of the switch peptide pulls the adjacent inhibitory peptide away from the actin–Tm complex (Kentish et al., 2001; Li et al., 2004; Rarick et al., 1999; Solaro and Rarick, 1998). As a result, Tm is released and it is able to roll across the surface of actin and expose myosin binding sites (Gordon et al., 2000).

In the mammalian heart, cTnI is a critical site for the regulation of thin filament function. It is targeted for phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and protein kinase D (PKD) following adrenergic stimulation (Kobayashi and Solaro, 2005; Cuello et al., 2007; Haworth et al., 2004). One domain of cTnI found in mammalian species that is targeted for phosphorylation is the N-terminal extension (residues 1–32), which contains two phosphorylatable residues (Ser23 and Ser24). This region of cTnI interacts with the N-terminal domain of cTnC and the strength of the interaction increases during Ca^{2+} activation (Robertson et al., 1982). When Ser23 and Ser24 are phosphorylated, this interaction between cTnI and cTnC is thought to weaken, leading to a reduction in the Ca^{2+} affinity of the regulatory domain of cTnC (Chandra et al., 1997; Robertson et al., 1982; Solaro et al., 2008). This translates into a decrease in the Ca^{2+} sensitivity of force generation and increased rates of muscle relaxation (Kentish et al., 2001; Li et al., 2004; Matsuba et al., 2009; Rarick et al., 1999; Solaro and Rarick, 1998). The change in cTnC Ca^{2+} affinity upon cTnI phosphorylation illustrates the fact that changes in the structural properties of cTnI can alter the ability of the cTn complex to be activated by Ca^{2+}.

The purpose of this study was to determine how trout cTnI influences the Ca^{2+} affinity of a troponin complex containing mammalian components (cTnC, cTnT, cTnI) and to characterize how PKA phosphorylation modulates this. To do this, we cloned cTnI from a trout heart cDNA library, expressed the protein and then complexed it with rat cTnT and the human cTnC mutant C35S, T53C, C84S human cTnC (T53C human cTnC) labeled with anilinonapthalenesulfote iodoacetamide (IAANS) at Cys53. IAANS is a fluorescent molecule and by titrating this complex with Ca^{2+} and measuring fluorescence we were able to monitor the change in cTnC conformation that occurs during Ca^{2+} activation. Using this technique we compared the Ca^{2+} affinity of the cTn complex containing trout cTnI with that of one containing rat cTnI. In addition, we characterized how the Ca^{2+} affinity of these two protein complexes is altered following PKA treatment.

MATERIALS AND METHODS

Cloning of TnI from trout cardiac cDNA

Nested PCR with degenerate primers was first utilized to clone an interior region of the cTnI cDNA and then RACE-PCR was used to clone the 5′ and 3′ ends of the gene. The degenerate primers were designed to target a region of high conservation identified using known cTnI sequences from the zebrafish (*Danio rerio*), and two species of puffer fish (*Takifugu rubripes* and *Tetraodon nigroviridis*). One forward (F) and two reverse primers (R1 and R2) were chosen. The primer sequences are as follows: cTnI-F: GAC RWR GTG GAT GAR GAG MGR TAY GA, cTnI-R1: CTT CTT CAC CKK CTT GAG GTK GG, cTnI-R2: CSA YRT TCT TRC GCC AGT CKS YCA C; where R, W, M, Y, K and S correspond to standardized coding used to indicate specific, multiple nucleotides in the same position within degenerate primers.

The first PCR reaction was performed using trout heart cDNA as the template with F and R2 as the primers. The second PCR reaction was performed using the product of the first PCR reaction with F and R1 as the primers. Following PCR, the DNA was inserted into the StrataClone™ PCR cloning vector pSC-A (Stratagene, Agilent Technologies Inc., La Jolla, CA, USA) for sequencing.

RACE-PCR

RACE-PCR was completed using primers cTnI-F and cTnI-R2 and the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). Two different samples of RACE-ready cDNA were created from different hearts and used as separate templates for each reaction. The products of each RACE-PCR reaction were inserted into the StrataClone™ PCR cloning vector and sequenced.

Generating full-length genes

Primers were designed for the 5′ and 3′ ends of each gene based on the sequences obtained *via* RACE-PCR. PCR was performed with multiple samples of trout heart cDNA and the products were cloned into the sequencing plasmid. Sequencing results demonstrate that each PCR product was the same.

Subcloning of trout cTnI

The full-length trout cTnI cDNA was inserted into a pET-24a(+) expression vector (Novagen, San Diego, CA, USA) by engineering *Nde*I and *Eco*RI restriction sites into the 5′ and 3′ ends of the gene, respectively. The sequence of the primers used was: cTnI-*Nde*I-F: C ATA TGC CAG AGC AAG TAC AAG AGA AGC G and cTnI-*Eco*RI-R: G AAT TCT ACT GAG GGG TGC CTT TGG C. The PCR product was subsequently digested with *Eco*RI and *Nde*I, then gel purified. The insert was ligated into linearized pET-24a(+) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The ligated plasmid was transfected into NovaBlue competent cells (Novagen). Liquid cultures were grown overnight and isolated plasmids were sent for sequencing to confirm the presence of the insert.

Confirmation of trout cTnI identity

To confirm that the protein resulting from the cloned gene corresponds to that expressed in the trout heart, western blotting and mass spectrometry were used to characterize the cTnI present in trout cardiac myofilament proteins. Multiple lanes of trout myofilament proteins were separated using SDS-PAGE with recombinant rat cTnI used as the standard. We took care to ensure that equal amounts of protein were loaded per lane. The SDS-PAGE gel was transferred onto Hybond-P PVDF membranes using an ECL semi-dry transfer unit (GE Healthcare, Piscataway, NJ, USA). The primary antibody was a goat polyclonal raised against the C-terminus of human cTnI (sc-8118, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). This antibody also binds fast skeletal (fsTnI) and slow skeletal TnI (ssTnI) from a number of vertebrate species (T.E.G., unpublished) (Santa Cruz Biotechnology product literature). The secondary antibody was a rabbit anti-goat IgG conjugated to horseradish peroxidase (AP106P; Millipore, Etobicoke, ON, Canada). The western blot identified rat cTnI on the membrane as well as a single protein band in the trout heart sample that was of lower mass (Fig. 1).

Mass spectometry analysis of trout cTnI

A sample of the trout myofilament proteins used in western blotting was separated using SDS-PAGE and then transferred onto Hybond-P PVDF membranes. The membrane was stained with Coomassie R250 and the single band, from multiple lanes, corresponding to that identified using western blotting was excised and prepared for trypsin digestion and mass spectroscopy analysis at the Advanced Analysis Center at the University of Guelph. (Note that there was only a single band on the protein gel in the region of the band that was bound by the western antibody; see Fig. 1.) Briefly, the excised gel bands were destained until colorless, and dried using a SpeedVac. Following reduction with dithiothreitol (DTT) and alkylation with iodoacetamide, the protein was digested with 10 ng of sequencing grade trypsin (Calbiochem, La Jolla, CA, USA) in 25 mmol l^{-1} NH_4HCO_3 (pH 7.6) at 37°C overnight. The proteolytic peptides were

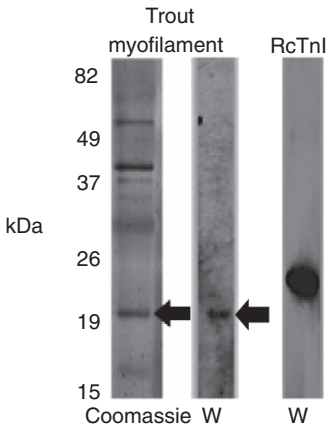

Fig. 1. Identification of native trout cTnI (ScTnI, where S refers to the fact that trout are salmonids) using a human cTnI antibody for western blotting. The first lane is an image from an SDS gel stained with Coomassie R250 on which trout myofilament proteins were separated. The second and third lanes are from a western blot (W) using a goat polyclonal antibody raised against human cTnI (sc-8118). Lane two corresponds to a sample of the trout myofilament proteins used in lane 1. Lane three corresponds to a sample of recombinant rat cTnI (RcTnI).

extracted, and cleaned up by a C18 Ziptip (Millipore). MALDI data were acquired using an Applied Biosystems/MDS Sciex QStar XL quadrupole time-of-flight (QqTOF) mass spectrometer under a nitrogen laser (337 nm), and 2,5-dihydroxybenzoic acid was used as the matrix. The peptide fingerprinting masses (*m*/*z*) on the MS spectrum were compared with the theoretical values generated *in silico* by MS-Digest, a ProteinProspector program developed at the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu/). Using this method the peptide fingerprint generated from the protein band was compared with those for proteins within the National Center for Biotechnology Information (NCBI) protein database, including trout cTnI.

Expression of troponin components

The cDNA for rat cTnI and rat cTnT were provided by Dr M. Regnier (University of Washington) while the cDNA for T53C human cTnC was provided by Dr J. P. Davis (University of Ohio). All proteins were expressed using BL21 Gold (DE3) competent *E. coli* cells (Agilent Technologies Inc.) with expression being initiated with IPTG (0.4 mmol l^{-1}). Each protein was purified using an AKTA FPLC (GE Healthcare) and standard procedures. T53C human cTnC was purified as detailed previously (Gillis et al., 2007), rat cTnT was purified as in Chandra et al. (Chandra et al., 2006), while rat cTnI and trout cTnI were purified as in Guo et al. (Guo et al., 1994). One modification to optimize the purification of trout cTnI was that all buffers used on the CM cation exchange column had a pH of 7.0 instead of 8.0. Following purification, the identity of each protein was confirmed using peptide sequence analysis as described above. All proteins were lyophilized and then stored at –20°C.

IAANS labeling of T53C human cTnC

Purified T53C human cTnC was labeled as in Davis et al. (Davis et al., 2007). In brief, T53C human cTnC was dialyzed into (in mmol l^{-1}): 50 Tris, 6000 urea, 90 KCl, 1 EGTA, pH 7.5. Protein concentration was determined using a Bradford assay and then 5-fold molar excess of IAANS was added to the solution. The reaction was allowed to occur for 8 h in the dark, and then stopped with 2 mmol l^{-1} DTT. IAANS-labeled T53C human cTnC (human $cTnC^{IAANS}$) was then dialyzed against (in mmol l^{-1}): 10 MOPS (pH 7.0), 4600 urea, 1 DTT, with 0.01% NaN_3. IAANS concentration was calculated from its absorbance at 325 nm using its extinction coefficient of 24,900 l mol^{-1} cm^{-1}. The efficiency of binding was calculated by dividing the concentration of human $cTnC^{IAANS}$ by the IAANS concentration (binding efficiency was 92%).

Tn complex formation

Tn complexes were prepared following Davis et al. (Davis et al., 2007). Tn subunits were dialyzed separately into (in mmol l^{-1}): 10 MOPS (pH 7.0), 4600 urea, 1 DTT, with 0.01% NaN_3. Bradford assays were utilized and the subunits were mixed for 20 min at a molar ratio of 1.5:1:1.5 (TnI:TnC:TnT). The control group contained rat cTnI/human $cTnC^{IAANS}$/rat cTnT (mammalian cTn^{IAANS}) and the experimental group contained trout cTnI/human $cTnC^{IAANS}$/rat cTnT (trout cTnI-cTn^{IAANS}). The sequence of rat cTnC varies from that of human cTnC by 1 residue out of 161. This difference is a methionine instead of an isoleucine at residue 119. This is a conservative substitution that should not alter the function of the protein. Therefore, the proteins in the mammalian cTn complex would be interacting with each other within a 'rat' conformation. The complexes were dialyzed against a series of buffers containing (in mmol l^{-1}): 10 MOPS (pH 7.0), 0.5 DTT, 3 $MgCl_2$, with 0.01% NaN_3. The first buffer contained 2 mol l^{-1} urea and 1 mol l^{-1} KCl; the second contained 1 mol l^{-1} KCl; and the third contained 0.15 mol l^{-1} KCl (repeated three times). The complexes were then dialyzed against a titration buffer containing (in mmol l^{-1}): 200 MOPS (pH 7.0), 150 KCl, 3 $MgCl_2$, 1 DTT, 2 EGTA, with 0.02% Tween-20.

PKA phosphorylation

The PKA treatment was completed when the proteins were in a complex so that they would be in a 'physiological' conformation interacting with cTnC and cTnT. This was done to ensure that only targets that are phosphorylated *in vitro* would be targeted in this experiment. Samples of trout cTnI-cTn^{IAANS} and mammalian cTn^{IAANS} were each diluted to 0.35 mg ml^{-1} by the addition of titration buffer. PMSF and Na_2ATP were then added to final concentrations of 0.25 and 10 mmol l^{-1}, respectively. The catalytic subunit of PKA (20 units) was added to 1 ml volumes of each protein complex. The mixtures were incubated for 1 h at 25°C and then dialyzed against titration buffer. The control groups were treated in the same way but without the addition of PKA or Na_2ATP. Phosphorylated trout cTnI-cTn^{IAANS} and mammalian cTn^{IAANS} will be designated as trout cTnI-$cTn^{IAANS*P}$ and mammalian $cTn^{IAANS*P}$.

Analysis of phosphoproteins

The protein complexes were separated using SDS-PAGE and the gels were stained using Pro-Q Diamond phosphoprotein stain following the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Stained gels were imaged using a Typhoon 9410 scanner (GE Healthcare) with an excitation wavelength of 532 nm and an emission filter of 560 nm. Following imagining of Pro-Q-stained gels, gels were stained for total protein using Coomassie R250.

Ca^{2+} titrations

The Ca^{2+} affinity of each Tn complex was measured as in Davis et al. (Davis et al., 2007) using a spectrofluorometer (model LS 55; Perkin Elmer, Waltham, MA, USA). In brief, 2 ml volumes of 0.15 μmol l^{-1} Tn were titrated with 1 μl volumes of $CaCl_2$ stock solutions. Quartz cuvettes were used for the titrations, temperature

was maintained at 15°C and a stir bar provided constant mixing. The excitation wavelength was 330 nm and fluorescence was monitored at 450 nm. Following each addition of $CaCl_2$, the sample was mixed for 1 min and a reading was then taken. As in the Davis et al. study (Davis et al., 2007), as Ca^{2+} bound to Tn, the resulting conformational change caused the intensity of IAANS fluorescence to decrease. The free Ca^{2+} concentrations were determined as described previously (Gillis et al., 2000) using MaxChelator (Bers et al., 1994). The free Ca^{2+} concentrations were plotted against the percentage maximal fluorescence (relative fluorescence) of IAANS. Curves were fitted to the Hill equation:

$$Y = F_{max}[x^{nH} / (K_{F1/2}{}^{nH} + x^{nH})] , \quad (1)$$

using Sigma Plot 9.0. (Systat Software, Richmond, CA, USA), where Y is the relative fluorescence, F_{max} is the maximum Ca^{2+}-dependent fluorescence equal to 1, x is the free [Ca^{2+}] (mol l^{-1}), n_H is the Hill coefficient and $K_{F1/2}$ is the Ca^{2+} concentration at half-maximum Ca^{2+}-dependent fluorescence. $K_{F1/2}$ was converted to pCa (–log[Ca^{2+}]) and used as a measure of Ca^{2+} affinity.

Data and statistical analysis

Differences between the $K_{F1/2}$ values and Hill coefficients (n_H) generated by fitting each titration curve with the Hill equation were analyzed using two-way ANOVA followed by Bonferonni *post hoc* tests (P<0.05). Data are represented as means ± s.e.

RESULTS

Sequencing results of trout cTnI

The cDNA for trout cTnI is 570 nucleotides (GenBank accession number HM012798). The protein encoded by this gene is 189 amino acids, which is 21 residues shorter than human cTnI. Interestingly, trout cTnI lacks the cardiac-specific N-terminal extension that contains the PKA/PKC/PKD phosphorylation targets (serine residues) at positions 23 and 24 (Fig. 2). All other cTnIs cloned from fish species also lack this extension (Shaffer and Gillis, 2010). Trout cTnI does, however, contain Ser residues at positions 14 and 16. The corresponding residues in human cTnI are the PKC targets Ser42 and Ser44 (Fig. 2). In addition, there is also a Ser at residue 115 in trout cTnI that corresponds to the PKC target Thr143 in human cTnI (Fig. 2). At the amino acid level trout cTnI displays 59% identity to the human cTnI paralog while it is 78% identical to human ssTnI.

Mass spectometry analysis of native trout cTnI

The single TnI band in the trout cardiac myofilament protein sample was identified as trout cTnI with a display score of 88% with 72% coverage. This was the best score obtained for any protein within the NCBI protein database. The identified peptides obtained from this analysis are mapped onto the protein sequence of trout cTnI in Fig. 2. This analysis clearly indicates that the TnI gene we cloned from trout heart cDNA corresponds to the TnI protein expressed in the heart.

PKA phosphorylation of trout cTnI-cTn and mammalian cTn

The treatment of trout cTnI-cTn and mammalian cTn with PKA resulted in the phosphorylation of trout cTnI and rat cTnI. Fig. 3 is a composite image establishing the phosphorylation state of rat cTnI and trout cTnI following PKA treatment. The lanes in Fig. 3A show recombinant rat cTnI and trout cTnI stained with Coomassie R250, demonstrating the difference in protein mass. The western blot in Fig. 3B identifies recombinant rat cTnI and trout cTnI using the human cTnI antibody sc-8118. Fig. 3C is a comparison image of control and phosphorylated rat cTnI and trout cTnI stained with Pro-Q Diamond stain and Coomassie R250. The levels of these images have not been modified. From Fig. 3C it is evident that there is no Pro-Q signal from either of the non-phosphorylated bands while there is a clear signal from the phosphorylated bands. From this it is evident that the phosphorylation of mammalian cTn and trout cTnI-cTn increases the strength of each TnI band on the Pro-Q stained gel. The Coomassie-stained bands indicate that there was equal loading of protein between the control and PKA-treated samples. As expected, there was no evidence that either cTnC or cTnT was phosphorylated by PKA treatment (data not shown). To determine whether there were differences in the levels to which trout cTnI and rat cTnI were phosphorylated by PKA, we utilized ImageJ (National Institutes of Health, Bethesda, MD, USA) to analyze the density of the TnI bands on the protein gels stained with Pro-Q and Coomassie R250. To correct for differences in the amount of protein in each band, we standardized the density of each band stained with Pro-Q to the density of the same band stained with Coomassie. This analysis demonstrated that the Pro-Q-stained bands of phosphorylated rat cTnI had a higher density than the Pro-Q-stained bands of phosphorylated trout cTnI. For rat cTnI the standardized density was 1.03±0.02 and that for trout cTnI was 0.85±0.07. This

Fig. 2. Amino acid sequence of trout cTnI (ScTnI) derived from the cloning and sequencing of ScTnI cDNA. Amino acid sequence comparison of ScTnI with human (H) cTnI (HcTnI); human fast skeletal TnI (HfsTnI) and human slow skeletal TnI (HssTnI). Important features of cTnI are indicated on the figure including the position of protein kinase A (PKA), protein kinase C (PKC) and protein kinase D (PKD) phosphorylation sites as well as the cardiac-specific N-terminal peptide. The results of QqTOF mass spectroscopy sequence analysis of trypsin-digested native ScTnI are mapped onto the ScTnI sequence. The boxes indicate peptides that matched between the sample and the sequence. A confidence level of 88% with 72% coverage was obtained, indicating that the sequence obtained *via* RACE-PCR corresponds with that of the native sample.

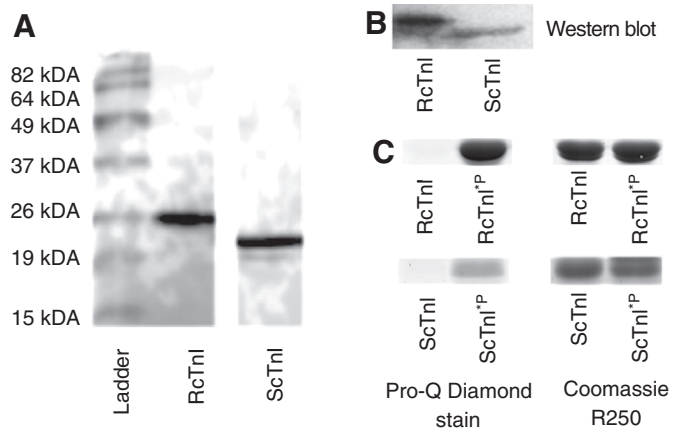

Fig. 3. Characterization of the effect of PKA treatment on the phosphorylation state of rat cTnI (RcTnI) and trout cTnI (ScTnI). (A) SDS gel containing recombinant RcTnI and ScTnI stained with Coomassie R250. (B) Western blot of recombinant RcTnI and ScTnI. (C) Bands of control and PKA-phosphorylated RcTnI ($RcTnI^{*P}$) and ScTnI ($ScTnI^{*P}$) from the same SDS gel stained with Pro-Q Diamond stain (phosphoprotein) and then Coomassie R250 (total protein). For all protein gels, we took care to ensure equal amounts of protein were loaded in each lane.

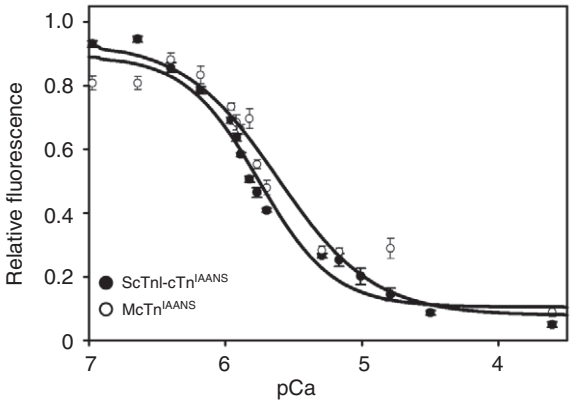

Fig. 4. Comparison of the Ca^{2+} titration curves of trout cTnI-cTn^{IAANS} (ScTnI-cTn^{IAANS}; N=8) and mammalian cTn^{IAANS} ($McTn^{IAANS}$; N=6). Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and are presented as means ± s.e. The curves generated by fitting the data with the Hill equation have been added for comparison with the data points. Comparison of the Ca^{2+} concentration at half-maximal fluorescence, $K_{F1/2}$, of the two curves indicates that the Ca^{2+} affinity of the cTn complex containing trout cTnI (ScTnI-$cTnI^{IAANS}$) is 1.8-fold more sensitive to Ca^{2+} than that containing rat cTnI ($McTn^{IAANS}$) (P<0.05).

indicates that the level of phosphorylation of the trout cTnI sample was ~82.5% that of the rat cTnI sample. As the same concentrations of the trout cTnI-cTn and mammalian cTn complexes were treated with the same number of units of PKA, this difference indicates that more phosphate groups were incorporated into rat cTnI than into trout cTnI. This can be interpreted as there being more phosphorylatable targets in rat cTnI than in trout cTnI. This result reflects the fact that trout cTnI does not contain the N-terminal extension but that it does contain other putative PKA targets.

Ca^{2+} titrations

The Ca^{2+} titration of each Tn complex produced sigmoidal curves that reached an asymptote upon saturation (Fig. 4). The data were fitted well by the Hill equation with a goodness of fit for all curves being greater than 0.89 (Table 1). The titration curve for trout cTnI-cTn^{IAANS} is positioned to the left of that for mammalian cTn^{IAANS} (Fig. 4). The $K_{F1/2}$ for trout cTnI-cTn^{IAANS} was significantly less than that of mammalian cTn^{IAANS} (1.46±0.01 *vs* 2.62±0.35 μmol l^{-1}) (Table 1). This difference indicates that the Ca^{2+} affinity of trout cTnI-cTn^{IAANS} is 1.80-fold that of mammalian cTn^{IAANS}. Phosphorylation of the trout cTnI-cTn^{IAANS} and mammalian cTn^{IAANS} caused the titration curves to shift to the right (Fig. 5), indicating that the Ca^{2+} affinity of the complexes was reduced by this treatment. The $K_{F1/2}$ of trout cTnI-$cTn^{IAANS*P}$ shifted 2.06 μmol l^{-1} while that for mammalian $cTn^{IAANS*P}$ shifted 4.51 μmol l^{-1}. This clearly shows that the presence of trout cTnI reduces the influence of PKA treatment on the Ca^{2+} affinity of the Tn complex. The Hill coefficients calculated for the titration curves of trout cTnI-cTn^{IAANS} and mammalian cTn^{IAANS} were less than 1 (Table 1) and there was no effect of PKA phosphorylation on these values.

The use of IAANS-labeled T53C human cTnC to study the Ca^{2+} activation of the cTn complex is a technique that was established by Davis et al. (Davis et al., 2007). These authors demonstrated that the conservative mutations (C35S, T53C, C84S) required to allow the labeling of Cys53 with IAANS and the subsequent attachment of IAANS to Cys53 did not significantly alter the functional characteristics of the Tn complex (Davis et al., 2007). The shift in IAANS fluorescence is caused by the change in the conformation of cTnC that occurs upon Ca^{2+} binding. The residue to which IAANS is attached (Cys53) is located within the BC subdomain of cTnC, which moves away from the NAD subdomain as the regulatory domain (N-terminus) of cTnC binds Ca^{2+} and interacts with cTnI (Davis et al., 2007). In the current model of the Ca^{2+} activation of cTnC, and subsequent interaction with cTnI, the BC domain does not interact with cTnI during Ca^{2+} activation (Takeda et al., 2003). Therefore, the change in fluorescence that was monitored reports on the change in conformation of cTnC and not on the interaction between cTnC and cTnI. In addition, the K_d of the labeled cTn complex (0.65 μmol l^{-1}) measured by these authors is within the

Table 1. $K_{F1/2}$ values and Hill coefficients of ScTnI-cTn^{IAANS}, ScTnI-$cTn^{IAANS*P}$, $McTn^{IAANS}$ $cTn^{IAANS*P}$ ($McTn^{IAANS*P}$) at 15°C, pH 7.0

	ScTnI-cTn^{IAANS} (N=8)	ScTnI-$cTn^{IAANS*P}$ (N=8)	$McTn^{IAANS}$ (N=6)	$McTn^{IAANS*P}$ (N=6)
$K_{F1/2}$ (μmol l^{-1})	1.46±0.01[a]	3.52±0.32[b]	2.62±0.35[c]	7.13±0.91[d]
Hill coefficient	0.88±0.06[a,b]	0.88±0.02[a]	0.77±0.04[b]	0.91±0.04[a,c]
R^2	0.94±0.02	0.97±0.01	0.89±0.03	0.97±0.01

ScTnI-cTn^{IAANS} is trout cTnI/human $cTnC^{IAANS}$/rat cTnT; $McTn^{IAANS}$ is rat cTnI/human $cTnC^{IAANS}$/rat cTnT; here, cTn is cardiac troponin and S is used to delineate trout cTnI as trout are salmonids. Phosphorylated complexes are indicated by the superscript ^{*P}.

Data are presented as means ± s.e. $K_{F1/2}$ is the Ca^{2+} concentration at half-maximum Ca^{2+}-dependent fluorescence. Values with the same superscript letters are not significantly different from each other (P<0.05). R^2 values, used as a goodness of fit, were generated when each titration curve was fitted with the Hill equation.

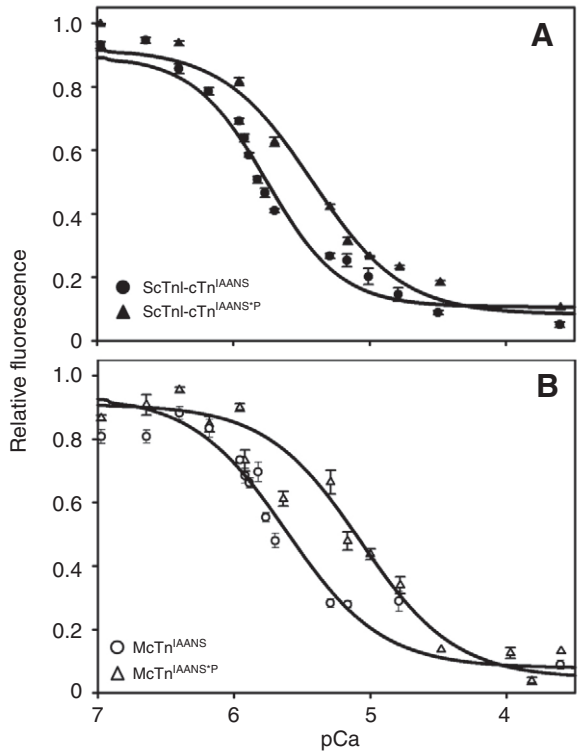

Fig. 5. Comparison of the effect of phosphorylation by PKA on the Ca^{2+} titration curves of trout cTnI-cTn^{IAANS} (ScTnI-cTn^{IAANS}) and mammalian cTn^{IAANS} (McTn^{IAANS}). Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and are presented as means ± s.e. The curves generated by fitting the data with the Hill equation have been added for comparison with the data points. (A) Titration of the fluorescence of ScTnI-cTn^{IAANS} (N=8) and PKA-phosphorylated ScTnI-cTn^{IAANS*P} (N=8). (B) Titration of fluorescence of McTn^{IAANS} (N=6) and PKA-phosphorylated McTn^{IAANS*P} (N=6). In both A and B the phosphorylation of the cTn complex caused $K_{F1/2}$ to increase (P<0.05). This indicates that the Ca^{2+} affinity of the complex has decreased.

range of intracellular Ca^{2+} concentrations during muscle activation and the rate of Ca^{2+} disassociation from the labeled complex (k_{off}) is almost identical to that of the native complex (36.0±0.8 *vs* 36.5±0.4 s^{-1}) measured using a fluorescent chelator (Quinn 2) (Davis et al., 2007). As a result, this method is an effective technique to monitor the Ca^{2+} activation of the cTn complex.

DISCUSSION

In this study we have demonstrated that the TnI expressed in the trout heart lacks the N-terminal extension present in cTnI from all other vertebrate groups. By replacing rat cTnI in a mammalian cTn complex with trout cTnI we have demonstrated that this protein increases the Ca^{2+} affinity of the complex and also reduces the influence of PKA phosphorylation on the Ca^{2+} affinity of the cTn complex. These results are significant as they provide insight into the role that trout cTnI plays in regulating thin filament function in a lower vertebrate and how this protein has changed during the evolution of the heart and its function in vertebrate species.

The influence of trout cTnI on the Ca^{2+} affinity of cTn

In the current study, the $K_{F1/2}$ (equivalent to K_d) of mammalian cTn^{IAANS} was 2.62±0.35 μmol l^{-1}. This value is within the range of intracellular Ca^{2+} concentrations during muscle activation. The difference between this value and that from the Davis et al. study (Davis et al., 2007) (0.65 μmol l^{-1}), where the same techniques were used, likely reflects the fact that the studies were completed in different laboratories using buffers made from different stock solutions. The shift in the $K_{F1/2}$ of the complex that occurred when rat cTnI was replaced with trout cTnI indicates that this protein increases the Ca^{2+} affinity of the cTn complex. A similar shift in $K_{F1/2}$ was found to occur when human cTnI was replaced in a cTn complex with mammalian ssTnI (Davis et al., 2007), which is consistent with previous experiments demonstrating that ssTnI increases the Ca^{2+} sensitivity of force generation in mammalian cardiac tissue (Fentzke et al., 1999; Metzger et al., 2003; Westfall and Metzger, 2007). The results of the current study therefore suggest that trout cTnI does play a role in the comparatively high Ca^{2+} sensitivity of the trout heart. The similar shift in $K_{F1/2}$ caused by the replacement of rat cTnI with either trout cTnI (present study) or rat ssTnI (Fentzke et al., 1999; Metzger et al., 2003; Westfall and Metzger, 2007) is also evidence that these two proteins are more similar to each other than trout cTnI is to rat cTnI. This result is interesting as both rat ssTnI and trout cTnI lack the N-terminal extension, present in rat cTnI, that interacts with the N-terminal domain of cTnC in a Ca^{2+}-dependent manner.

To more completely characterize the influence of trout cTnI on the Ca^{2+} affinity of the Tn complex, further work needs to be completed with this protein complexed with trout cTnC and trout cTnT. Previous work has illustrated that each of the troponin components influences the Ca^{2+} binding characteristics of the Tn complex (Abbott et al., 2001; Davis et al., 2007; Liang et al., 2008; McAuliffe et al., 1990; McCall et al., 2006; Robertson et al., 1982). It may be, therefore, that all trout components need to be working together for the full effect of the functional characteristics of the proteins to be realized. In addition, the reconstituted troponin may differ in unpredictable ways from a native trout or rat complex because of potential differences in interactions between heterologous subunits.

Following a phylogenetic study of all known TnI isoforms, we have recently proposed that the three muscle-specific isoforms of TnI (cardiac, ss and fs) were produced *via* two genome duplications that occurred in an ancestor of all extant vertebrate groups (Shaffer and Gillis, 2010). Our analysis indicated that a fsTnI 'like' paralog is the ancestral form of the protein, which was then replicated to produce two isoforms *via* genome duplication. One of these evolved to become cTnI. The gene for cTnI was subsequently replicated and one of these evolved to become ssTnI. A similar scheme for the evolution of other contractile proteins has also been suggested by Oota and Saitou (Ota and Saitou, 1999). We have also suggested that the similarity between cTnI from fish species and ssTnI indicates that there has been little change in the sequence of fish cTnI since this group diverged from the vertebrate lineage (Shaffer and Gillis, 2010). The results of the current study demonstrating that trout cTnI increases the Ca^{2+} affinity of the cTn complex, as does ssTnI, support this hypothesis.

The influence of PKA phosphorylation on Ca^{2+} affinity

The result that PKA treatment causes a reduction in the Ca^{2+} affinity of mammalian cTn^{IAANS} is supported by previous studies characterizing cTn function in solution (Chandra et al., 1997; Robertson et al., 1982). In a study by Robertson and colleagues (Robertson et al., 1982) where native cTnC was labeled with IAANS, the pCa_{50} (equivalent to the –log of $K_{F1/2}$) of the cTn complex decreased from 6.71 to 6.51. This is equal to a 1.14 μmol l^{-1} increase

in the amount of Ca^{2+} required to half-saturate the protein complex upon PKA phosphorylation. In the current study, this increase in $K_{F1/2}$ was equal to 4.51 μmol l^{-1}. The difference between the two studies is likely due to differences in the position of the IAANS probe within the cTn complex. In the Robertson et al. study (Robertson et al., 1982), native bovine cTnC was used, so IAANS would have been bound to Cys35 and Cys84. In the current study, IAANS was attached to residue 53, which, because of its location within the molecule, does not interact with other regions of cTnC or with cTnI/cTnT during Ca^{2+} activation (Davis et al., 2007). The decrease in the Ca^{2+} affinity of the mammalian cTn complex with PKA treatment is consistent with the decrease in Ca^{2+} sensitivity of force generation that occurs when mammalian cardiac tissue is treated with PKA (Fentzke et al., 1999; Matsuba et al., 2009). This change in cTn function is thought to be responsible for the increased rate of muscle relaxation that occurs following β-adrenergic stimulation in the heart (Kentish et al., 2001; Li et al., 2004; Rarick et al., 1999; Solaro and Rarick, 1998).

The treatment of trout cTnI-cTn with PKA results in the phosphorylation of trout cTnI and causes a decrease in the Ca^{2+} affinity of the cTn complex. Because of the experimental design it was not possible to determine whether this shift in $K_{F1/2}$ (2.06 μmol l^{-1}) was significantly different from that caused by similar treatment of mammalian cTn (4.51 μmol l^{-1}). As trout cTnI lacks the PKA targets at positions 23 and 24, this indicates that another residue or residues was/were targeted for phosphorylation and this caused the change in Ca^{2+} affinity. The most likely candidates are the serines at residues 14 and 16 of trout cTnI (Fig. 2). These residues correspond to Ser43 and Ser45 in mammalian cTnI isoforms, which are targets for phosphorylation by PKC (Fig. 2). Analysis of trout cTnI protein sequence using NetPhos (Blom et al., 1999) gives each of these residues a high score (>98%), indicating that these are putative phosphorylation sites. In mouse cardiac preparations the phosphorylation, or pseudo-phosphorylation, of these sites causes a decrease in the Ca^{2+} sensitivity of force generation, a decrease in maximum tension and a reduction in thin filament sliding speed (Burkart et al., 2003; Noland et al., 1995). These effects at the cellular level are due to the cTn complex losing its ability to be activated by Ca^{2+}. The fact that the Ca^{2+} affinity of the trout cTnI-cTn shifted by a smaller value following PKA treatment suggests that the function of the complex is affected to a lesser degree than that containing rat cTnI.

In the accompanying study (Gillis and Klaiman, 2011), we demonstrated that the treatment of skinned trout cardiac trabeculae with PKA reduces force generation and the rate of cross-bridge cycling at maximum Ca^{2+} activation. There was no influence, however, on the Ca^{2+} sensitivity of force generation. When the trabeculae were analyzed using Pro-Q diamond stain it was found that the only protein to increase in phosphate content was myosin regulatory light chain. There was no change in the level of cTnI phosphorylation. The accompanying study demonstrates that trout cardiac tissue does not respond to PKA treatment in the same way as mammalian tissue and this is likely because trout cTnI is not phosphorylated by PKA when functioning within the trout contractile element. Our result in the current study demonstrating that trout cTnI is phosphorylated by PKA is likely due to the protein being more accessible to PKA when it is functioning in solution than when it is functioning within an intact contractile element.

Conclusions and perspectives

The results of this study demonstrate that trout cTnI can increase the Ca^{2+} affinity of a mammalian cTn complex and that it also reduces the influence of PKA phosphorylation on cTn function. These results provide evidence that cTnI from fish can be viewed as ancestral or primitive forms of the paralogous protein in mammalian cardiac tissue. This hypothesis is supported by previous phylogenetic studies of vertebrate TnI (Shaffer and Gillis, 2010). The sensitizing effect of trout cTnI on the mammalian cTn complex suggests that this protein is involved in the comparatively high Ca^{2+} sensitivity of the trout heart and therefore helps enable cardiac function at low temperatures. The limited response to PKA phosphorylation of the cTn complex containing trout cTnI is due to the lack of the N-terminal extension in the protein. The N-terminal extension present in cTnI from all other vertebrate groups allows for greater regulation of cTn function and it evolved after fish diverged from the vertebrate lineage (Shaffer and Gillis, 2010). The lack of this protein domain in cTnI therefore limits the ability of the animal to regulate cardiac contractility.

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