The Journal of Experimental Biology 214, 1989-1996

doi:10.1242/jeb.052084

RESEARCH ARTICLE

The influence of PKA treatment on the Ca^{2+} activation of force generation by trout cardiac muscle

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Accepted 2 November 2010

SUMMARY

β-Adrenergic stimulation of the mammalian heart increases heart rate, the strength of contraction as well as the kinetics of force generation and relaxation. These effects are due to the phosphorylation of select membrane and thin filament proteins by cAMP-activated protein kinase (PKA). At the level of the sarcomere, it is typically the phosphorylation of cardiac myosin binding protein C (cMyBP-C) and cardiac troponin I (cTnI) that is responsible for the change in the kinetics of contraction and relaxation. Trout cTnI (ScTnI) lacks two critical PKA targets within the N-terminus of the protein that, when phosphorylated in mammalian cTnI, cause a reduction in myofilament Ca^{2+} affinity. To determine what role the contractile element plays in the response of the trout heart to β-adrenergic stimulation, we characterized the influence of PKA treatment on the Ca^{2+} activation of skinned preparations dissected from ventricular trabeculae. In these experiments, isometric force generation and the rate of force development were measured over a range of Ca^{2+} concentrations. The results demonstrate that PKA treatment does not influence the Ca^{2+} sensitivity of force generation but it decreases maximum force generation by 25% and the rate of force re-development at maximal activation by 46%. Analysis of the trabeculae preparations for phosphoproteins revealed that PKA treatment phosphorylated myosin light chain 2 but not cTnI or cMyBP-C. These results indicate that the function of the trout cardiac contractile element is altered by PKA phosphorylation but in a manner different from that in mammalian heart.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/12/1989/DC1

Key words: Ca^{2+} activation, adrenergic stimulation, force generation, force redevelopment, phosphorylation, protein kinase A.

INTRODUCTION

The response of the vertebrate heart to β-adrenergic stimulation is a critical component of the fight or flight response. In mammals, this reaction includes an increase in heart rate, an increase in the strength of contraction and increases in the rate of myocyte contraction and relaxation (Wehrens et al., 2006). The common modulator of these cellular functions is cAMP-activated protein kinase (PKA), which targets select proteins that are key regulators of each of these processes. For example, the phosphorylation of the ryanodine receptor and phospholamban increases the amount and rate of Ca^{2+} cycling through the sarcoplasmic reticulum (Wehrens et al., 2006), while the increased kinetics of myocyte contraction and relaxation are due to the phosphorylation of cardiac myosin binding protein C (cMyBP-C) and cardiac troponin I (cTnI), respectively (Stelzer et al., 2007). Together, these changes in myocyte function, caused by the activity of PKA, result in an increase in cardiac output.

Using phylogenetic analysis we have recently shown that the number of PKA targets in cMyBP-C and cTnI increased in vertebrates after fish diverged from the vertebrate lineage (Shaffer and Gillis, 2010). For example, the two targets for PKA (Ser23 and Ser24) in human cTnI are within the N-terminal extension (Kobayashi and Solaro, 2005). This peptide, and associated PKA targets, is present in cTnI from all vertebrates, except fish (Shaffer and Gillis, 2010). It is also not found in any isoform of slow skeletal TnI (ssTnI) or fast skeletal TnI (fsTnI). This suggests that the phosphorylation of cTnI by PKA as a mechanism to regulate cardiac function evolved in the lineage that led to amphibians, reptiles, birds and mammals after fish diverged (Shaffer and Gillis, 2010).

Previous work has demonstrated that the function of the teleost fish heart is sensitive to β-adrenergic stimulation (Farrell, 1985; Gesser et al., 1982; Graham and Farrell, 1989; Keen et al., 1993; Vornanen, 1998). The application of adrenaline to the trout heart increases heart rate and the strength of contraction (Aho and Vornanen, 2001), as well as the rate of contraction and relaxation (Shiels et al., 1998). These changes in cardiac function have been shown to be due, at least in part, to increased current (2.3-fold) through the L-type Ca^{2+} channels (Vornanen, 1998). However, it is not known what role the regulatory proteins of the contractile element play in these effects of adrenergic stimulation on the trout heart.

The purpose of the current study was to characterize how PKA treatment influences the response of the trout contractile element to Ca^{2+}. To do this, we developed a skinned cardiac preparation utilizing cardiac trabeculae dissected from the spongy ventricle wall of the trout heart. This preparation was then used to characterize the Ca^{2+} sensitivity of force generation and the rate of force re-development. Ca^{2+} activation was measured over a range of Ca^{2+} concentrations, enabling the Ca^{2+} sensitivity of force generation to be characterized. These measurements were made in control preparations as well as in preparations phosphorylated with PKA for 1 h. The sarcomere length of each preparation was set to 2.2 μm. This length was chosen to enable the results to be compared with previous studies of mammalian cardiac tissues that utilized similar methods.

MATERIALS AND METHODS

Cardiac trabecular mechanics

Trabecular dissection

Mechanical experiments were conducted on permeabilized trabeculae preparations dissected from the spongy ventricular wall of rainbow trout hearts. Adult rainbow trout, *Oncorhynchus mykiss* Walbaum 1792 (males and females, ~1 kg), were maintained in 10±1°C circulating ground water for the period of the study. Water was kept at normoxic levels with a 12 h:12 h photoperiod. All fish were fed *ad libitum* for the duration of the experiment and protocols were approved by the University of Guelph Animal Care Committee. Fish were removed from the tank by dip net and then rapidly killed by a blow to the head and severing of the cerebral spinal column. The time from netting of fish until removal of the heart was less than 1 min. Following removal, the heart was immediately placed in ice-cold physiological saline [in mmol l^{-1}: 94 NaCl, 24 $NaCO_3$, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 0.7 $CaCl_2$, pH 7.6 at 15°C]. All blood was washed from the heart and then the heart was placed onto a dissection plate on a chilled dissection stage containing ice-cold relaxing solution (in mmol l^{-1}: 100 KCl, 10 MOPS, 5 K_2EGTA, 9 $MgCl_2$, 4 NaATP, 20 2,3-butanedione monoxime (BDM), pH 7.0 at 4°C) (Gillis et al., 2007). BDM inhibits cross-bridge formation and was used to reduce injury due to contracture. The protease inhibitors phenylmethanesulphonylfluoride (PMSF), leupetin and bezamidine were also present in all relaxing and skinning solutions. The ventricle wall was exposed and sheets of trabeculae (4.0×2.0 mm) were dissected and then pinned out in a dissection dish. The trabeculae were skinned overnight, on ice, in relaxing solution containing glycerol (50% v:v) and Triton X-100 (1%) with one solution change, as previously described (Gillis et al., 2007). The following morning the skinning solution was replaced with the same solution minus the Triton X-100. The skinned trabeculae were then dissected on a chilled microscope stage to produce preparations that were ~1.7×0.35 mm. Preparations were only used if it was obvious that the muscle fibers ran in parallel down its length. These were visualized using polarized light. The ends of the preparations were wrapped in homemade aluminium foil 'T-clips'. These T-clips were 1.0 mm in length and 1 mm in width with a small triangular hole cut in one end to allow the preparation to be mounted to the experimental apparatus *via* steel hooks (Fig. 1A).

Treatment of trabecular preparations with PKA

Skinned and wrapped trabecular preparations were transferred into 2 ml of relaxing solution containing 1 mmol l^{-1} dithiothreitol (DTT) and 100 U ml^{-1} of the catalytic subunit of PKA from bovine heart (Sigma-Aldrich, St Louis, MO, USA). This concentration of PKA was selected to mimic conditions used by Patrick et al. (Patrick et al., 2010). The preparations were then incubated at 15°C for 1 h. Control preparations were incubated in the same solution without the PKA under the same conditions. Following incubation, the buffer was replaced with 2 ml relaxing solution without PKA, then incubated at 15°C for 15 min with gentle agitation.

Muscle mechanics instrument

The mechanical measurements were made using a custom-designed instrument from Aurora Scientific (Aurora, ON, Canada) mounted on the stage of an inverted microscope (Model Eclipse TE 2000U, Nikon, Tokyo, Japan). The instrument is composed of a permeabilized fiber test system (Model 802B, Aurora Scientific), a force transducer (Model 400A, 2.0 kHz resonant frequency, Aurora Scientific) and a servo-motor (Model 308C, Aurora Scientific) tuned for a 250 µs step response. The fiber test system consists of an 8-well stainless steel bath attached to two stepper motors. The stepper motors allow the preparation, mounted to the force transducer and servomotor, to be moved from bath to bath with an excursion time of 1.4 s. The temperature of the plate is regulated *via* three thermoelectric coolers and well temperature is measured *via* a thermocouple. Temperature is maintained within 1°C of the set temperature. Sarcomere length (SL) was measured by fast Fourier transform (FFT) analysis using the high-speed video sarcomere length system (Model 901A, Aurora Scientific) with a CCD camera (Model VGA-210-LMCN, Imperx, FL, USA) with 640×480 resolution. The camera images the preparation through a 40× objective with a 3 mm working distance (Nikon).

Solutions for mechanical measurements

Solution composition was determined according to an iterative computer program that calculates the equilibrium concentration of ligands and ions based on published affinity constants. Activation solutions contained (in mmol l^{-1}): 15 phosphocreatine, 15 EGTA, at least 40 MOPS, 1 free Mg^{2+}, 135 Na^{+}+K^{+}, 1 DTT, 250 U ml^{-1} creatine phosphokinase (CPK; Sigma) and 5 mmol l^{-1} NaATP, pH 7.0 at 15±1°C. Ionic strength was 0.17 mol l^{-1}. The Ca^{2+} level (expressed as pCa=–log[Ca^{2+}]) was varied between pCa 9 and pCa 5.2 by adjusting $Ca(propionate)_2$ as previously described (Gillis et al., 2007).

Experimental protocol

The ability of the preparation to be activated by Ca^{2+} was measured by moving the preparation through the series of wells containing activation solutions with different pCa values. When the force trace for each pCa reached an asymptote, steady-state force and the rate of isometric tension redevelopment (k_{tr}) were measured. The activation solutions had pCa values between 9 and 5.2. These pCa values were used because preliminary experiments demonstrated that the trabeculae were maximally activated by pCa 5.3 for both the control and PKA-phosphorylated preparations. In addition, the specific pCa values used were selected so that we could effectively describe the Ca^{2+} activation curve. Between each activation solution of different pCa, the preparation was exposed to the pre-activation solution (in

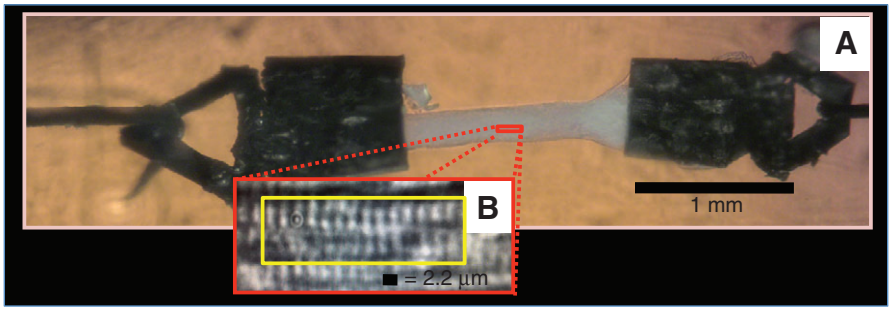

Fig. 1. Images of a skinned trout cardiac trabeculae preparation mounted to the muscle mechanics system. (A) Trabeculae preparation attached to force transducer and servomotor using aluminium foil clips. (B) Image of sarcomere pattern through 40× objective. The yellow box represents a typical sarcomere pattern that was analyzed using fast Fourier transformation to measure sarcomere length (SL).

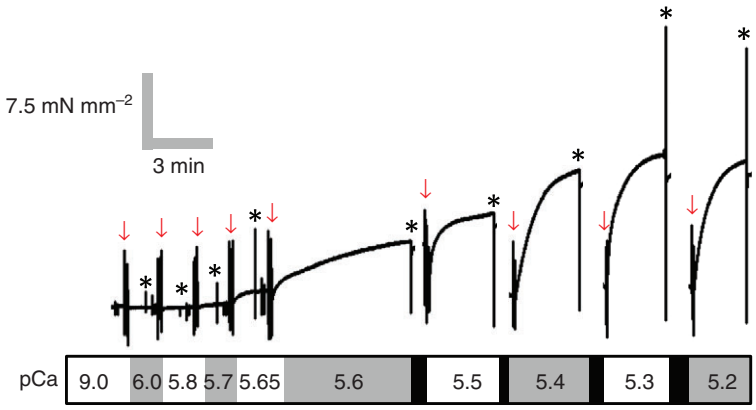

Fig. 2. Sample force trace resulting from the Ca^{2+} activation of a trout cardiac trabeculae preparation at 15°C and SL 2.2 µm. The noise on the force trace is due to motor movements during rate of force redevelopment (k_{tr}) measurements (*) and movement of preparation between solution wells (↓). Force is displayed as mN mm^{-2} and pCa values are indicated along the bottom of the trace.

mmol l^{-1}: 100 KCl, 10 MOPS, 0.1 EGTA, 9.0 $MgCl_2$, 4 NaATP) for ~5 s. The purpose of this Ca^{2+}-free solution was to enable the rapid penetration of Ca^{2+} into the muscle upon subsequent exposure and to prevent Ca^{2+} contamination between wells. Prior to being mounted on the apparatus, each preparation was incubated on ice in 2 ml relaxation buffer without BDM for at least 15 min. The preparation was then mounted on the apparatus by attaching the T-clips on the muscle to steel hooks attached to the force transducer and servomotor (Fig. 1A). During this time the preparation was immersed in a well containing activation solution with a pCa of 9 and no BDM. The mean length (ML) and width of the preparation were then measured using an eyepiece reticle and the 4× objective. The mean length of the preparations used in this experiment was 1.7±0.1 mm and the mean width was 0.34±0.01 mm. To standardize force generation the cross-sectional area was calculated from the diameter assuming a circular geometry. To 'set' the clips on the hooks, the preparation was then activated using pCa 5.3 and allowed to generate 0.5 mN of force. The preparation was then moved back into pCa 9 to relax. This was done before the preparation was maximally activated. SL was then set to 2.2 µm by stretching the preparation accordingly using the *XYZ* micrometer translation stage, to which the servomotor was attached. Between each length adjustment, SL was measured by analyzing the sarcomere pattern at multiple locations (>5) throughout the preparation using FFT. An example of such a pattern is shown within the yellow box in Fig. 1B. The measurement generated, in µm, was a mean of the multiple sarcomeres (>10) in each analyzed pattern. If a consistent SL pattern could not be obtained throughout the preparation, the preparation was discarded. The passive force at pCa 9 was then measured as described previously (Gillis et al., 2007) using a release–restretch protocol. This value was subtracted from total force measured at higher Ca^{2+} concentrations. The preparation was then maximally activated and force generation was measured. This value was used to characterize the effect of the subsequent activation protocols on muscle function. Steady-state isometric force and k_{tr} following rapid (<4 ML s^{-1}) release–restretch (15% ML) was measured as described previously (Gillis et al., 2007). As the rate of force redevelopment is a function of cross-bridge formation, this measure is used as an estimate of the rate of cross-bridge cycling during Ca^{2+} activation. The preparation was then returned to pCa 9 and allowed to relax. Once relaxed, the experiment began. Fig. 2 is a sample force trace of a preparation being activated over a range of pCa values.

Phosphorylation detection

Individual trabeculae were solubilized by sonicating them (3×10 s on ice) in 2× SDS loading buffer. Samples were then separated on 10% SDS-PAGE gels using the miniVE system (GE Healthcare, Piscataway, NJ, USA) and then stained with Pro-Q Diamond phosphoprotein gel stain following the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Briefly, gels were fixed overnight in 50% methanol, 10% acetic acid, and were stained for 75 min, in light-excluding containers. Gels were destained using 20% acetonitrile and 50 mmol l^{-1} sodium acetate (pH 4.0) and imaged using a Typhoon 9410 scanner (GE Healthcare) with an excitation wavelength of 532 nm and emission filter of 560 nm. Gels were then stained for total protein using SYPRO-Ruby protein stain (Bio-Rad, Hercules, CA, USA). To ensure that the Pro-Q stain was working and to compensate for non-specific binding, the 'peppermint stick' (Molecular Probes) protein ladder, which contains two phosphorylated proteins, was run on each gel according to the manufacturer's instructions. Once gels had been imaged for the Pro-Q signal, levels were reduced until only the two phosphorylated protein bands were visible in the ladder lane. Any bands that were still visible in the sample lanes were then characterized as phosphorylated. To determine whether PKA treatment altered the level of phosphorylation of individual proteins, the program ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to analyze the density of the Pro-Q-stained protein bands. The Pro-Q signal was standardized to the SYPRO-Ruby signal for the same band to account for differences in protein loading between lanes. Individual myofilament proteins were identified on the SDS gel using mass spectroscopy and western blotting. Details of this analysis are available in the Appendix. Accompanying western blot images are available in the supplementary material Fig. S1.

Analysis

Each force–pCa curve was fitted with the Hill equation:

$$F = F_{max}\,(1 + 10^{n_H(pCa - pCa_{50})})^{-1}\,, \quad (1)$$

where F_{max} is the force at high [Ca^{2+}] (low pCa), pCa_{50} is the pCa needed to achieve 50% of F_{max} (used here as a measure of the Ca^{2+} sensitivity of force), and n_H, the Hill coefficient, reflects the steepness of the relationship. The reported values for each of these parameters represent the means of the values from the individual fits ±s.e. Student's *t*-tests were used for statistical comparisons of the different parameters between the two treatment groups.

The data produced by the k_{tr} protocol describing the increase in force following the rapid release–restretch was well fitted by a single exponential equation. The calculated error values, used as a goodness of fit indicator, were within an acceptable range. In addition, each data fit was examined by eye to confirm a good fit.

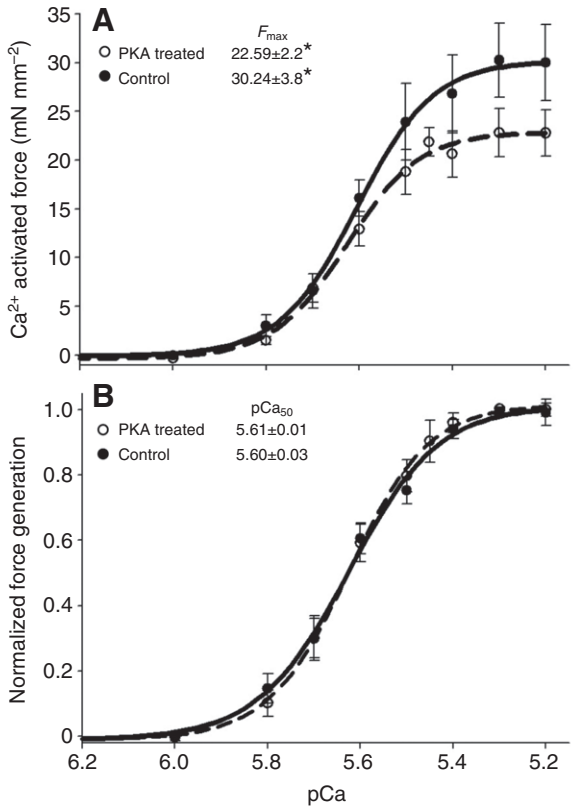

Fig. 3. PKA treatment reduces the force generated by skinned trout cardiac trabeculae during maximum activation but it does not change the Ca^{2+} sensitivity of force generation. (A) Actual force generated. (B) Relative force generated. Measurements made at 15°C and a SL of 2.2 µm. For control preparations, N=10; for PKA-treated preparations, N=8. The difference in maximum force (F_{max}) between the control and PKA-treated preparations is significant (P<0.05). This is indicated by an asterisk. The difference between pCa_{50} values is not significant.

RESULTS

Ca^{2+} activation of trabeculae

The exposure of the trout cardiac trabeculae preparations to increasing levels of Ca^{2+} resulted in increased levels of force generation (Fig. 2). When the force produced was plotted against pCa, the resulting curve was sigmoidal in shape and reached an asymptote by pCa 5.3 (Fig. 3A). The pCa_{50} calculated for control preparations was 5.60±0.03. This pCa_{50} value is lower (greater Ca^{2+} concentration) than that previously reported (6.28±0.06) for the Ca^{2+} activation of force generation by skinned single trout cardiac myocytes at a SL of 2.3 µm (Patrick et al., 2010). It is also higher than the amplitude of intracellular Ca^{2+} concentrations measured during myocyte activation using Fura-2 at 14°C (pCa 6.9). However, it is very difficult to compare measurements made between labs because of differences in experimental conditions and buffer design/composition. For example, the study by Patrick and colleagues (Patrick et al., 2010) was completed at 22°C, where the sensitivity of the preparation to Ca^{2+} would be greater due to the influence of temperature on the Ca^{2+} affinity of cTnC (Gillis et al., 2000). In addition, the pCa of a solution for force activation or the calibration of a Ca^{2+} binding dye is calculated using an iterative process that takes into account ionic strength, total Ca^{2+} concentration, solution pH, experimental temperature, and the concentration of Ca^{2+} chelators. If any of these vary within the solution from what was used to calculate pCa, due to differences in moisture content or purity of the chemical components, the actual pCa will be significantly different. This in turn would translate into a difference in the pCa_{50} determined when the solutions are used in an experiment. For example, the pCa_{50} of rat trabeculae measured at 15°C with a SL of 2.2 µm using the same recipes and techniques as in the current study was 5.41±0.01 (Regnier et al., 2004), while that of single rat myocytes at 22°C and a SL of 2.3 µm measured by Patrick and colleagues (Patrick et al., 2010) was 6.05±0.04. Both of these pCa_{50} values are for cardiac tissue from the same species but measured at different temperatures with different solutions. Therefore, while the pCa_{50} reported in the current study is different from what might be considered physiological, this difference is likely due to variations in experimental conditions and buffer composition between studies.

As mentioned above, each preparation was maximally activated prior to the protocol used to generate the force–pCa curve to allow for any 'run-down' to be characterized. During this initial activation, the maximum force generated was measured, as was the k_{tr}. These initial values were then compared with those collected during the maximal activation at the end of the Ca^{2+} activation protocol. For the control preparations, the rundown was equal to 9% of the mean initial value, while for the phosphorylated preparations the rundown was equal to 7% the mean initial value. There were also relatively minor, non-significant, differences in k_{tr} for both the treatment and control between the first maximal activation and the second. For the control preparations, the initial k_{tr} at maximum activation was 2.79±0.7 s^{-1} and that during the final maximal activation was 3.14±0.5 s^{-1} (Table 1). For the phosphorylated preparation, the initial k_{tr} at maximum activation was 2.37±0.4 s^{-1} and that during the final maximal activation was 1.70±0.3 s^{-1} (Table 1). Together, these limited changes in preparation function indicate that there was minimal 'run down' during the duration of the experiment.

k_{tr} was measured at each Ca^{2+} activation and these values were plotted against pCa (Fig. 4). Interestingly, the highest k_{tr} values were measured at a relatively low Ca^{2+} concentration when the amount of force being generated was less than half maximal (Fig. 4). For the control preparation this value was 5.25±0.9 s^{-1} at pCa 5.6 while that for the phosphorylated trabeculae was 6.5±0.9 s^{-1} at pCa 5.7. These values were greater than those obtained during the initial maximum activation at pCa 5.3. When the preparations were subsequently exposed to higher Ca^{2+} concentrations, causing force generation to increase towards a maximum, k_{tr} decreased (Fig. 4). In the control tissue the k_{tr} at maximal activation was 3.14±0.5 s^{-1}

Table 1. The influence of PKA treatment on the Ca^{2+} activation of trout cardiac trabeculae

	F_{max} (mN mm^{-2})	pCa_{50}	n_H	k_{tr} at F_{max} (s^{-1})	Passive force (mN mm^{-2})
Control (N=10)	30.24±3.8^a	5.60±0.03^a	6.22±0.90^a	3.14±0.51^a	1.10±0.18^a
PKA treated (N=8)	22.59±2.2^b	5.61±0.01^a	6.72±0.70^a	1.70±0.25^b	1.11± 0.15^a

F_{max}, maximum force generated; pCa_{50}, Ca^{2+} concentration measured as pCa at half-maximum force generation; n_H, Hill coefficient; k_{tr} at F_{max}, rate of force generation at maximum activation. Significant differences between values in the same column are indicated by different superscripts (P<0.05).

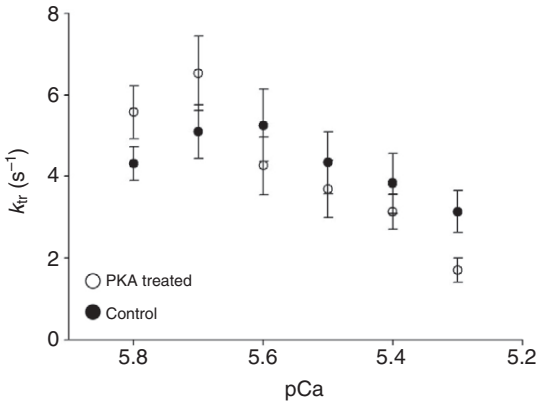

Fig. 4. PKA treatment reduces the k_{tr} in skinned trout cardiac trabeculae preparations at maximum activation. For control preparations, N=10; for PKA-treated preparations, N=8. The difference in k_{tr} between the control and PKA-treated preparations at maximum activation (pCa 5.3) is significant (P<0.05) at pCa 5.3.

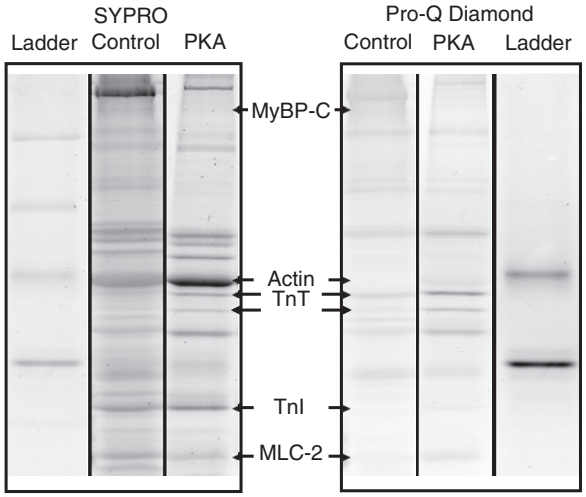

Fig. 5. PKA treatment of skinned cardiac trabeculae increases the phosphorylation state of myosin light chain 2 (MLC-2). (A) SYPRO-Ruby-stained gel showing total protein content of control and PKA-treated preparations. (B) Pro-Q Diamond-stained gel indicating presence of phosphorylated proteins in control and PKA-treated preparations. The positions of myosin binding protein C (MyBP-C), actin, troponin I (TnI), troponin T (TnT) and MLC-2 are indicated on the gel. The bands representing MyBP-C, TnI, TnT and MLC-2 were identified using western blotting or mass spectrometry. The position of actin is inferred from its highly conserved mass and the fact that it is the most prevalent protein in muscle. The peppermint stick ladder was used, which contains two phosphorylated proteins at 23.6 and 45 kDa. The level of phosphorylation of each protein was compared between treatment and control samples *via* the ratio of the density of the Pro-Q/SYPRO-stained bands using ImageJ.

(Table 1). This is significantly less (P<0.05) than that at pCa 5.6. The same result was seen in the phosphorylated preparations, with the k_{tr} at maximal activation (1.7±0.9 s^{-1}) being significantly less than that at pCa 5.7 (P<0.05). It is unlikely that the decrease in k_{tr} at higher rates of force generation is due to run down as the k_{tr} values obtained during the initial maximum activation were not statistically different from those obtained during the second maximum activation. It is also unlikely that the decease in k_{tr} was due to limiting concentrations of ATP in the solutions. All solutions contained 5 mmol l^{-1} ATP as well as 15 mmol l^{-1} phosphocreatine and 250 U ml^{-1} CPK. This ensured that any ADP produced was recycled back to ATP. It is likely, therefore, that this decease in the rate of cross-bridge cycling is a physiologically relevant phenomenon.

Influence of PKA on trabeculae preparation function

The treatment of the trabeculae preparations with PKA caused the maximum force generated to decrease from 30.3±3.8 to 22.59±2.2 mN mm^{-2} (Table 1, Fig. 3A). This represents a 25% decrease in maximum force generation. There was, however, no difference in the amount of force generated by the control and treatment preparations at their pCa_{50} values of 5.60±0.013 and 5.61±0.01, respectively. In addition, there was no effect of PKA treatment on the Ca^{2+} sensitivity of the preparation. This is evident when the force–pCa curves for the control and treatment groups are standardized and then superimposed (Fig. 3B). In addition, the pCa_{50} values for the PKA-treated and control preparations were virtually identical (Table 1). There was also no influence of PKA treatment on the Hill equation generated from the force–pCa curves, indicating that the co-operativity of the contractile element was not altered by the treatment (Table 1). PKA treatment was found to decrease the k_{tr} at maximal activation (Fig. 4). In the phosphorylated preparations k_{tr} was 46% less than that of the control fibers (Table 1). Finally, there was no effect of PKA treatment on the passive force of the preparations. These values were 1.11±0.15 and 1.10±0.18 mN mm^{-2} for the control and PKA-treated preparations, respectively.

Influence of PKA treatment on phosphorylation state of contractile proteins

The treatment of the trabeculae with PKA increased the phosphorylation level of myosin light chain 2 (MLC-2) (Fig. 5). The standardized density of the MLC-2 band stained with Pro-Q Diamond increased from 0.78±0.09 to 1.00±0.07; this is equal to a ~28% increase in the level of phosphorylation. A Pro-Q band was detected for cTnI and cMyBP-C but these did not increase with PKA treatment, indicating that there was no change in the level of phosphorylation (Fig. 5).

DISCUSSION

The limited effect of PKA phosphorylation on the contractility of trout cardiac tissue is in significant contrast to what occurs when mammalian cardiac tissue is similarly treated (Fentzke et al., 1999; Holroyde et al., 1979; Matsuba et al., 2009; Mope et al., 1980; Solaro et al., 2008; Stelzer et al., 2007). For example, the exposure of porcine ventricle fiber bundles to PKA shifted the pCa_{50} by 0.1 pCa units (Matsuba et al., 2009) while the exposure of mouse cardiac myocytes to PKA shifted the pCa_{50} of force generation by 0.2 pCa units (Fentzke et al., 1999). This change in the Ca^{2+} sensitivity of force generation in skinned mammalian cardiac preparations is due to the phosphorylation of the N-terminal extension of cTnI (Matsuba et al., 2009; Yasuda et al., 2007). As trout cTnI lacks these phosphorylation sites it is not surprising that treatment of the trout trabeculae with PKA had no impact on the Ca^{2+} sensitivity of force generation. These results indicate that the Ca^{2+} sensitivity of the trout heart cannot be regulated to the same degree as the mammalian heart.

The reduction in force generation and cross-bridge cycling rates at maximal activation that occurred in the trout preparations following PKA treatment is not predicted by previous studies with skinned mammalian tissue. In mammalian tissue following PKA

treatment there is no change in force generation (Zhang et al., 1995) while the rate of force development increases (Stelzer et al., 2007). The physiological significance of this finding is limited as it is unlikely that the trout heart would ever be maximally activated. There was also no influence of PKA treatment on force generation at sub-maximal levels.

In mammalian hearts the accelerated rate of force development with PKA treatment is caused by the phosphorylation of cMyBP-C (Stelzer et al., 2007). This is thought to be in response to changes in the interaction between this protein and the myosin head (Stelzer et al., 2007). In the current study we did not detect a change in the phosphorylation state of cMyBP-C but we did for MLC-2. Previous work has also reported that MLC-2 is phosphorylated in trout cardiac tissue (Patrick et al., 2010). MLC-2 is located at the neck region of the myosin molecule and, together with the essential light chain, stabilizes the α-helical neck of the myosin head (Szczesna-Cordary et al., 2004). The phosphorylation of this regulatory protein influences the flexibility of the myosin head and therefore alters its ability to bind to actin, thereby affecting the kinetics of cross-bridge formation (Buck et al., 1999; Olsson et al., 2004). MLC-2 is thought to be primarily targeted by myosin light chain kinase; previous work has, however, demonstrated that rat MLC-2 is phosphorylatable by protein kinase C (PKC) (Noland and Kuo, 1993; Venema and Kuo, 1993; Venema et al., 1993) and PKA (Venema and Kuo, 1993). In mammalian tissue the phosphorylation of MLC-2 increases the activity of actin–myosin ATPase (AM ATPase) (Scruggs et al., 2009) and the Ca^{2+} sensitivity of force generation (Morano et al., 1988; Morano and Ruegg, 1986; Olsson et al., 2004; Sweeney and Stull, 1986). Comparison of the amino acid sequence of rat cardiac MLC-2 with that from a salmonid heart reveals that there are phosphorylatable residues in the fish protein at the same positions as known phosphorylation targets in rat MLC-2. However, the amino acid sequences of the two proteins are only 69% identical. It is not too surprising, therefore, that the phosphorylation of trout MLC-2 has a different effect on contractile function from phosphorylation of MLC-2 in the mammalian heart. The changes in contractile function caused by the phosphorylation of MLC-2 in the trout trabeculae are likely caused by changes in the functional characteristics of the myosin heads, influencing the formation of force-generating cross-bridges.

In a related study, we have demonstrated that the phosphorylation of a cardiac troponin (cTn) complex containing trout TnI (ScTnI, where S refers to the fact that trout are salmonids), rat cTnT and human cTnC caused a reduction in the Ca^{2+} affinity of the complex (Kirkpatrick et al., 2011). The only protein that was phosphorylated by this treatment was ScTnI. In the current study, PKA treatment did not alter the phosphorylation state of ScTnI. This suggests that when the proteins are functioning within an intact contractile element the phosphorylation sites are not accessible to the kinase because of the protein conformation.

The treatment of the trabeculae preparations with PKA did not alter the passive force measured at pCa 9. Passive force was measured by a release–restretch protocol that measures the force difference before and after all the cross-bridges are knocked off under relaxing conditions. Previous work by Patrick and colleagues (Patrick et al., 2010) has shown that the passive tension of single trout cardiac myocytes is determined by the content of titin, a phosphorylatable protein. As a multi-cellular preparation was used in the present study, if a decrease in tension was caused by the phosphorylation of titin it was likely dampened by the connections between myocytes.

Experiments with rat cardiac trabeculae completed at the same temperature and at a SL of 2.25 μm generated a maximum force of 20.2±4.0 mN mm^{-2} (Regnier et al., 2004). In the current study, this value for the control preparation was equal to 30.24±3.8 mN mm^{-2}. This suggests that the force being generated by these preparations is at least equal to that generated by mammalian tissue under the same conditions. The k_{tr} values generated during maximal activation of the control trout preparations are less than those obtained during the maximal activation of rat cardiac trabeculae. In the current study, the fastest k_{tr} value (5.25±0.4 s^{-1}, Fig. 4) corresponded to sub-maximal activation of force at pCa 5.6. Using similar methods, Regnier and colleagues (Regnier et al., 2004) have reported k_{tr} values in rat trabeculae as high as 10.6±4.0 s^{-1} during maximal activation. This indicates faster cross-bridge cycling rates for the rat heart than for the trout heart (Brenner, 1988). The higher level of steady-state force production in the trout than in the rat corresponds to a greater cross-bridge binding and force production in the trout. This increased force response would also correlate with a slower myosin detachment rate in the trout, if the attachment rate was similar for the two preparations (Hancock et al., 1997). In contrast with observations of mammalian k_{tr}, which increases with [Ca^{2+}], the trout k_{tr}–pCa relationship decreased with increased Ca^{2+} activation and force generation. This implies that, in the trout preparations, an increase in Ca^{2+} caused a decrease in the rate of cross-bridge detachment.

One factor to consider is that the higher rates of cross-bridge cycling in the trout preparations were obtained at sub-maximal levels of activation. As it is not expected that an intact preparation would ever be maximally activated, it is likely that the differences between the preparations at these levels of activation are more meaningful. The higher k_{tr} values obtained at the lower Ca^{2+} concentrations likely reflect the fact that the preparation functions optimally at these concentrations. The rate of cross-bridge cycling also appeared to be higher at these sub-maximal levels of activation in the PKA-treated preparations but the difference was not statistically significant. This does, however, suggest that PKA has a different impact on cross-bridge cycling rate at sub-maximal levels of activation than at maximal levels of activation.

Physiological significance

The results of the current study suggest that the contractile element plays a comparatively smaller role in the functional changes caused by β-adrenergic stimulation in the trout heart. These functional changes are an increase in heart rate, an increase in the strength of contraction (Aho and Vornanen, 2001), as well as increases in the rate of contraction and relaxation (Shiels et al., 1998). The increase in heart rate is caused by increased chronotropy regulated by the sinoatrial node, while the increased force generation is due to increased current through the L-type Ca^{2+} channels (Aho and Vornanen, 2001; Vornanen, 1998). In the trout heart the L-type Ca^{2+} channels play a comparatively significant role, delivering activator Ca^{2+} for myocyte contraction (Vornanen, 1998). Any increase in the current through this channel, therefore, has significant potential to increase contractility. In the current study we demonstrated that there was a decrease in force generation by the skinned preparation at maximum activation in response to PKA treatment. In the intact myocyte, the parallel increase in activator Ca^{2+} entering through the L-type channel would likely compensate for this loss of myofilament function that occurs with PKA phosphorylation.

In the mammalian heart the decrease in Ca^{2+} sensitivity of the contractile element caused by the phosphorylation of cTnI by PKA translates into faster rates of muscle relaxation following contraction

and an increase in the kinetics of cross-bridge cycling (Kentish et al., 2001; Li et al., 2004; Rarick et al., 1999; Solaro and Rarick, 1998; Yasuda et al., 2007). The result of the current study demonstrating that PKA treatment does not affect the Ca^{2+} sensitivity of trout cardiac tissue illustrates the fact that the contractile element does not play a role in this effect of PKA phosphorylation on contractility of the trout heart. This limited response of the trout contractile element to PKA treatment correlates with the results of a recent phylogenetic study where we demonstrated that the number of PKA targets in cTnI and cMyBP-C increased during vertebrate evolution (Shaffer and Gillis, 2010). For example, the N-terminal peptide, containing two PKA sites, did not appear in vertebrate cTnI until after fish diverged from the vertebrate lineage. Its absence in fish cTnI is therefore a consequence of the evolutionary history of the cardiac proteins in this vertebrate group. Accordingly, the isoforms of cTnI and cMyBP-C found in the trout heart can be viewed as ancestral or primitive forms of the proteins and their limited role in the regulation of the trout cardiac contractile element is a consequence of this.

APPENDIX

Identification of contractile proteins on protein gels

Methods

Western blotting

Western blotting was used to identify cTnI, cTnT and cMyBP-C. For western blot analysis, native trout cardiac muscle proteins were separated on an SDS-PAGE gel. The SDS-PAGE gel was then transferred onto Hybond-P PVDF membranes using an ECL Semi-Dry Transfer Unit (GE Healthcare). The primary antibodies used were sc-8118 for cTnI and sc-19 for cTnT (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). These are polyclonal goat anti-human antibodies synthesized using the C-terminus of each protein as the epitope. The primary antibody used for cMyBP-C was a customized polyclonal rabbit antibody for rat cMyBP-C, kindly donated by Dr Samantha Harris (University of California Davis). For the detection of cTnI and cTnT, the secondary antibody was a rabbit anti-goat IgG conjugated to horseradish peroxidase (AP106P; Millipore, Etobicoke, ON, Canada). For cMyBP-C, the secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (G21234; Invitrogen, Eugene, OR, USA).

Mass spectroscopy

To identify MLC-2, the band corresponding to the correct mass was cut from the gel then exposed to trypsin in-gel digestion. The resulting peptides were analyzed using nano-LC quadrupole time-of-flight (QTOF) mass spectrometry. This identification was completed at the Mass Spectrometry Facility at the University of Guelph.

Results

Western blotting

This analysis successfully identified cTnI, cTnT and cMyBP-C. Supplementary material Fig. S1 is a composite image created from the results of the western blots for each muscle protein. This identified single bands for cTnI and cMyBP-C. The cTnT antibody identified two different protein bands indicating that there are two isoforms of TnT in the trout heart.

Mass spectroscopy

Comparison between the peptide sequences obtained from the trypsin digestion of native myosin regulatory light chain 2 and the amino acid sequence of Atlantic salmon myosin regulatory light chain 2 resulted in a display score of 79% and 48% coverage, indicating a positive match (supplementary material Fig. S2).

ACKNOWLEDGEMENTS

We thank Drs Albert Gordon and Bertrand Tanner for helpful discussions during the preparation of this manuscript. We also acknowledge the generous donations of myosin binding protein C antibodies from Dr Samantha Harris (University of California, Davis). This work was supported by an Ontario Graduate Scholarship to J.M.K. T.E.G. is supported by a Discovery grant from NSERC and equipment grants from NSERC and the Canadian Foundation for Innovation.

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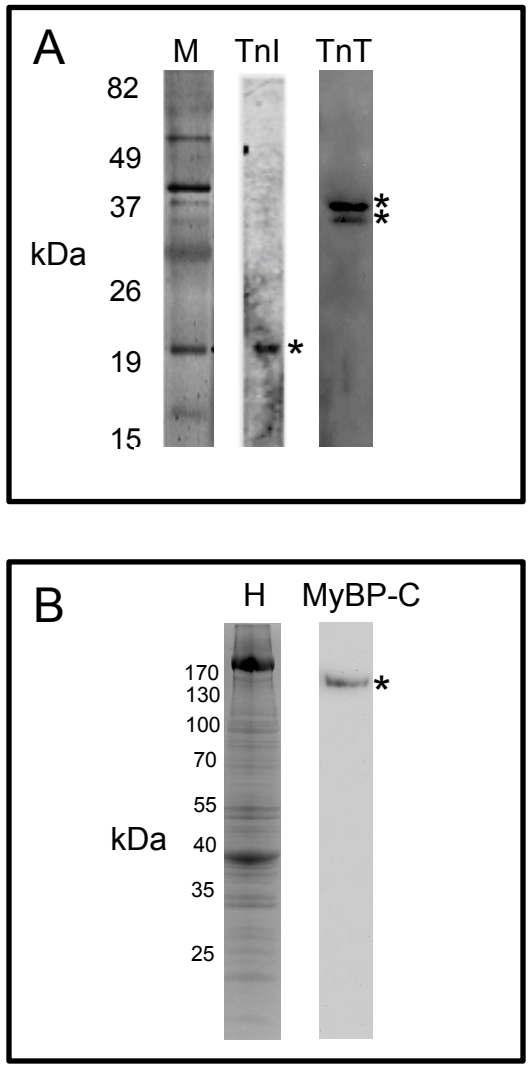

Figure 1. **Composite image showing results of western blotting to identify cTnI, cTnT and MyBP-C.** Panel A: lane 1 is trout cardiac myofilament proteins (M) stained with coomassie blue for total protein, lane 2 is western blot of trout myofilament proteins probed with antibody for mammalian cardiac troponin I, lane 3 is western blot of trout myofilament proteins probed with antibody for mammalian cardiac troponin T. Panel B, lane 1 is whole trout heart homogenate (H) stained with coomassie blue, lane 2 is a western blot of whole trout heart homogenate with antibody for rat cardiac myosin binding protein C. * is used to identify protein band

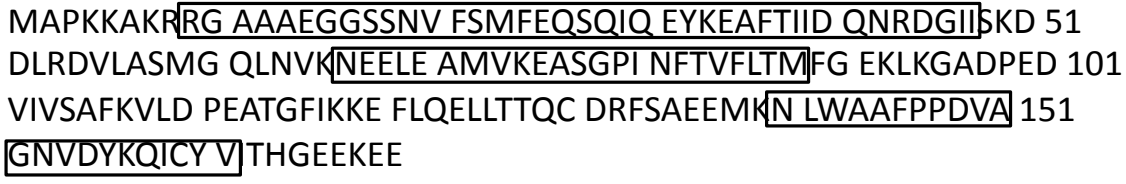

Figure 2. **The results of Nano-LC-QTOF mass spectroscopy sequence analysis of in gel trypsin digested native trout cardiac myosin regulatory light chain 2 mapped onto the amino acid sequence of Atlantic salmon cardiac myosin regulatory light chain 2.** The boxes indicate peptides that matched between the sample and the sequence. A display score of 79% with 48% coverage was obtained indicating a positive match.