Chapter 6
Evolution of the Regulatory Control of the Vertebrate Heart: The Role of the Contractile Proteins

Todd E. Gillis

Introduction

Evolutionary pressures have driven the vertebrate heart to transform from the simple, single-chambered structure found in the tunicates to the four-chambered organ with multiple valves and septa found in mammals and birds. Between these two extremes are intermediate forms found in other vertebrate groups. This includes the teleost heart where there is a single ventricle and a single atrium and the amphibian heart with a single ventricle but two atria. Along with these morphological changes, there is also significant variation in the functional capacity of hearts from different vertebrate groups. For example, the heart rate of a Brazilian hummingbird, *Amazilia lacteal*, is 1,056 beats per minute (bpm) (Bishop and Butler 1995), that of a mouse heart is greater than 600 bpm (Burggren et al. 1997; Farmer 1999; Suzuki et al. 2002), and that of a trout is 93 bpm at 15°C (Priede 1974). These differences are due to significant variation in pacemaker firing rate and in the rate of Ca^{2+} flux through the myocytes. There is also variation in the ability of the contractile element to sense, and respond to, changes in intercellular Ca^{2+}.

The evolution of endothermy in vertebrates represented a significant challenge to contractile function. This is because an increase in temperature dramatically increases the Ca^{2+} sensitivity of the heart (Harrison and Bers 1989). Such an increase in Ca^{2+} sensitivity caused by an increase in physiological temperature would reduce the rate of contraction and possibly cause the heart to remain in contracture. It was therefore necessary for the Ca^{2+} sensitivity of the heart to decrease with the evolution of endothermy in the vertebrates.

In addition to modifications that allow for high rates of contraction at high temperatures there have also been changes to the components of the cardiac myocytes

T.E. Gillis, Ph.D. (✉)
Department of Integrative Biology, University of Guelph,
Guelph, ON, Canada N1G 2W1
e-mail: tgillis@uoguelph.ca

D. Sedmera and T. Wang (eds.), *Ontogeny and Phylogeny of the Vertebrate Heart*,
DOI 10.1007/978-1-4614-3387-3_6,

that allow for greater regulatory control. Every myocyte within the vertebrate heart contracts with each heartbeat. This differs from skeletal muscle where the number of cells activated increases with the force required. As the oxygen requirements of an animal are not constant, but influenced by factors such as activity level, stress level, and reproductive status, it is important that animals can accommodate cardiac output to the fluctuating need. The mechanisms that enable such control include the Frank–Starling response where force generation of the heart increases as sarcomere length increases and the stimulatory effect of β-adrenergic stimulation (β-AS) on cardiac function. (α-adrenergic stimulation [α-AS] is also active within the vertebrate heart but it does not play as a significant role in regulating cardiac output.) As a result of these regulatory control mechanisms, there is a significant dynamic range in the functional capacity of the vertebrate heart. While these mechanisms are present in skeletal muscle, they have a larger influence on the function of cardiac muscle.

In mammals, the activation of β-receptors leads to an increase in heart rate, the strength of contraction, the rate of contraction, as well as the rate of relaxation between beats (Bers 2001; Kobayashi and Solaro 2005; Stelzer et al. 2007; Weisberg and Winegrad 1996). These changes result in a dramatic increase in cardiac output as more blood is being pumped at a faster rate. The stimulus of contractile function following β-AS is due, in part, to modifications in the function of specific Ca^{2+} handling and contractile proteins within the vertebrate heart caused by phosphorylation by protein kinase A (PKA). This kinase targets specific residues within the responsible proteins. One reason why the heart is more sensitive to β-AS than other muscle types is that there are targets for PKA in the cardiac isoforms of a number of regulatory proteins including cardiac (c) troponin I (cTnI) and cardiac myosin-binding protein C (cMyBP-C) (Shaffer and Gillis 2010). The isoforms of these proteins that are found in slow skeletal (ss) and fast skeletal (fs) muscle lack these target residues. The functional properties of the proteins and therefore of the muscle types are not as impacted as cardiac muscle following β-AS (Shaffer and Gillis 2010).

The stimulation of α-adrenergic receptors in the mammalian heart leads to a reduction in contractile function and force generation (Braz et al. 2004). These functional changes are due to the activation of protein kinase C (PKC) that in turn influences the function of a number of regulatory proteins either directly through phosphorylation or indirectly by initiating a signal cascade resulting in a change in phosphorylation state (Braz et al. 2004).

Interestingly, phylogenetic analyses of a number of different contractile proteins suggest that the ancestral form of the contractile proteins was “fast skeletal like” and that the cardiac and slow skeletal isoforms are the product of two subsequent genome duplications (Ota and Saitou 1999; Shaffer and Gillis 2010). Previous work by Cleto et al. (2003) identified a single gene for TnI expressed in the heart, the body wall, and the embryonic larval tail of the tunicate *Ciona intestinalis*. As tissue-specific TnI isoforms have been identified in teleost fast skeletal, slow skeletal, and cardiac muscle, this indicates that the gene duplication events occurred in a common ancestor to all vertebrates after the urochordates diverged from the vertebrate lineage (500 million years ago), but before the teleosts branched off (440 million years ago) (Shaffer and Gillis 2010). This means that the amino acid sequence of the TnI isoform that evolved and became specialized to function within the vertebrate heart most likely

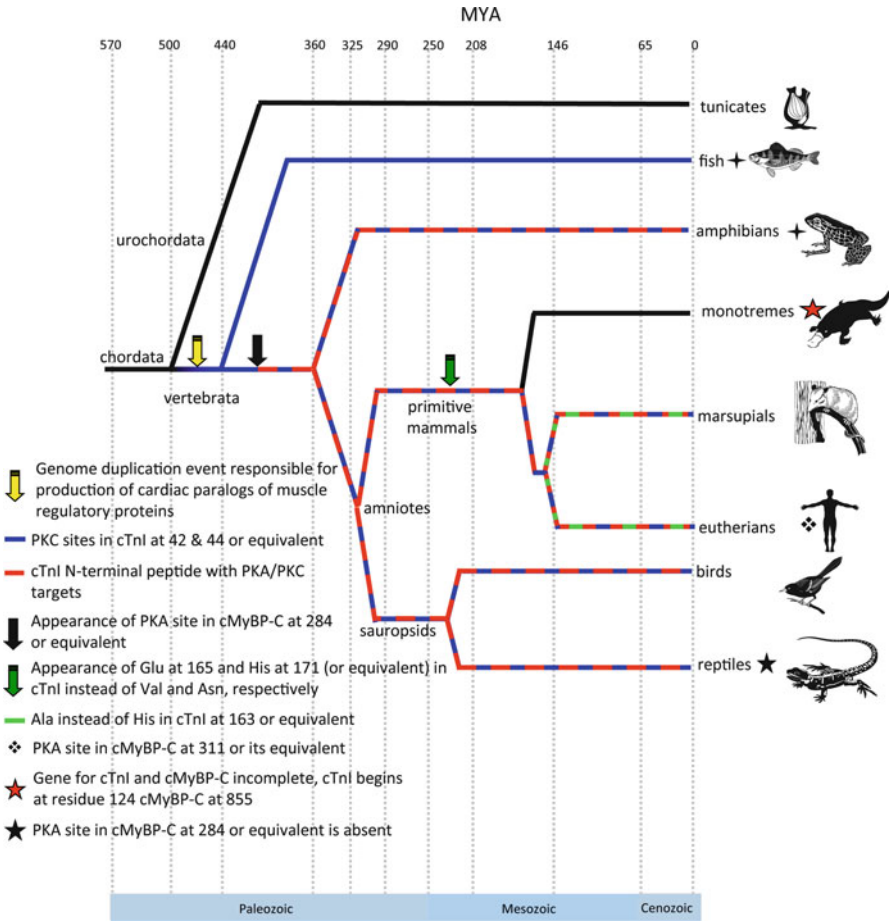

Fig. 6.1 A vertebrate phylogeny onto which critical changes in the sequence of cTnI and cMyBP-C have been mapped. Figure modified from Shaffer and Gillis (2010)

was similar to fast skeletal (fs) TnI of extant vertebrates. It was then subsequent changes to this protein that produced the tissue-specific isoform, with unique functional properties, found in the vertebrate heart today (Fig. 6.1).

The goal of this chapter is to examine the relationship between the contractile element and the functional changes that have occurred during the evolution of the vertebrate heart. This includes examining the mechanistic basis for the influence of β-AS on contractile function as well as the changes that have occurred to enable cardiac function at relatively high temperatures in endothermic animals. To accomplish this, phylogenetic data are integrated with the results of functional studies examining the Ca^{2+} activation of cardiac tissue as well as cardiac proteins from different species. It is hoped that this work will provide new insight into the role that the contractile element plays in regulating cardiac function and how this has evolved in vertebrates.

The Contractile Reaction in Mammals

The generation of force by cardiac muscle involves a series of conformational changes through the component proteins of the contractile element that results in the formation of cross-bridges between actin and myosin. When the myocyte is relaxed tropomyosin, a rod-shaped protein, sterically blocks myosin-binding sites along the length of the actin filament (Fig. 6.2) (Kentish et al. 2001; Li et al. 2004; Rarick et al. 1999; Solaro and Rarick 1998). It is locked in this position by an interaction between cTnI, via the inhibitory peptide (residues 128–147 in human cTnI), and actin (Kobayashi and Solaro 2005). Following the systolic rise in intracellular Ca^{2+}, Ca^{2+} binds to the regulatory domain of cTnC (site II) (Fig. 6.2). The resultant conformational change triggers the movement of the switch peptide of cTnI (residues 147–163 in human cTnI) toward 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 (Fig. 6.2) (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 exposing myosin-binding sites (Gordon et al. 2000) (Fig. 6.2). This allows the myosin heads to bind and results in the formation of force-generating cross-bridges (Fig. 6.2). Each of the steps that lead to the formation of a cross-bridge is dependent on changes in protein–protein interactions and/or protein conformation. This process is therefore highly influenced by the functional characteristic of the components involved. As a result, changes in protein structure by genetic mutation or post-translational modification has had a significant influence on contractile function during the evolution of the vertebrate heart.

The Influence of β-Adrenergic Stimulation on Cardiac Function

In vertebrates, β-receptors (β_1 and β_2 subtypes) are activated by norepinephrine which is released from sympathetic nerve ganglia. These receptors are coupled to G proteins that activate adenyl cyclase within the cell. Once activated, adenyl cyclase catalyzes the conversion of ATP to cyclic AMP, which then activates the catalytic subunit of PKA. It is the catalytic subunit of PKA that phosphorylates specific proteins within the cell by targeting individual residues (serine, tyrosine, threonine). For each of the different changes in contractile function that occur with β-AS, there is/are separate protein(s) involved (Table 6.1). By targeting the specific proteins responsible for each of these different functions, PKA acts to coordinate the response of the cell to β-AS.

The increase in heart rate caused by β-AS is due to the stimulation of β-receptors on the surface membrane of the cells that compose the sinoatrial (SA) node. The SA node is the primary pacemaker that generates action potentials at a rate that sets heartbeat frequency. Following β-receptor activation, there is an increase in the rate of action potential firing. This is caused primarily by the phosphorylation of the

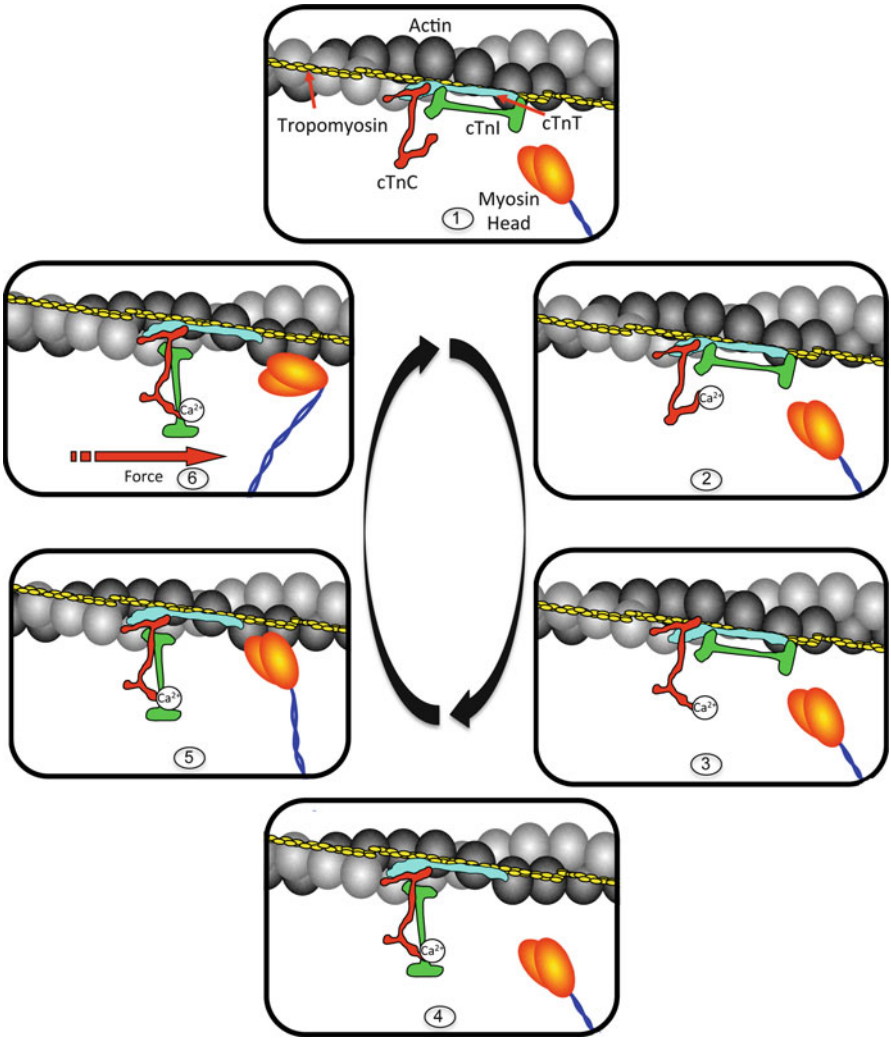

Fig. 6.2 The Ca^{2+} activation of the cardiac contractile element. (1) In the relaxed state, tropomyosin blocks the myosin-binding sites on actin. It is locked in this position by troponin I (cTnI) interacting with actin. (2) Ca^{2+} enters the cell and binds to the regulatory domain of troponin C (cTnC). (3) The conformation of cTnC changes exposing a hydrophobic patch. (4) The hydrophobic patch pulls cTnI away from the actin, releasing tropomyosin to move across the thin filament and expose myosin-binding sites. (5) The myosin head binds to actin. (6) The myosin head flexes and force is generated

hyperpolarization-activated cation channel I_f (DiFrancesco 1993). The phosphorylation of this channel increases the slope of diastolic depolarization thereby reducing the time required to reach the threshold for action potential generation (DiFrancesco 1993; Irisawa et al. 1993). As a result, the time between each action potential is reduced. This results in an increased frequency of membrane depolarization leading

Table 6.1 Summary of the specific changes in cardiac function caused by β-adrenergic stimulation and the specific proteins and cellular responses responsible

Change in heart function	Target protein(s)	Location	Cellular response
Increased heart rate	Hyperpolarization-activated cation channel I_f	Pacemaker cells of sinoatrial node	Increased frequency of membrane depolarization
Increased strength of contraction	Phospholamban (PLB)	Sarcoplasmic reticulum	Increased SR Ca^{2+} loading
Increased rate of contraction	Myosin-binding protein (MyBP-C)	Contractile element	Increased rate of cross-bridge cycling rates
Increased rate of relaxation	Troponin I (cTnI)	Contractile element	Increased rate of Ca^{2+} disassociation (k_{off}) from the troponin complex
	Phospholamban (PLB)	Sarcoplasmic reticulum	Increased rate of Ca^{2+} loading into SR

to an increase in heart rate. This control of heart rate by β-AS is in place early during embryonic development. We have recently demonstrated that isoproterenol, a β-agonist, has a stimulatory effect on heart rate in trout embryos at 15 days post-fertilization, while propranolol, a β-antagonist, has an inhibitory affect on heart rate at the same stage (Fig. 6.3) (Miller et al. 2011). Day 15 is just after heart beat is first detected in the embryos (Miller et al. 2011).

The increase in the strength of contraction caused by β-AS is due to an increase in the amount of Ca^{2+} released intercellularly following each membrane depolarization (Bers 2001). This is caused by an increase in the amount of Ca^{2+} in the sarcoplasmic reticulum (SR) (Kashimura et al. 2010). The greater Ca^{2+} loading is due to an increase in activity of the Ca^{2+} pump, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA 2a) (Kashimura et al. 2010). This protein pumps Ca^{2+} back into the SR following a contraction and it is under the regulatory control of phospholamban (PLB) (Bers 2001). When not phosphorylated, PLB acts as a break that limits the activity of SERCA and therefore the rate at which Ca^{2+} is pumped back into the SR (McIvor et al. 1988). Once PLB is phosphorylated, the break is released and more Ca^{2+} is pumped back into the SR at a faster rate. This increased load is then available for release into the cytosol following the next membrane depolarization (Kashimura et al. 2010). The end result is an increase in the number of troponin complexes activated leading to more cross-bridges formed with each contraction. This translates into greater force production.

The increase in the rate of force generation with β-AS is caused by phosphorylation of cMyBP-C (Stelzer et al. 2007; Weisberg and Winegrad 1996). cMyBP-C is located in the A-band of the sarcomeres, where it binds to actin (Shaffer et al. 2009) and myosin (Gruen et al. 1999) via the M-domain (Fig. 6.4) (Barefield and Sadayappan 2010). This interaction is thought to limit cross-bridge formation (Ababou et al. 2008; Shaffer et al. 2009, p. 1406). The phosphorylation of the M-domain causes the release of myosin (Gruen et al. 1999) and actin (Shaffer et al. 2009) (Fig. 6.4). This change is thought to result in a change in the orientation of the

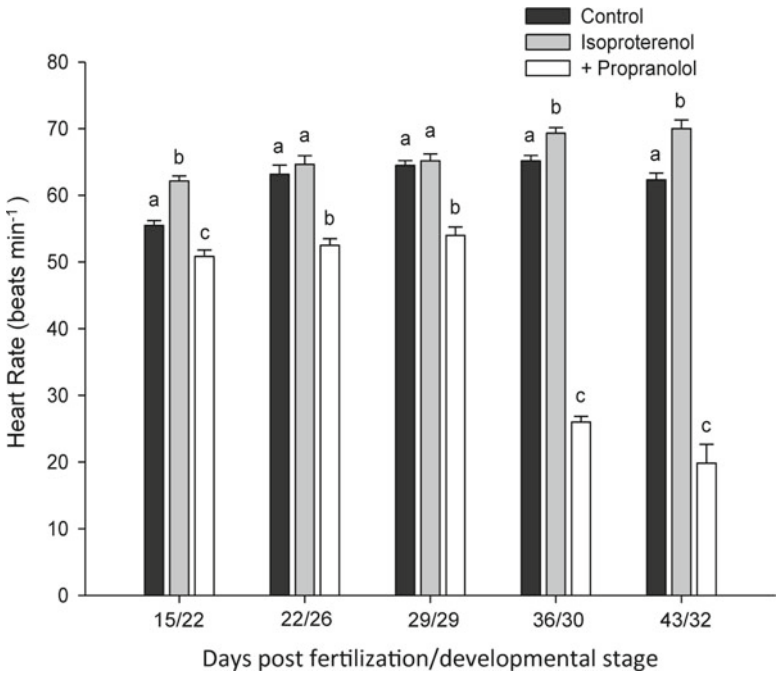

Fig. 6.3 The trout heart is responsive to adrenergic stimulation during early embryonic development. Heart rate measurements of rainbow trout embryos and larvae were measured in control conditions or following exposure to 0.1 mmol l^{-1} isoproterenol or combined 0.1 mmol l^{-1} isoproterenol + 1 mmol l^{-1} propranolol at different stages of development (Series I). Data are presented as means ± SEM ($n = 6$). *Different letters* indicate statistically significant differences between treatments at a given stage ($p < 0.05$). Figure modified from Miller et al. (2011)

myosin head and enables an increase in the rate of cross-bridge cycling (Stelzer et al. 2007; Weisberg and Winegrad 1996; Barefield and Sadayappan 2010). This leads to faster force generation within a single contraction.

The increase in the rate of muscle relaxation following a cardiac contraction with β-AS is due to the phosphorylation of cTnI (Fentzke et al. 1999; Kobayashi and Solaro 2005) and PLB (McIvor et al. 1988). On the N-terminus of cTnI cloned from mammalian hearts, there is a domain that contains two target residues of PKA/PKC (Fig. 6.5) (Noland et al. 1996). This domain, called the N-terminal extension, is only found in cTnI isoforms. It is not present in the TnI isoforms found in either slow skeletal muscle (ssTnI) or fast skeletal muscle (fsTnI). When the two PKA/PKC sites in the N-terminal extension are phosphorylated, the Ca^{2+} affinity of the troponin complex is reduced (Noland et al. 1996). This is due to changes in the interaction between cTnI and cTnC influencing the ability of cTnC to bind Ca^{2+} (Fig. 6.5) (Kobayashi and Solaro 2005). This reduction in Ca^{2+} affinity is caused by an increased rate of Ca^{2+} dissociation (k_{off}) from the regulatory domain of cTnC (Dong et al. 2007) and leads to a reduction in the Ca^{2+} sensitivity of force generation

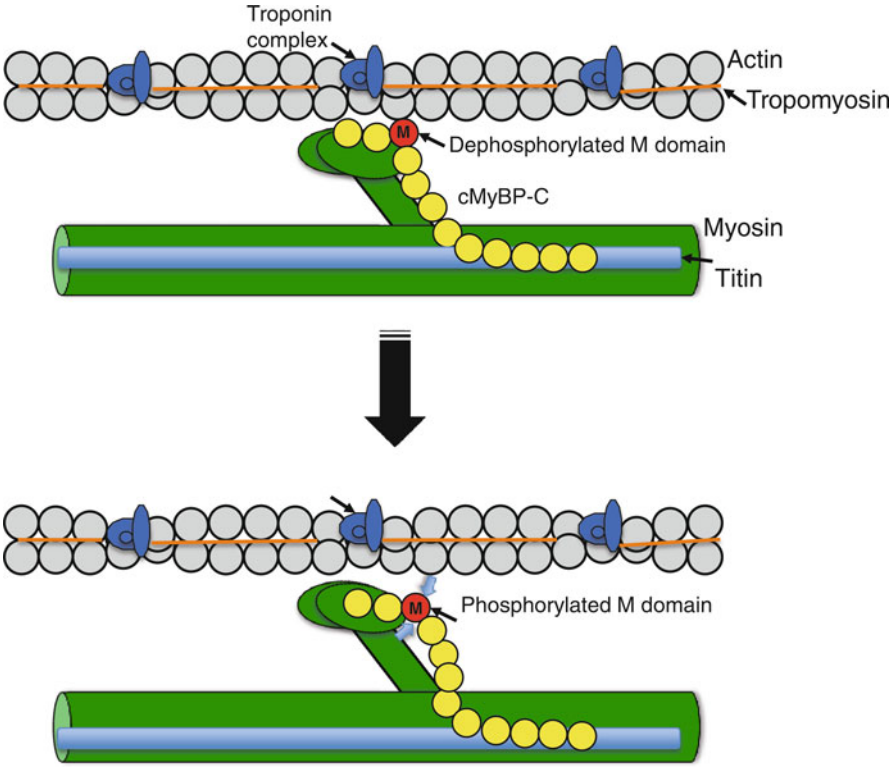

Fig. 6.4 The phosphorylation of cMyBP-C's M domain influences the interaction between myosin and actin within the contractile element. When not phosphorylated, the M domain of cMyBP-C in the N-terminus of the protein acts as a tether linking myosin and actin together. This interaction regulates the ability of the myosin head to bind to the actin filament during cross-bridge cycling. The C terminus of the cMyBP-C is anchored to titin. The phosphorylation of the M domain releases its interaction with myosin and actin. This removes the tether and enables a change in the orientation of the myosin head resulting in an increase in the rate of force production by the cardiac myocyte. Figure modified from Barefield and Sadayappan (2010)

(Fig. 6.6) (Fentzke et al. 1999). This effect of PKA activity translates into an increase in the rate at which the contractile element can be deactivated when Ca^{2+} is pumped back into the SR between cardiac contractions.

When cTnI is replaced with ssTnI within mouse (or murine) hearts using transgenic approaches, the effect of PKA activity on the Ca^{2+} sensitivity of force generation is eliminated (Fig. 6.6) (Fentzke et al. 1999). As described above, the phosphorylation of PLB increases the rate at which Ca^{2+} is pumped back into the SR via SERCA 2A following a contraction (Bers 2001). This causes an increase in the rate at which intercellular Ca^{2+} decreases between contractions. When this effect is coupled with the increased rate of Ca^{2+} dissociation from the Tn complex, caused by the phosphorylation of cTnI, the rate of muscle relaxation between contractions increases (Bers 2001).

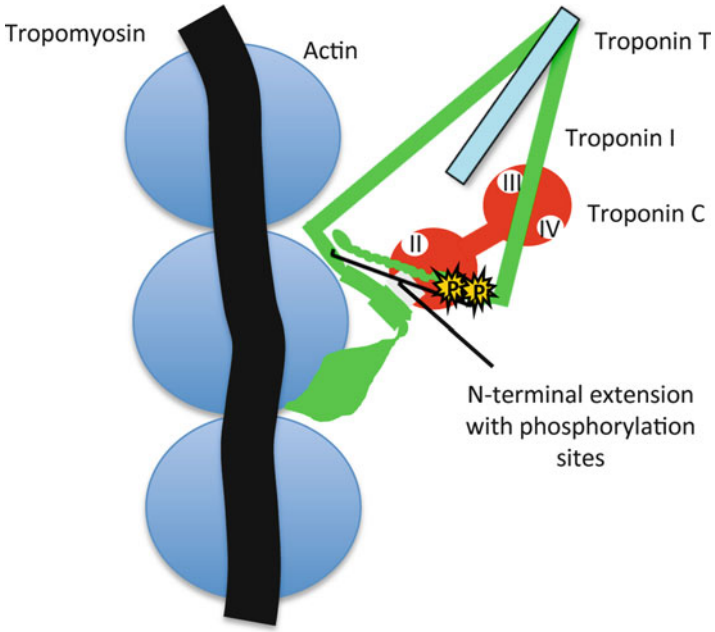

Fig. 6.5 Schematic of the interactions between the components of the troponin complex during diastole. Here the N-terminal extension of the mammalian isoform of cardiac troponin I (cTnI) is phosphorylated at serines 23 and 24, and as a result is interacting with the N-terminus of cardiac Troponin C (cTnC). This interaction regulates the ability of the cTnC to bind Ca^{2+}. cTnC is illustrated in the apo-state where site II in the regulatory domain is not bound by Ca^{2+}. Figure is modified from Solaro et al. (2008)

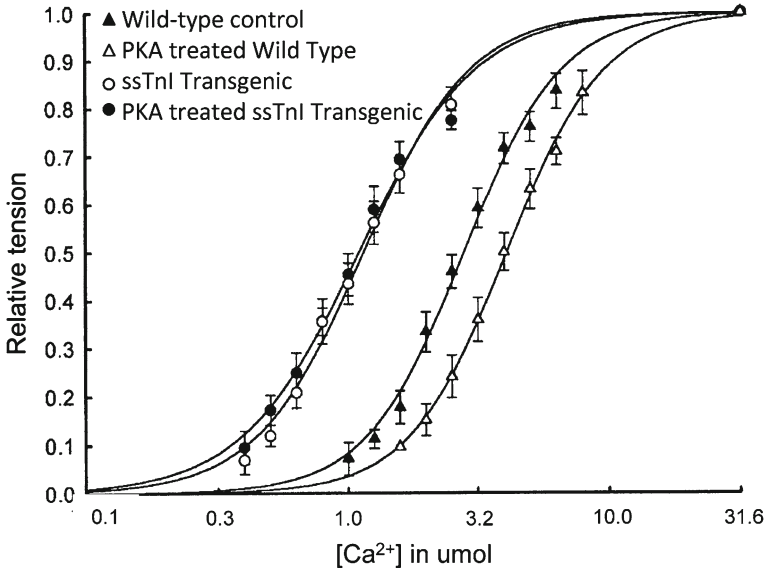

Fig. 6.6 The influence of PKA treatment on the Ca^{2+}-tension relationships of wild-type and transgenic cardiomyocytes expressing slow skeletal troponin I (ssTnI). PKA treatment caused the Ca^{2+} concentration at half maximum force, used as a measure of Ca^{2+} sensitivity, to increase by 1.5-fold. There was no influence of PKA treatment on the Ca^{2+} sensitivity of the transgenic preparations. Figure modified from Fentzke et al. (1999)

In addition to the proteins mentioned above, PKA also targets titin within the vertebrate heart (Yamasaki et al. 2002). This protein is the primary mechanism by which passive force is regulated within the mammalian heart (Fukuda et al. 2008). Two isoforms of cardiac titin are found within the adult heart that vary in their stiffness. These are N2B and N2BA. N2B is a shorter molecule with limited extensibility, while N2BA is more compliant (Fukuda et al. 2008). However, the extensibility of the N2B isoform can be increased via phosphorylation by PKA or cGMP-dependent kinase (Yamasaki et al. 2002). This leads to a reduction in the passive tension and greater extensibility of the myocardium (Kruger and Linke 2009). The result of this is greater diastolic filling in response to β-AS (Fukuda et al. 2008). Work by Shiels and co-workers (Patrick et al. 2010; Shiels et al. 2006) has demonstrated that trout cardiac myocytes are functional over a greater range of sarcomere lengths and this allows for greater length activation of the trout myocardium. These authors suggest that these properties of the trout myocardium are due in part to differences in the titin molecule as well as the phosphorylation of the molecule by PKA. Further work is required to establish this relationship.

The Evolution of TnI and MyBP-C in the Vertebrate Heart

The differences in the number and location of the phosphorylation sites within the tissue-specific isoforms of TnI and MyBP-C are an example of how the proteins have become specialized to function within a specific muscle type. The use of phylogenic analysis to examine when specific phosphorylation sites appeared during vertebrate evolution reveals a distinct pattern. This pattern is most apparent in the cardiac isoforms where the number of phosphorylation sites increased as the heart became more complex in structure and function (Fig. 6.1) (Shaffer and Gillis 2010). For example, cTnI from teleost species lack the N-terminal extension that contains the two PKA/PKC phosphorylation sites (Fig. 6.7) (Shaffer and Gillis 2010). As mentioned above, this peptide is present in all mammalian cTnI isoforms. It is also present in cTnI cloned from any tetrapod including reptiles, amphibians, and birds (Fig. 6.7). It is not known, however, if the N-terminal peptide is present within monotremes, as only the C-terminus of the cTnI gene in the platypus has been sequenced (residues 154–210) (Shaffer and Gillis 2010). This indicates that the N-terminal peptide appeared within the vertebrate lineage after the teleost fish diverged (Fig. 6.1). The appearance of this peptide may coincide with the emergence of vertebrates onto land where environmental conditions, such as temperature, and resources are more variable compared to that in water. An increased ability to regulate contractile function thereby increasing energy efficiency would have been of significant benefit.

The increase in the number of PKA sites within cMyBP-C during the evolution of the vertebrate heart occurred within the M-domain. The phosphorylation of residues within this region is thought to regulate cross-bridge cycling rates by interacting with actin and myosin (Gautel et al. 1995; Gruen et al. 1999; Shaffer et al. 2009). In mammalian cMyBP-C isoforms, this domain contains four PKA targets; in birds and

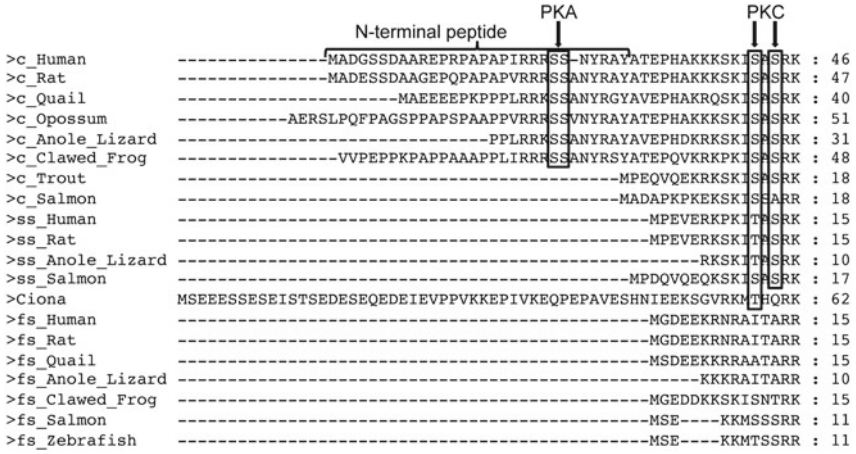

Fig. 6.7 Sequence alignment of the N-terminus of TnI sequences from cardiac, slow skeletal, and fast skeletal muscle from select vertebrate species. Human cTnI protein kinase A (PKA) and protein kinase C (PKC) targets are indicated as is the N-terminal extension in mammalian troponin I sequences. Figure modified from Shaffer and Gillis (2010)

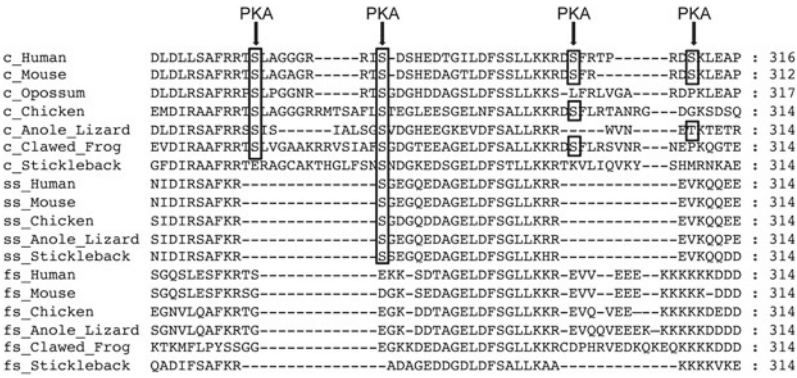

Fig. 6.8 Sequence alignment of the MyBP-C motif (M-domain) of cardiac, slow skeletal, and fast skeletal muscle from select vertebrate species. Human cMyBP-C PKA targets are indicated. Figure modified from Shaffer and Gillis (2010)

amphibians there are three within the same domain; and in fish there are two (Fig. 6.8). The additional sites present in birds and mammals appeared in cMyBP-C after fish diverged from the vertebrate lineage as they are not present in fast skeletal or slow skeletal isoforms of the protein. In fact there is no evidence that fast skeletal and slow skeletal isoforms of TnI and MyBP-C are phosphorylated by PKA. This analysis indicates that the regulation of contractile function via the phosphorylation of TnI and MyBP-C by PKA is only possible within cardiac myocytes.

While there are differences in the number and location of PKA sites within cTnI and cMyBP-C, there are two putative PKC sites that are found in TnI from all

muscle types at the same relative position within the protein. In human cTnI, these are located at positions 42 and 44 (Fig. 6.7). Phosphorylatable residues are found at the corresponding positions within most ssTnI isoforms and fsTnI from fish species (Fig. 6.7). The presence of these putative phosphorylatable targets at corresponding residues in all TnI isoforms is likely the result of an ancestral sequence motif being maintained within each of the tissue-specific isoforms. These residues are not present in TnI from tunicates, suggesting that they evolved after the tunicates diverged from the vertebrate lineage, but before the genome duplication event that gave rise to the three tissue-specific isoforms of the proteins (Fig. 6.1) (Shaffer and Gillis 2010). Previous studies have demonstrated that the phosphorylation of these specific residues decreases maximum Ca^{2+}-activated force, actomyosin ATPase activity, and desensitizes the myofilaments to Ca^{2+} (Burkart et al. 2003; Noland et al. 1995). The role that they play in regulating muscle contractility following α-AS does, however, remains to be established.

The Role of PKA in Regulating the Functional Properties of Cardiac Muscle

In mammals, as mentioned above, β-AS increases both the rate of force generation and the rate of relaxation. This is caused by the phosphorylation of MyBP-C and TnI/phospholamban, respectively. Work with trout has demonstrated that the application of adrenalin 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). While Zaar et al. (2007) have demonstrated that the application of adrenaline to muscle strips dissected from the python, *Python regius*, heart increases twitch force and the speed of contraction. Vornanen (1998) has suggested that the change in function with adrenergic stimulation in the trout heart is due, at least in part, to increased current (2.3-fold) through the L-type Ca^{2+} channels. To determine the role of the contractile proteins in "lower" vertebrates in this response, we recently completed a study that examined the influence of PKA phosphorylation on the Ca^{2+} activation of chemically skinned trout cardiac trabeculae (Gillis and Klaiman 2011). Here, we measured the kinetics of Ca^{2+} activation, the Ca^{2+} sensitivity of force generation, and how this is influenced by PKA activation. As the preparations were skinned, the Ca^{2+} levels that the contractile elements were exposed to were controlled via the experimental solutions. There was no influence, therefore, of the Ca^{2+} handling proteins. Results demonstrated that PKA treatment does not influence the Ca^{2+} sensitivity of force generation, but it decreases maximum force generation by 25% (Fig. 6.9) and the rate of force re-development at maximal activation by 46% (Gillis and Klaiman 2011). These results are quite different to those of similar experiments completed with mammalian tissues where the Ca^{2+} sensitivity of force generation is reduced (Fig. 6.6), and there is no change in force generated at maximal activation (Fentzke et al. 1999; Kobayashi and Solaro 2005). Analysis of the PKA-treated cardiac tissue in the trout experiments for phospho-protein content revealed

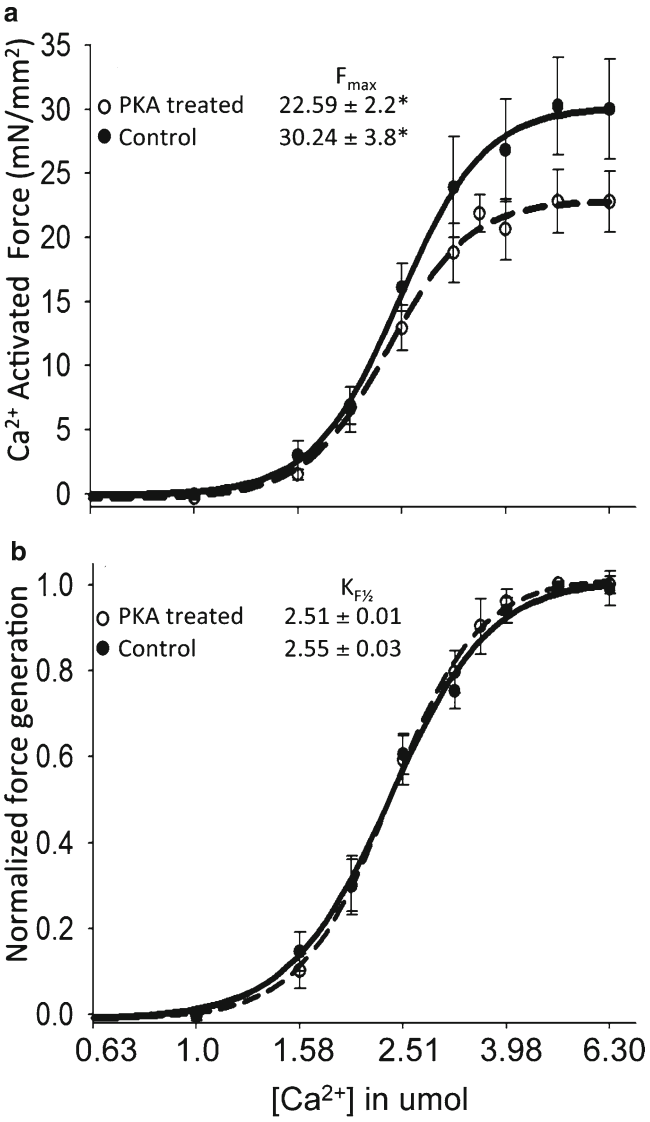

Fig. 6.9 Protein kinase A (PKA) treatment reduces the force generated by skinned trout cardiac trabeculae during maximum activation, but it does not change the Ca^{2+} sensitivity of force generation. (**a**) Actual force generated. (**b**) Relative force generated. Measurements made at 15°C and a sarcomere length of 2.2 μm. For the control preparations $n = 10$, for the PKA-treated preparations $n = 8$. The difference in maximum force generated (F_{max}) between the control and PKA-treated preparations is significantly different ($P < 0.05$). This is indicated by the *superscript asterisk*. The difference between the Ca^{2+} concentration at half maximum tension ($K_{F1/2}$), used as a measure of Ca^{2+} sensitivity, is not significant. Figure modified from Gillis and Klaiman (2011)

that the only protein affected by the PKA treatment was myosin light chain 2 (MLC2) (Gillis and Klaiman 2011). The finding that MLC2 is a target for phosphorylation is supported by work by Patrick et al. (2010), where the skinning of trout cardiac tissue caused an increase in the phosphorylation of MLC2. There was no change in the level of phosphorylation of cTnI or cMyBP-C. The phosphorylation of MLC2 can influence the flexibility of the myosin head and therefore alter the rate of cross-bridge formation (Buck et al. 1999; Olsson et al. 2004). These results, therefore, demonstrate that cTnI and cMyBP-C do not play a significant role in the response of trout cardiac myocytes to β-AS.

The results of the trout trabeculae study are not completely supported by those of a recent solution study where we found that PKA does phosphorylate trout cTnI when it is in a troponin complex, and that such treatment decreases the Ca^{2+} affinity of the complex (Fig. 6.9) (Kirkpatrick et al. 2011). In this study Ca^{2+} activation of the complex, made from recombinant proteins, was measured by monitoring a fluorescent reporter engineered into cTnC. Interestingly, the change in Ca^{2+} affinity was not as significant as when a troponin complex containing rat cTnI was treated with PKA (Fig. 6.10) (Kirkpatrick et al. 2011). As trout cTnI does not contain the N-terminal extension, the change in Ca^{2+} affinity caused by PKA treatment was due to the phosphorylation of residues elsewhere in the protein (Kirkpatrick et al. 2011). Two candidates for this are Ser^{14} and Ser^{16} whose positions correspond to PKC targets within mammalian isoforms of cTnI (Fig. 6.7). However, as we found no change in the phosphorylation state of trout cTnI when trout cardiac tissue is treated with PKA it is likely that these residues are not phosphorylated when the protein is functioning within the muscle. This conflicting result clearly demonstrates the need to integrate solution studies with in vitro measurements when working to characterize the molecular regulation of the contractile reaction.

Modifications to the Cardiac Contractile Proteins During the Evolution of Endothermy

When the Ca^{2+} sensitivity of rat cardiac tissue is compared to that of a trout with each at their respective physiological temperature, the Ca^{2+} sensitivities are similar (Gillis et al. 2007). However, when the Ca^{2+} sensitivities of the two hearts are compared at the same temperature the Ca^{2+} sensitivity of rat cardiac tissue is one-tenth that of the trout. If the rat heart had the same Ca^{2+} sensitivity as the trout heart it would likely be stuck in contracture at 37°C as Ca^{2+} would remain bound to cTnC. The comparatively lower Ca^{2+} sensitivity of the mammalian heart is due at least in part to functional changes to the contractile element. For example, the Ca^{2+} affinity of trout cTnC is ~2.3-fold that of human cTnC when the function of the two molecules is compared at the same temperature (Gillis et al. 2000). This means that the protein is activated by Ca^{2+} at a lower concentration.

However, when the Ca^{2+} affinities of trout cTnC and human cTnC are compared at temperatures close to their respective physiological temperatures the Ca^{2+} affinities

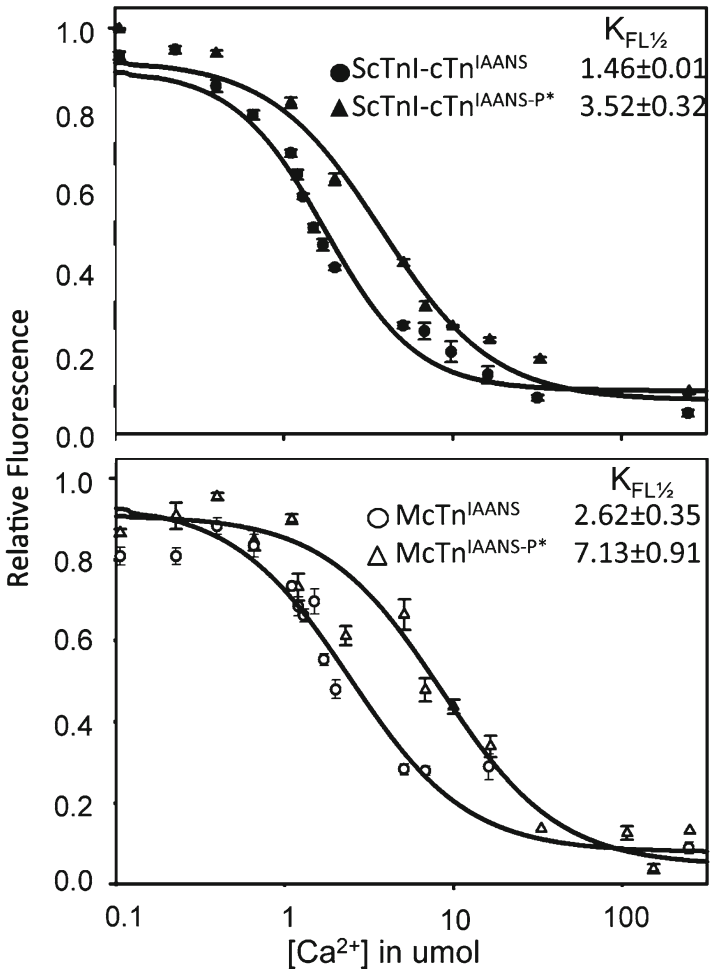

Fig. 6.10 Comparison of the effect of protein kinase A (PKA) phosphorylation on the Ca^{2+} titration curves of trout cTnI-cTn^{IAANS} (ScTnI-cTn^{IAANS}) and mammalian cTn^{IAANS} ($McTn^{IAANS}$). Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and presented as means ± SE. The curves generated by fitting the data with the Hill equation have been added for comparison with the data points. (**a**) Titration of 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 the Ca^{2+} concentration at half-maximal fluorescence, ($K_{FL1/2}$), to increase ($P<0.05$). This indicates that the Ca^{2+} affinity of the complex decreased. Figure modified from Kirkpatrick et al. (2011)

are more similar. This demonstrates that the Ca^{2+} affinity of the cTnC molecule increases with environmental temperature. In addition, if the pH of the experimental solutions used at the different temperatures (7°C trout cTnC, 30°C human cTnC) are buffered to mimic that which occur with α-stat regulation (pH 7.6 at 7°C, pH 7.0 at 30°C), the Ca^{2+} affinity of trout cTnC is significantly greater than that of human cTnC

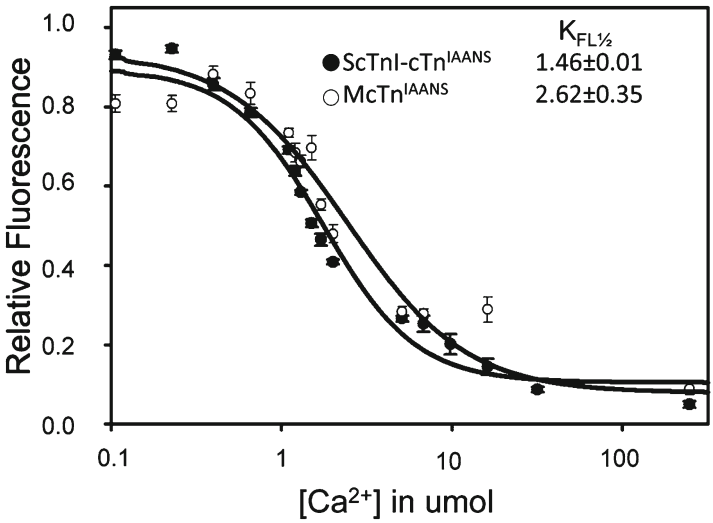

Fig. 6.11 Comparison of the Ca^{2+} titration curves of trout cTnI-cTn^{IAANS} (ScTnI-cTn^{IAANS}) ($n=8$) and mammalian cTn^{IAANS} ($McTn^{IAANS}$) ($n=6$). Data are normalized with respect to the maximal fluorescence of each Ca^{2+} titration and presented as means ± SE. The curves generated by fitting 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 ($ScTnI\text{-}cTnI^{IAANS}$) is 1.8-fold more sensitive to Ca^{2+} than that containing rat cTnI ($McTn^{IAANS}$) ($p<0.05$). An "S" is used to delineate trout cTnI as trout are salmonids. Figure modified from Kirkpatrick et al. (2011)

(Gillis et al. 2000). The specific residues responsible for the higher Ca^{2+} affinity of the trout isoform are Asn^{2}, Ile^{28}, Gln^{29}, and Asp^{30} (Gillis et al. 2005). When human cTnC was mutated to contain these residues, its Ca^{2+} affinity increased to that of trout cTnC (Gillis et al. 2005). In addition, when this mutant human cTnC was inserted into rabbit cardiac myocytes, the Ca^{2+} sensitivity of force generation increased by ~2-fold relative to controls containing recombinant human cTnC (Gillis et al. 2005). This means it is a reduction in the Ca^{2+} affinity of cTnC in mammalian cardiac tissues that is responsible, in part, for the comparatively lower Ca^{2+} sensitivity of the mammalian heart.

In a recent study, we demonstrated that the replacement of rat cTnI in a cTn complex with trout cTnI increases the Ca^{2+} affinity of the complex by ~1.8-fold (Fig. 6.11) (Kirkpatrick et al. 2011). The Ca^{2+} activation of the cTn complexes was monitored by measuring the signal of a fluorescent reporter engineered into cTnC. The increase in Ca^{2+} affinity caused by the replacement of rat cTnI with trout cTnI is thought to be due to the presence of His^{163}, Val^{165}, and Asn^{172} in trout cTnI (Kirkpatrick et al. 2011).

The corresponding residues are Ala, Glu, and His, respectively, in rat cTnI. Interestingly, His^{163}, Val^{165}, and Asn^{172} are present in all isoforms of fsTnI and ssTnI with few exceptions. These residues are also present in all cTnI isoforms until the

lineage that led to mammals diverged from that which led to the birds and reptiles approximately 300 million years ago (Shaffer and Gillis 2010). Previous work has demonstrated that the insertion of any of these residues (Ala^{163}, Glu^{165}, and His^{172}) at their respective positions into rat ssTnI reduces the Ca^{2+} sensitivity of myocyte contraction (Westfall and Metzger 2007). This means that the presence of Ala^{163}, Glu^{165}, and His^{172} in mammalian cTnI translates into a reduction in the Ca^{2+} sensitivity of force generation. Together with the lower inherent Ca^{2+} affinity of mammalian cTnC, the lower Ca^{2+} affinity of the troponin complex containing mammalian cTnI would be responsible, at least in part, for the comparatively lower Ca^{2+} sensitivity of mammalian cardiac tissue. The reduction in Ca^{2+} sensitivity, caused by changes in the sequence of cTnI and cTnC, has likely evolved to offset the sensitizing effects of increasing temperature of contractile function.

Conclusions and Perspectives

The functional characteristics of the vertebrate heart have been tuned through changes in the functional properties of the contractile element. This includes changes in the Ca^{2+} binding characteristics to enable cardiac contraction at high temperatures as well as modifications to protein sequence to allow for targeting by PKA and greater regulatory control of contractile function. The appearance of PKA target residues in cTnI and cMyBP-C after fish diverged from the vertebrate lineage brings the contractile machinery under the regulatory influence of β-AS. The resulting changes in contractile function following β-AS neatly integrate with the changes already in place caused by the phosphorylation of the Ca^{2+} handling proteins. These changes in contractile function include the increased k_{off} of Ca^{2+} from the cardiac troponin complex, caused by the phosphorylation of cTnI, coupling with the increased rate of Ca^{2+} sequestration into the SR caused by the phosphorylation of PLB to enable a faster rate of muscle relation. This increased rate of relaxation and the increased rate of contraction integrate with the faster rate of myocyte stimulation caused by increased activity of the SA node. As a result, the functional coupling between the contractile stimulus and contractile machinery is increased. It is likely only through such coupling that heart rates of 600 bpm are attainable within the mouse heart. Coupled with the increased strength of contraction, the end result is a dramatic increase in cardiac output enabling extremely high rates of aerobic activity at high temperatures. Interestingly, there has been no study of how the Ca^{2+} handling proteins targeted by PKA following β-AS has evolved within striated muscle.

Future Work

To date, there has been limited examination of how myocyte contraction in reptilian and amphibian hearts is regulated by the specific contractile proteins and how this

is influenced by β-AS. This represents a significant gap in our knowledge of how the control of the vertebrate heart evolved. The cTnI present in the hearts of these vertebrate groups posses the N-terminal extension that contains the two target residues of PKA. In addition, the cMyBP-C in amphibians and reptiles are also intermediate between teleosts and endotherms in regards to the number of PKA targets within the M-domain. Whether these are actually targeted is unknown. Functional data from these animals would be extremely interesting, as they are ectothermic but have some of the protein sequence motifs that are also present in mammalian species. Such studies would increase our understanding of the stepwise changes that occurred to the functional properties of the vertebrate heart with the evolution of endothermy. In addition, as significant changes occurred between the divergence of teleosts from the vertebrate lineage and when amphibians diverged, studying this transition would provide insight into how/if the regulation of the cardiovascular system was modified as vertebrates moved from the water onto land.

Studies of the regulation of contractile function in nonmammalian, nonmodel organisms are extremely useful for increasing our basic understanding of the molecular mechanisms that regulate contractility. The hearts of these species function under a wide range of physiological conditions such as low temperature, high temperature, or low oxygen. Understanding how the regulatory systems have evolved to enable cardiac function under such conditions provides insight into the adaptive capability of the vertebrate heart. Such knowledge is also potentially useful in the study and treatment of cardiac dysfunction caused by changes in the function of a number of the regulatory proteins, including cTnI and cMyPB-C. Such changes in function initiate compensatory changes in the heart that ultimately lead to pathological conditions and heart failure.

Acknowledgments This work was supported by operating grants from the Natural Sciences and Engineering Research Council (NSERC), and equipment grants from NSERC and the Canadian Foundation for Innovation. The author thanks Drs H.A. Shiels and S.L. Alderman for comments on an earlier version.

References

Ababou A, Rostkova E, Mistry S, Le Masurier C, Gautel M, Pfuhl M (2008) Myosin binding protein C positioned to play a key role in regulation of muscle contraction: structure and interactions of domain C1. J Mol Biol 384:615–630

Aho E, Vornanen M (2001) Cold acclimation increases basal heart rate but decreases its thermal tolerance in rainbow trout (Oncorhynchus mykiss). J Comp Physiol B 171:173–179

Barefield D, Sadayappan S (2010) Phosphorylation and function of cardiac myosin binding protein-C in health and disease. J Mol Cell Cardiol 48:866–875

Bers D (2001) Excitation-contraction coupling and cardiac contractile force. Kluwer Academic, London

Bishop C, Butler P (1995) Physiological modelling of oxygen consumption in birds during flight. J Exp Biol 198:2153–2163

Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, Kimball TF, Lorenz JN, Nairn AC, Liggett SB et al. (2004) PKC-alpha regulates cardiac contractility and propensity toward heart failure. Nat Med 10:248–254

Buck SH, Konyn PJ, Palermo J, Robbins J, Moss RL (1999) Altered kinetics of contraction of mouse atrial myocytes expressing ventricular myosin regulatory light chain. Am J Physiol 276:H1167–H1171

Burggren W, Farrell AP, Lillywhite H (1997) Vertebrate cardiovascular systems. In: Dantzler WH (ed) Handbook of physiology, vol 1. Oxford University Press, New York, pp 215–308

Burkart EM, Sumandea MP, Kobayashi T, Nili M, Martin AF, Homsher E, Solaro RJ (2003) Phosphorylation or glutamic acid substitution at protein kinase C sites on cardiac troponin I differentially depress myofilament tension and shortening velocity. J Biol Chem 278:11265–11272

Cleto CL, Vandenberghe AE, MacLean DW, Pannunzio P, Tortorelli C, Meedel TH, Satou Y, Satoh N, Hastings KE (2003) Ascidian larva reveals ancient origin of vertebrate-skeletal-muscle troponin I characteristics in chordate locomotory muscle. Mol Biol Evol 20:2113–2122

DiFrancesco D (1993) Pacemaker mechanisms in cardiac tissue. Annu Rev Physiol 55:455–472

Dong WJ, Jayasundar JJ, An J, Xing J, Cheung HC (2007) Effects of PKA phosphorylation of cardiac troponin I and strong crossbridge on conformational transitions of the N-domain of cardiac troponin C in regulated thin filaments. Biochemistry 46:9752–9761

Farmer CG (1999) Evolution of the vertebrate cardio-pulmonary system. Annu Rev Physiol 61:573–592

Fentzke RC, Buck SH, Patel JR, Lin H, Wolska BM, Stojanovic MO, Martin AF, Solaro RJ, Moss RL, Leiden JM (1999) Impaired cardiomyocyte relaxation and diastolic function in transgenic mice expressing slow skeletal troponin I in the heart. J Physiol 517:143–157

Fukuda N, Granzier HL, Ishiwata S, Kurihara S (2008) Physiological functions of the giant elastic protein titin in mammalian striated muscle. J Physiol Sci 58:151–159

Gautel M, Zuffardi O, Freiburg A, Labeit S (1995) Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: a modulator of cardiac contraction? EMBO J 14:1952–1960

Gillis TE, Klaiman J (2011) The influence of PKA treatment on the Ca2+ activation of force generation by trout cardiac muscle. J Exp Biol 214:1989–1996

Gillis TE, Liang B, Chung F, Tibbits GF (2005) Increasing mammalian cardiomyocyte contractility with residues identified in trout troponin C. Physiol Genomics 22:1–7

Gillis TE, Marshall CR, Tibbits GF (2007) Functional and evolutionary relationships of troponin C. Physiol Genomics 32:16–27

Gillis TE, Marshall CR, Xue XH, Borgford TJ, Tibbits GF (2000) Ca^{2+} binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. Am J Physiol Regul Integr Comp Physiol 279:R1707–R1715

Gordon AM, Homsher E, Regnier M (2000) Regulation of contraction in striated muscle. Physiol Rev 80:853–924

Gruen M, Prinz H, Gautel M (1999) cAPK-phosphorylation controls the interaction of the regulatory domain of cardiac myosin binding protein C with myosin-S2 in an on-off fashion. FEBS Lett 453:254–259

Harrison SM, Bers DM (1989) Influence of temperature on the calcium sensitivity of the myofilaments of skinned ventricular muscle from the rabbit. J Gen Physiol 93:411–428

Irisawa H, Brown HF, Giles W (1993) Cardiac pacemaking in the sinoatrial node. Physiol Rev 73:197–227

Kashimura T, Briston SJ, Trafford AW, Napolitano C, Priori SG, Eisner DA, Venetucci LA (2010) In the RyR2(R4496C) mouse model of CPVT, beta-adrenergic stimulation induces Ca waves by increasing SR Ca^{2+} content and not by decreasing the threshold for Ca waves. Circ Res 107:1483–1489

Kentish JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, Solaro RJ (2001) Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 88:1059–1065

Kirkpatrick K, Robertson AS, Klaiman J, Gillis TE (2011) The influence of trout cardiac troponin I and PKA phosphorylation on the Ca^{2+} affinity of the cardiac troponin complex. J Exp Biol 214:1981–1988

Kobayashi T, Solaro RJ (2005) Calcium, thin filaments, and the integrative biology of cardiac contractility. Annu Rev Physiol 67:39–67

Kruger M, Linke WA (2009) Titin-based mechanical signalling in normal and failing myocardium. J Mol Cell Cardiol 46:490–498

Li MX, Wang X, Sykes BD (2004) Structural based insights into the role of troponin in cardiac muscle pathophysiology. J Muscle Res Cell Motil 25:559–579

McIvor ME, Orchard CH, Lakatta EG (1988) Dissociation of changes in apparent myofibrillar Ca2+ sensitivity and twitch relaxation induced by adrenergic and cholinergic stimulation in isolated ferret cardiac muscle. J Gen Physiol 92:509–529

Miller SC, Gillis TE, Wright PA (2011) The ontogeny of regulatory control of the rainbow trout (Oncorhynchus mykiss) heart and how this is influenced by chronic hypoxia exposure. J Exp Biol 214:2065–2072

Noland TA Jr, Guo X, Raynor RL, Jideama NM, Averyhart-Fullard V, Solaro RJ, Kuo JF (1995) Cardiac troponin I mutants. Phosphorylation by protein kinases C and A and regulation of Ca^{2+}-stimulated MgATPase of reconstituted actomyosin S-1. J Biol Chem 270:25445–25454

Noland TA Jr, Raynor RL, Jideama NM, Guo X, Kazanietz MG, Blumberg PM, Solaro RJ, Kuo JF (1996) Differential regulation of cardiac actomyosin S-1 MgATPase by protein kinase C isozyme-specific phosphorylation of specific sites in cardiac troponin I and its phosphorylation site mutants. Biochemistry 35:14923–14931

Olsson MC, Patel JR, Fitzsimons DP, Walker JW, Moss RL (2004) Basal myosin light chain phosphorylation is a determinant of Ca2+ sensitivity of force and activation dependence of the kinetics of myocardial force development. Am J Physiol Heart Circ Physiol 287:H2712–H2718

Ota S, Saitou N (1999) Phylogenetic relationship of muscle tissues deduced from superimposition of gene trees. Mol Biol Evol 16:856–867

Patrick SM, Hoskins AC, Kentish JC, White E, Shiels HA, Cazorla O (2010) Enhanced length-dependent Ca2+ activation in fish cardiomyocytes permits a large operating range of sarcomere lengths. J Mol Cell Cardiol 48:917–924

Priede IG (1974) The effect of swimming activity and section of the vagus nerves on heart rate in rainbow trout. J Exp Biol 60:305–319

Rarick HM, Tang HP, Guo XD, Martin AF, Solaro RJ (1999) Interactions at the NH2-terminal interface of cardiac troponin I modulate myofilament activation. J Mol Cell Cardiol 31:363–375

Shaffer JF, Gillis TE (2010) Evolution of the regulatory control of vertebrate striated muscle: the roles of troponin I and myosin binding protein-C. Physiol Genomics 42:406–419

Shaffer JF, Kensler RW, Harris SP (2009) The myosin-binding protein C motif binds to F-actin in a phosphorylation-sensitive manner. J Biol Chem 284:12318–12327

Shiels HA, Calaghan SC, White E (2006) The cellular basis for enhanced volume-modulated cardiac output in fish hearts. J Gen Physiol 128:37–44

Shiels HA, Stevens ED, Farrell AP (1998) Effects of temperature, adrenaline and ryanodine on power production in rainbow trout oncorhynchus mykiss ventricular trabeculae. J Exp Biol 201:2701–2710

Solaro RJ, Rarick HM (1998) Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. Circ Res 83:471–480

Solaro RJ, Rosevear P, Kobayashi T (2008) The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. Biochem Biophys Res Commun 369:82–87

Stelzer JE, Patel JR, Walker JW, Moss RL (2007) Differential roles of cardiac myosin-binding protein C and cardiac troponin I in the myofibrillar force responses to protein kinase A phosphorylation. Circ Res 101:503–511

Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-Sekine Y, Tamagawa M, Seino S, Marban E, Nakaya H (2002) Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. J Clin Invest 109:509–516

Vornanen M (1998) L-type Ca^{2+} current in fish cardiac myocytes: effects of thermal acclimation and beta-adrenergic stimulation. J Exp Biol 201:533–547

Weisberg A, Winegrad S (1996) Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle. PNAS 93:8999–9003

Westfall MV, Metzger JM (2007) Single amino acid substitutions define isoform-specific effects of troponin I on myofilament Ca2+ and pH sensitivity. J Mol Cell Cardiol 43:107–118

Yamasaki R, Wu Y, McNabb M, Greaser M, Labeit S, Granzier H (2002) Protein kinase A phosphorylates titin's cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. Circ Res 90:1181–1188

Zaar M, Overgaard J, Gesser H, Wang T (2007) Contractile properties of the functionally divided python heart: two sides of the same matter. Comp Biochem Physiol A Mol Integr Physiol 146:163–173