Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 360 (2007) 27–32
BBRC
www.elsevier.com/locate/ybbrc

DCM troponin C mutant Gly159Asp blunts the response to troponin phosphorylation

Laura C. Preston [a,b], Christopher C. Ashley [b], Charles S. Redwood [a,*]

[a] Department of Cardiovascular Medicine, University of Oxford, Wellcome Trust Centre of Human Genetics, Oxford OX3 7BN, UK
[b] Department of Physiology, University of Oxford, Oxford, UK

Received 22 May 2007
Available online 11 June 2007

Abstract

Dilated cardiomyopathy (DCM) can be caused by a Gly159Asp mutation in cardiac troponin C (cTnC). Our previous work found that partial replacement of endogenous troponin in skinned muscle fibres with human cardiac troponin containing Gly159Asp cTnC had no significant effect on maximum force generation, Ca^{2+}-sensitivity or cooperativity, but halved the activation rate. In order to examine whether the mutant affected contractility when troponin was phosphorylated, Gly159Asp cTnC was introduced in the presence of a phosphomimic of protein kinase A phosphorylation of troponin I (Ser23Asp,Ser24Asp). The increased force production of the muscle fibres caused by this phosphomimic was significantly depressed. Furthermore, in the presence of the protein kinase C phosphomimic of troponin T (Thr203Glu), Gly159Asp mutant significantly reversed the decrease in Ca^{2+}-sensitivity. We conclude that this DCM mutant significantly blunts the contractile response to phosphorylation and this novel mechanism may contribute to its pathogenic effect.

Keywords: Cardiomyopathy; Contractility; Calcium regulation; Mutation; Thin filament; Phosphorylation; Troponin; Protein kinase A; Protein kinase C

Dilated cardiomyopathy (DCM) is characterized by a thinning of the left ventricular wall and dilation of the ventricles, resulting in impaired systolic contractility and a reduction of ejection volume from the heart. This leads to serious arrhythmias and thromboembolic events, explaining the substantial level of premature death from this disease [1]. Inherited DCM has been estimated to account for at least 20% of cases [2] and is most commonly an autosomal dominant trait with most of the identified DCM-causing genes encode components of either the cytoskeleton or the sarcomere [3]. Of the latter, DCM-causing mutations have been identified in genes encoding thin filament proteins including α-actin [4], α-tropomyosin [5], cardiac troponin T (cTnT) [6,7] and cardiac troponin C (cTnC) [7].

The trimeric troponin complex (subunits C, I, and T) and the α-helical coiled coil tropomyosin dimer located on the thin filament constitute the principal mechanism by which cardiac contractility is regulated in response to sarcoplasmic Ca^{2+} concentration [8]. During activation, cTnC binds Ca^{2+} at its single low affinity, regulatory, site II, bringing about conformational changes within the troponin–tropomyosin that result in the cooperative activation of the thin filament. Troponin subunits are also an important target of a number of protein kinases within the cardiomyocyte that act to modify the response to Ca^{2+}. The best characterized of these is the β-adrenergic-induced phosphorylation of cardiac troponin I (cTnI) at serines 23 and 24 by cAMP-dependent protein kinase (PKA). PKA phosphorylation reduces the affinity of troponin for Ca^{2+}, causing a rightward shift in the force–pCa curve and an increased crossbridge cycling rate and unloaded shortening velocity [9] contributing to increased inotropy [10]. More recently, it has been shown that cTnT is a target for protein kinase C (PKC). PKC phosphorylation of cTnT also results in decreased Ca^{2+}-sensitivity [11].

Current biochemical and physiological studies have shown that DCM mutations in thin filament regulatory

* Corresponding author. Fax: +44 1865 287586.
E-mail address: credwood@well.ox.ac.uk (C.S. Redwood).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.05.221

proteins generally result in a decrease in the Ca^{2+}-sensitivity of contractility [12,13]. However, we have previously shown that the unique DCM mutation in cTnC, Gly159Asp, when exchanged into rabbit skinned muscle fibres had no effect on isometric force generation, though it caused a significant decrease in the rate of activation and apparent thin filament cooperativity [14]. Existing studies have not examined the effect of DCM-causing mutations in conjunction with the modification of contractile behavior through the phosphorylation of the thin filament proteins. In this study, we generated recombinant human cardiac TnI and TnT containing acidic residues at the known PKA and PKC phosphorylation sites (Ser23, Ser24 of cTnI, and Thr203 of cTnT) in order to mimic the addition of a phosphate group at these residues. We then measured the effect of Gly159Asp cTnC on the generation of steady state force in whole cardiac troponin exchanged into rabbit skinned muscle fibres, when complexed with the phosphomimics Ser23Asp/Ser24Asp cTnI or Thr203Glu cTnT, compared with Gly159Asp cTnC complexed with the wild-type troponin subunits. We found that Gly159Asp cTnC blunted some of the effects of both phosphomimic proteins on contractile regulation and we suggest that this may contribute to the pathogenic effect of this DCM mutation.

Materials and methods

Expression and purification of recombinant proteins. Site-directed mutagenesis was used to introduce adidic residues at the phosphorylatable serine and threonine residues to mimic phosphorylation of cTnI by PKA (Ser23Asp,Ser24Asp) and phosphorylation of cTnT by PKC (Thr203Glu). Recombinant wild-type and mutant human troponin subunits were overexpressed in BL21(DE3)pLys *Escherichia coli* and subsequently purified as previously described [15,16]. Trimeric troponin complexes were reconstituted using our established protocol involving extensive dialysis and gel filtration [12].

Force measurements. All relaxing and activating solutions contained a final concentration (in mM) of: 2 free Mg^{2+}, 4.4 free MgATP, 14.5 creatine phosphate, 7 EGTA, and 10 imidazole. Ionic strength was adjusted to 0.18 mM with potassium propionate, and pH adjusted to 7.0 using potassium hydroxide (calculated at 20 °C). Activating solutions were made by adding $CaCl_2$ to give final free Ca^{2+} concentrations of pCa values between 6.4 and 4.5. Pre-rigor solution contained (in mM): 10 imidazole, 2.5 EGTA, 15 EDTA, pH 7.0. Rigor solution contained (in mM): 10 imidazole, 2.5 EGTA, 2.5 EDTA, pH 7.0. The whole troponin exchange solution contained (in mM): 10 imidazole, 170 NaCl, 5 $MgCl_2$, 5 EGTA, 5 dithiothreitol, pH 6.8.

Steady state force measurements were carried out on troponin-exchanged psoas fibres from female New Zealand White rabbits (killed by lethal injection of barbiturate in accordance with UK Home Office guidelines) as previously described [14,17]. Briefly, glycerinated fibre bundles (4 mm long by 0.15–0.2 mm in diameter) were attached at one end to an Akers 801 piezo-electric force transducer and at the other to a moving-coil galvanometer. All measurements were made using a rotating chamber assembly [18] at 20 °C. The fibre bundle was skinned for 2 min in relaxing solution containing 1% v/v Triton-X100, and then immersed in relaxing solution. The initial sarcomere length was set to 2.5 μm and force/pCa relationships were determined by immersing the fibre bundle alternately between activating solutions of increasing calcium concentration (pCa 6.4–4.5) and relaxing solution, and the isometric tension generation recorded.

Whole troponin replacement was achieved using our modification of the method first described by Brenner and colleagues [19]. Briefly, fibres were brought to rigor through washes in ATP-free pre-rigor solution and rigor solution. The fibres were then incubated in 2.2 mg/ml recombinant human cardiac troponin exchange solution for 2 h at 20 °C. Following this fibres were washed in fresh exchange buffer and relaxing solution to remove excess protein. The proportion of human cardiac troponin incorporated into the psoas fibre was determined by SDS–PAGE and immunoblotting as previously described.

Maximum Ca^{2+}-activated force is expressed as % of force prior to troponin exchange. Both force and pCa_{50} data are expressed as means ± SEM Student's *t* test was used for statistical analysis of the data. A *P* value of <0.05 was set as the confidence level.

Results

Exchange of recombinant whole troponin into rabbit skinned muscle fibres

In order to study the combined effects of the Gly159Asp cTnc DCM mutant and troponin phosphorylation, six different troponin complexes were reconstituted for this study: wild-type human cardiac troponin composed of all three wild-type subunits; DCM mutant troponin consisting of Gly159Asp cTnC and wild-type cTnI and cTnT; cTnI PKA phosphomimic troponin composed of Ser23Asp,Ser24Asp cTnI and wild-type cTnC and cTnT along with DCM/cTnI phosphomimic troponin containing Gly159Asp cTnC, Ser23Asp,Ser24Asp cTnI and wild-type cTnT; cTnT PKC phosphomimic troponin composed of Thr203Glu cTnT and wild-type cTnC and cTnI along with DCM/cTnT phosphomimic troponin containing Gly159Asp cTnC, wild-type cTnI and Thr203Glu cTnT. Each troponin was reconstituted as a 1:1:1 complex by our established protocol [12] and the presence of the mutant subunits was found not to have a significant effect on troponin assembly. To study the effects of Gly159Asp cTnC on the modification of muscle contractility by thin filament phosphorylation in situ, we exchanged endogenous troponin in rabbit skinned psoas muscle fibres for recombinant human cardiac troponin using our recently described method [14]. The total mass of troponin relative to actin was not altered following the exchange process. Wild-type troponin was incorporated at $55.0\% \pm 1.3\%$ ($n = 6$) of total troponin after exchange and the incorporation of each of the other five complexes was not significantly different.

The increase in maximum Ca^{2+}-activated force (but not the decrease in Ca^{2+}-sensitivity) caused by the cTnI PKA phosphomimic is attenuated by Gly159Asp cTnC

Incorporation of human cardiac troponin containing the Ser23Asp,Ser24Asp cTnI phosphomimic into rabbit skinned fibres was found, in comparison to the exchange of wild-type human cardiac troponin, to cause a significant reduction in the Ca^{2+}-sensitivity (WT Tn pCa_{50} 5.64 ± 0.03 vs. Ser23Asp/Ser24Asp cTnI pCa_{50} 5.43 ± 0.01 $P < 0.0001$) and significant increase in the

cooperativity (WT Tn nH 2.03 ± 0.21 vs. Ser23Asp/Ser24Asp cTnI nH 5.31 ± 0.24 $P < 0.0001$) of isometric force production (Fig. 1). Incorporation of Ser23Asp/Ser24Asp cTnI also resulted in a significant increase in maximal Ca^{2+}-activated force at pCa 5.0 (WT Tn $62.5\% \pm 7.7\%$ vs. Ser23Asp/Ser24Asp TnI $101.0\% \pm 13.6\%$ $P < 0.05$) (Fig. 2).

We have previously shown that unlike other DCM-causing mutations Gly159Asp cTnC has no significant effect on the Ca^{2+}-sensitivity of force production, cooperativity in muscle fibres or the maximum isometric force generation per cross-sectional area of the fibres [14]. When troponin containing both Ser23Asp/Ser24Asp cTnI and Gly159Asp cTnC was exchanged into fibres, the Ca^{2+}-regulation of isometric force was indistinguishable from cTnI phosphomimic troponin and similar changes in comparison to wild-type in pCa50 (Ser23Asp/Ser24Asp cTnI, WT cTnC pCa_{50} 5.43 ± 0.01 vs. Ser23Asp/Ser24Asp cTnI, Gly159Asp cTnC pCa_{50} 5.42 ± 0.01) and cooperativity (Ser23Asp/Ser24Asp cTnI, WT cTnC nH 5.31 ± 0.24 vs. Ser23Asp/Ser24Asp cTnI, Gly159Asp cTnC nH 5.99 ± 0.24) were observed (Fig. 1). However, the presence of the Gly159Asp cTnC abolished the significant increase in maximal force that resulted from the incorporation of the cTnI phosphomimic in the presence of wild-type cTnC (Fig. 2). Therefore we conclude that the DCM-causing mutation Gly159Asp cTnC blunts the effect of PKA phosphorylation of Ser23/Ser24 cTnI on maximum Ca^{2+}-activated force in muscle fibres.

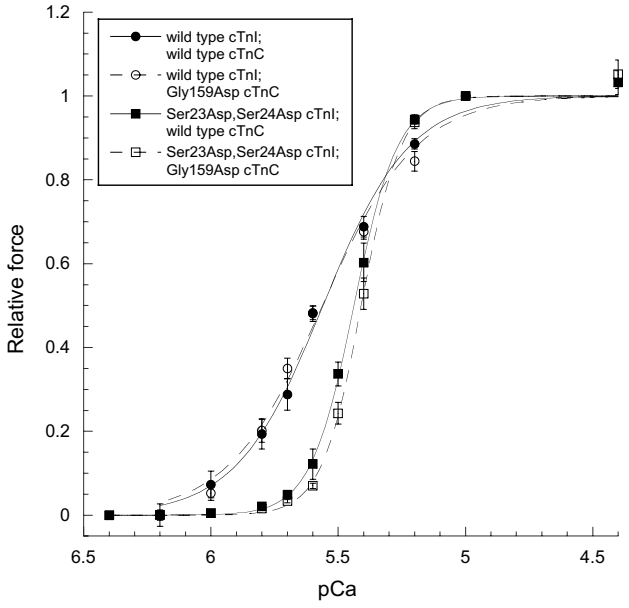

Fig. 1. Ca^{2+} regulation of isometric force in fibres containing human cardiac troponin composed of cTnI phosphomimic and DCM mutant cTnC. Force–pCa curves of rabbit psoas fibres following whole troponin exchange with either wild-type human cardiac troponin or troponin containing cTnI phosphomimic and/or Gly159Asp cTnC. Individual points represent mean ± SEM ($n = 8$); curves were normalized to pCa4.0 and fitted to a standard log-sigmoidal curve using the equation $1/(1 + 10^{((pCa-pCa50) \times nH)})$.

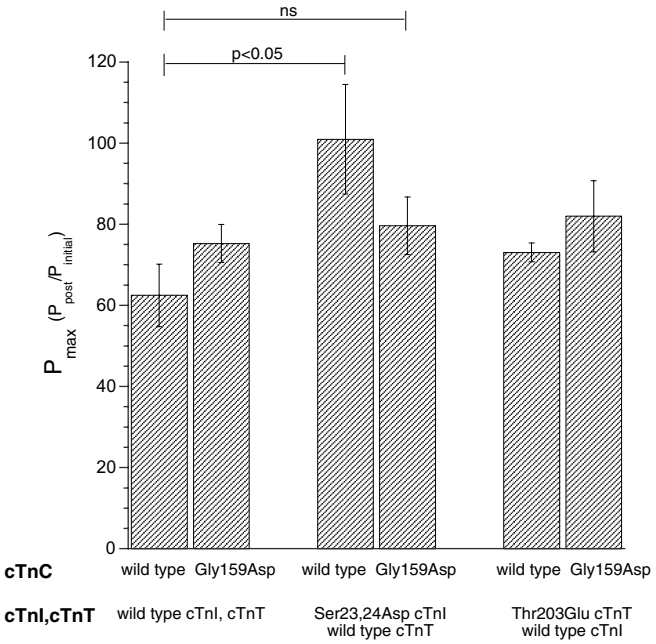

Fig. 2. Maximum Ca^{2+}-activated force in fibres containing human cardiac troponin composed of cTnI phosphomimic, cTnT phosphomimic and DCM mutant cTnC. Maximum Ca^{2+}-activated force expressed as % of force prior to exchange for fibres containing human cardiac troponin composed of either cTnI phosphomimic, cTnT phosphomimic or DCM mutant cTnC. Error bars show SEM.

The presence of Gly159Asp cTnC blunts the Ca^{2+}-sensitivity decrease caused by Thr203Glu cTnT

In order to mimic PKC phosphorylation of cTnT, troponin containing Thr203Glu cTnT was exchanged into rabbit skinned muscle fibres. Compared with wild-type troponin, Thr203Glu TnT significantly reduced the Ca^{2+}-sensitivity of isometric force production (WT troponin pCa_{50} 5.64 ± 0.03 vs. Thr203Glu TnT pCa_{50} 5.32 ± 0.02, $n = 12$, $P < 0.0001$), and significantly increased the cooperativity (WT troponin nH 2.03 ± 0.21 vs. Thr203Glu TnT nH 4.61 ± 0.29 $P < 0.0001$) (Fig. 3). No significant effect by Thr203Glu TnT was seen on restored P_{max} force (Fig. 2). In comparison with these data, troponin complex containing both Thr203Glu cTnT and the DCM mutant Gly159Asp TnC significantly reversed the fall in Ca^{2+}-sensitivity caused by the PKC phosphorylation-mimic Thr203Glu TnT, restoring significantly the pCa_{50} towards the value observed for the wild-type troponin complex (Thr203Glu TnT pCa_{50} 5.31 ± 0.03 vs. Thr203Glu TnT, Gly159Asp TnC pCa_{50} 5.43 ± 0.01 vs. WT troponin pCa_{50} 5.64 ± 0.03). Additionally Thr203Glu TnT, Gly159Asp TnC significantly increased the cooperativity of the fibres compared with Thr203Glu TnT on its own (Thr203Glu TnT nH 4.61 ± 0.29 vs. Thr203Glu TnT, Gly159Asp TnC nH 7.74 ± 0.55 $P < 0.0001$) (Fig. 3).

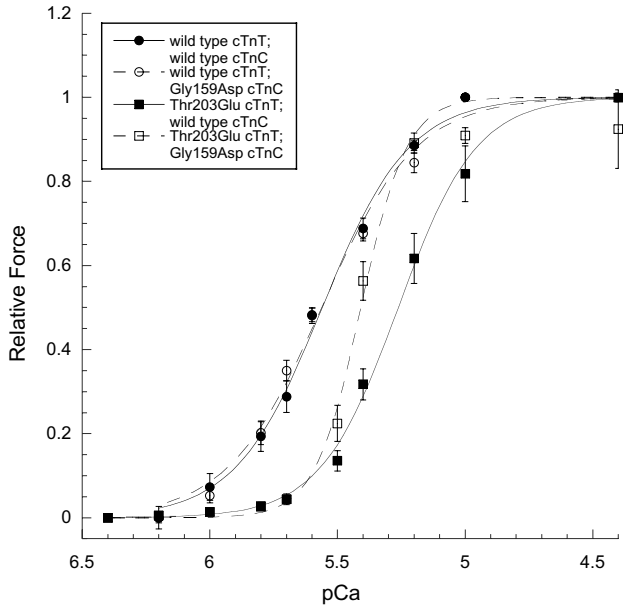

Fig. 3. Ca^{2+} regulation of isometric force in fibres containing human cardiac troponin composed of cTnT phosphomimic and DCM mutant cTnC. Force–pCa curves of rabbit psoas fibres following whole troponin exchange with either wild-type human cardiac troponin or troponin containing cTnT phosphomimic and/or Gly159Asp cTnC. Individual points represent mean ± SEM ($n = 8$); curves were normalized to pCa4.0 and fitted to a standard log-sigmoidal curve using the equation $1/(1 + 10^{((pCa-pCa50) \times nH)})$.

Discussion

This study has examined how isometric force production regulated by reconstituted recombinant human cardiac troponin incorporating mimics of either PKA phosphorylation of cTnI or PKC phosphorylation of cTnT is affected by the DCM mutant cTnC Gly159Asp. We have shown that Gly159Asp cTnC, when exchanged into rabbit skinned psoas muscle fibres blunts both the observed increase in maximum force caused by Ser23Asp,Ser24Asp cTnI, and the reduction in Ca^{2+}-sensitivity brought about by Thr203Glu cTnT. This novel finding provides important functional information on this DCM mutant protein and suggests a likely mechanism that may contribute to its pathogenic action.

The modification of cTnI by PKA has been extensively investigated and the principal sites of phosphorylation shown to be Ser23 and Ser24 of human cTnI, present in the cardiac-specific 30 amino acid N-terminal extension [20]. It has been shown that phosphorylation of Ser23 and Ser24 of cTnI reduces the Ca^{2+}-sensitivity of the myofilament and increases crossbridge cycling [21–23]. Furthermore, such modification results in increased power output at both the myocyte [10] and whole heart levels [24]. Replacing a phosphorylatable serine or threonine with an acidic amino acid can serve to mimic the incorporation of negative charge that occurs upon phosphorylation and this approach has proved successful in studies of troponin phosphorylation. Many studies have used the double aspartate mutant at serines 23 and 24 to mimic PKA phosphorylation of cTnI and it has been shown to reduce the Ca^{2+}-sensitivity (compared with wild-type cTnI) of skinned fibres by a similar amount to actual PKA phosphorylation [25]. In agreement with previously published data, the work presented here demonstrates that incorporation of recombinant human cardiac troponin containing Ser23Asp/Ser24Asp TnI into skinned fibres resulted in a fall in the Ca^{2+}-sensitivity compared with the presence of wild-type cardiac troponin (Fig. 1). The PKA phosphomimic also enhanced cooperativity (Fig. 1) and produced a significant increase in the maximum Ca^{2+}-activated force when measured as a proportion of pre-exchange force (Fig. 2). It has been unclear as to whether PKA phosphorylation of cTnI contributes to the positive inotropic response *in vivo*, or whether this was solely the effect of the phosphorylation of the sarcolemmal L-type Ca^{2+}-channels in generating an increased inward Ca^{2+}-current. The present results however support a recent study of transgenic mice with pseudo-phosphorylated cTnI PKA sites that had significantly enhanced systolic function at baseline, and greater frequency-dependent enhancement of systolic function *in vivo* [24]. When the cTnI PKA phosphomimic was investigated in combination with the Gly159Asp cTnC DCM mutant, the Ca^{2+}-sensitivity decrease was unaffected but the observed increase in maximum force was suppressed. This suggests that the DCM cTnC mutant is likely to suppress β-adrenergic stimulation of systolic function. Structural studies have suggested that the N-terminal extension of cTnI binds to and stabilizes the Ca^{2+} bound N-terminal domain of TnC and that bisphosphorylation interferes with this interaction leading to reduced Ca^{2+} affinity [26]. This may provide a structural basis for our observation that the DCM mutation, present in the C-terminal domain of cTnC, does not affect the alteration of Ca^{2+}-sensitivity by the cTnI phosphomimic but does perturb the effect on maximum force, the latter effect likely to be transmitted to the whole thin filament via the interaction of the C-terminal cTnC domain with cTnT and hence to tropomyosin.

PKC phosphorylation of cTnT has been reported to cause depression in maximum actomyosin ATPase rate *in vitro* [27] and the principal phosphorylation site identified as residue Thr206 in mouse cTnT (equivalent to Thr203 in the human sequence) [28]. Mutating this residue to Glu resulted in a decrease in the Ca^{2+}-sensitivity, cooperativity, and maximum Ca^{2+}-activated force in permeabilized mouse left ventricular papillary muscle fibre bundles [11]. In our troponin-exchanged fibre experiments, the PKC phosphorylation mimic Thr203Glu cTnT caused a significant decrease in Ca^{2+}-sensitivity of force production compared to wild-type troponin (Fig. 3), though with no change in cooperativity (Fig. 3) or maximum force (Fig. 2). The additional presence of Gly159Asp cTnC partially reversed the decrease in Ca^{2+}-sensitivity generated by the PKC phosphomimic and also increased the cooperativity of force generation. Gly159Asp TnC had no significant

effect however on the maximal tension developed in the muscle fibres containing Thr203Glu cTnT. Thr203 is within the C-terminal T2 domain that interacts with both cTnI and cTnC and the crystal structure of human cardiac troponin reveals that this residue lies within a short helix 202–227 [29]. NMR studies have shown that this region binds to the C-lobe of cTnC, including cTnC residue Gly159 [30], and this interaction hence may be involved in the transduction of the Ca^{2+} signal. PKC phosphorylation of cTnT may therefore alter this interaction and thus decrease the Ca^{2+}-sensitivity of force production and the Gly159Asp DCM mutation may further modulate the effect.

Although to date Gly159Asp cTnC is only DCM mutant to be tested in combination with troponin phosphorylation, studies on mutations in cTnI that cause hypertrophic cardiomyopathy (HCM) have revealed additional effects of troponin modification. The presence of the HCM cTnI mutation Arg145Gly has been shown to antagonize both PKA and PKC phosphorylation of cTnI [31,32]. These published results and the data presented here emphasise that different phosphorylation states of the thin filament should be examined when evaluating the functional effects of disease-causing mutations in thin filament regulatory proteins. The results obtained with Gly159Asp cTnC suggest that this DCM mutant may act to blunt the effects of both PKA and PKC phosphorylation *in vivo* and this may contribute to its pathogenic mechanism.

Acknowledgments

This work was funded by the British Heart Foundation. We thank Paul Robinson for preparation of some of the troponin subunits used in this study and Hugh Watkins for critical reading of the manuscript.

References

[1] J.N. Cohn, M.R. Bristow, K.R. Chien, W.S. Colucci, O.H. Frazier, L.A. Leinwand, B.H. Lorell, A.J. Moss, E.H. Sonnenblick, R.A. Walsh, S.C. Mockrin, L. Reinlib, Report of the National Heart, Lung, and Blood Institute Special Emphasis Panel on heart failure research, Circulation 95 (1997) 766–770.

[2] V.V. Michels, P.P. Moll, F.A. Miller, A.J. Tajik, J.S. Chu, D.J. Driscoll, J.C. Burnett, R.J. Rodeheffer, J.H. Chesebro, H.D. Tazelaar, The frequency of familial dilated cardiomyopathy in a series of patients with idiopathic dilated cardiomyopathy, N. Engl. J. Med. 326 (1992) 77–82.

[3] J.G. Seidman, C. Seidman, The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms, Cell 104 (2001) 557–567.

[4] T.M. Olson, V.V. Michels, S.N. Thibodeau, Y.S. Tai, M.T. Keating, Actin mutations in dilated cardiomyopathy, a heritable form of heart failure, Science 280 (1998) 750–752.

[5] T.M. Olson, N.Y. Kishimoto, F.G. Whitby, V.V. Michels, Mutations that alter the surface charge of alpha-tropomyosin are associated with dilated cardiomyopathy, J. Mol. Cell Cardiol. 33 (2001) 723–732.

[6] M. Kamisago, S.D. Sharma, S.R. DePalma, S. Solomon, P. Sharma, B. McDonough, L. Smoot, M.P. Mullen, P.K. Woolf, E.D. Wigle, J.G. Seidman, C.E. Seidman, Mutations in sarcomere protein genes as a cause of dilated cardiomyopathy, N. Engl. J. Med. 343 (2000) 1688–1696.

[7] J. Mogensen, R.T. Murphy, T. Shaw, A. Bahl, C. Redwood, H. Watkins, M. Burke, P.M. Elliott, W.J. McKenna, Severe disease expression of cardiac troponin C and T mutations in patients with idiopathic dilated cardiomyopathy, J. Am. Coll. Cardiol. 44 (2004) 2033–2040.

[8] L.S. Tobacman, Thin filament-mediated regulation of cardiac contraction, Annu. Rev. Physiol. 58 (1996) 447–481.

[9] J. Layland, R.J. Solaro, A.M. Shah, Regulation of cardiac contractile function by troponin I phosphorylation, Cardiovasc. Res. 66 (2005) 12–21.

[10] T.J. Herron, F.S. Korte, K.S. McDonald, Power output is increased after phosphorylation of myofibrillar proteins in rat skinned cardiac myocytes, Circ. Res. 89 (2001) 1184–1190.

[11] M.P. Sumandea, W.G. Pyle, T. Kobayashi, P.P. de Tombe, R.J. Solaro, Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T, J. Biol. Chem. 278 (2003) 35135–35144.

[12] P. Robinson, M. Mirza, A. Knott, H. Abdulrazzak, R. Willott, S. Marston, H. Watkins, C. Redwood, Alterations in thin filament regulation induced by a human cardiac troponin T mutant that causes dilated cardiomyopathy are distinct from those induced by troponin T mutants that cause hypertrophic cardiomyopathy, J. Biol. Chem. 277 (2002) 40710–40716.

[13] M. Mirza, S. Marston, R. Willott, C. Ashley, J. Mogensen, W. McKenna, P. Robinson, C. Redwood, H. Watkins, Dilated cardiomyopathy mutations in three thin filament regulatory proteins result in a common functional phenotype, J. Biol. Chem. 280 (2005) 28498–28506.

[14] L.C. Preston, S. Lipscomb, P. Robinson, J. Mogensen, W.J. McKenna, H. Watkins, C.C. Ashley, C.S. Redwood, Functional effects of the DCM mutant Gly159Asp Troponin C in skinned muscle fibres, Pflugers Arch. 453 (2007) 771–776.

[15] K. Elliott, H. Watkins, C.S. Redwood, Altered regulatory properties of human cardiac troponin I mutants that cause hypertrophic cardiomyopathy, J. Biol. Chem. 275 (2000) 22069–22074.

[16] C. Redwood, K. Lohmann, W. Bing, G.M. Esposito, K. Elliott, H. Abdulrazzak, A. Knott, I. Purcell, S. Marston, H. Watkins, Investigation of a truncated cardiac troponin T that causes familial hypertrophic cardiomyopathy: Ca(2+) regulatory properties of reconstituted thin filaments depend on the ratio of mutant to wild-type protein, Circ. Res. 86 (2000) 1146–1152.

[17] S. Lipscomb, L.C. Preston, P. Robinson, C.S. Redwood, I.P. Mulligan, C.C. Ashley, Effects of troponin C isoform on the action of the cardiotonic agent EMD 57033, Biochem. J. 388 (2005) 905–912.

[18] P. Griffith, A. Jones, A simple device for transfer of single muscle fibres by rotation between 70 μl chambers while making optical measurements, J. Physiol. 480 (1994) 5P.

[19] B. Brenner, T. Kraft, L.C. Yu, J.M. Chalovich, Thin filament activation probed by fluorescence of *N*-((2-(Iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1, 3-diazole-labeled troponin I incorporated into skinned fibers of rabbit psoas muscle, Biophys. J. 77 (1999) 2677–2691.

[20] K. Swiderek, K. Jaquet, H.E. Meyer, L.M. Heilmeyer Jr., Cardiac troponin I, isolated from bovine heart, contains two adjacent phosphoserines. A first example of phosphoserine determination by derivatization to *S*-ethylcysteine, Eur. J. Biochem. 176 (1988) 335–342.

[21] J.C. Kentish, D.T. McCloskey, J. Layland, S. Palmer, J.M. Leiden, A.F. Martin, J. Solaro, Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle, Circ. Res. 88 (2001) 1059–1065.

[22] K.T. Strang, N.K. Sweitzer, M.L. Greaser, R.L. Moss, β-Adrenergic receptor stimulation increases unloaded shortening velocity of skinned single ventricular myocytes from rats, Circ. Res. 74 (1994) 542–549.

[23] Y. Pi, D. Zhang, K.R. Kemnitz, H. Wang, J.W. Walker, Protein kinase C and A sites on troponin I regulate myofilament Ca^{2+} sensitivity and ATPase activity in the mouse myocardium, J. Physiol. 552.3 (2003) 845–857.

[24] E. Takimoto, D.G. Soergel, P.M. Janssen, L.B. Stull, D.A. Kass, A.M. Murphy, Frequency- and afterload-dependent cardiac modulation in vivo by troponin I with constitutively active protein kinase A phosphorylation sites, Circ. Res. 94 (2004) 496–504.

[25] C. Dohet, E. al-Hillawi, I.P. Trayer, J.C. Ruegg, Reconstitution of skinned cardiac fibres with human recombinant cardiac troponin-I mutants and troponin-C, FEBS Lett. 377 (1995) 131–134.

[26] D.G. Ward, S.M. Brewer, M.J. Calvert, C.E. Gallon, Y. Gao, I.P. Trayer, Characterization of the interaction between the N-terminal extension of human cardiac troponin I and troponin C, Biochemistry 43 (2004) 4020–4027.

[27] T.A. Noland, J.F. Kuo, Protein kinase C phosphorylation of cardiac troponin I and troponin T inhibits Ca^{2+}-stimulated MgATPase activity in reconstituted actomyosin and isolated myofibrils, and decreases actin–myosin interactions, J. Mol. Cell. Cardiol. 25 (1993) 53–65.

[28] M.P. Sumandea, W.G. Pyle, T. Kobayashi, P.P. De Tombe, J. Solaro, Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T, J. Biol. Chem. 278 (2003) 35135–35144.

[29] S. Takeda, A. Yamashita, K. Maeda, Y. Maeda, Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form, Nature 424 (2003) 35–41.

[30] T.M. Blumenschein, B.P. Tripet, R.S. Hodges, B.D. Sykes, Mapping the interacting regions between troponins T and C. Binding of TnT and TnI peptides to TnC and NMR mapping of the TnT-binding site on TnC, J. Biol. Chem. 276 (2001) 36606–36612.

[31] Y. Deng, A. Schmidtmann, A. Redlich, B. Westerdorf, K. Jaquet, R. Thieleczek, Effects of phosphorylation and mutation R145G on human cardiac troponin I function, Biochemistry 40 (2001) 14593–14602.

[32] T. Kobayashi, W.-J. Dong, E.M. Burkart, H.C. Cheung, J. Solaro, Effects of protein kinase C dependent phosphorylation and a familial hypertrophic cardiomyopathy-related mutation of cardiac troponin I on structural transition of troponin C and myofilament activation, Biochemistry 43 (2004) 5996–6004.