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Original Article

Phosphorylation of human cardiac troponin I G203S and K206Q linked to familial hypertrophic cardiomyopathy affects actomyosin interaction in different ways

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Abstract

cAMP-dependent protein kinase (PKA)-dependent phosphorylation of the two serine residues in the amino terminal region unique to cardiac troponin I (cTnI) is known to cause two effects: (i) decrease of the maximum Ca^{2+}-controlled thin filament-activated myosin S1-ATPase (actoS1-ATPase) activity and mean sliding velocity of reconstituted thin filaments; (ii) rightward shift of the Ca^{2+} activation curves of actoS1-ATPase activity, filament sliding velocity, and force generation. We have studied the influence of phosphorylation of human wild-type cTnI and of two mutant cTnI (G203S and K206Q) causing familial hypertrophic cardiomyopathy (fHCM) on the secondary structure by circular dichroism spectroscopy and on the Ca^{2+} regulation of actin–myosin interaction using actoS1-ATPase activity and in vitro motility assays. Both mutations slightly influence the backbone structure of cTnI but only the secondary structure of cTnI-G203S is also affected by bis-phosphorylation of cTnI. In functional studies, cTnI-G203S behaves similarly to wild-type cTnI, i.e. the mutation itself has no measurable effect and bis-phosphorylation alters the actoS1-ATPase activity and the in vitro thin filament motility in the same way as does bis-phosphorylation of wild-type cTnI. In contrast, the mutation K206Q leads to a considerable increase in the maximum actoS1-ATPase activity as well as filament motility compared to wild-type cTnI. Bis-phosphorylation of this mutant cTnI still suppresses the maximum actoS1-ATPase activity and filament sliding velocity but does no longer affect the Ca^{2+} sensitivity of these processes. Thus, these two fHCM-linked cTnI mutations, although reflecting similar pathological situations, exert different effects on the actomyosin system per se and in response to bis-phosphorylation of cTnI.

Keywords: Human cardiac troponin I phosphorylation; cTnI CD spectroscopy; Familial hypertrophic cardiomyopathy

1. Introduction

The contractile function of a cardiac myocyte is modulated by signalling processes which occur synchronously with the autonomous beating of the heart [1]. One of these functionally important signalling pathways involves cAMP-dependent protein kinase (PKA) which is activated upon β-adrenergic stimulation and thereby phosphorylates several proteins involved in the contractile process including cardiac troponin I (cTnI). cTnI is the inhibitory component of the cardiac troponin (cTn) complex. At low cytosolic [Ca^{2+}], it strongly binds to cardiac troponin C (cTnC) and to actin through the inhibitory region (amino acids [1] 128–148) [2], and a carboxy terminal region (amino acids 170–182) [3] resulting in inhibition of the actomyosin interaction. Binding of Ca^{2+} to cTnC leads to a cascade of conformational changes involving cTnI, cardiac troponin T (cTnT), and tropomyosin, which eventually allow a strong actin–myosin interaction [4]. Activation of PKA via the β-adrenergic pathway leads to the consecutive phosphorylation of two serine

Abbreviations: ActoS1-ATPase, thin filament-activated myosin S1-ATPase; CD, circular dichroism; cTn, cardiac troponin; cTnI, cTnC, and cTnT, cardiac troponin I, C, and T, respectively; cTnI-WT and cTnI-WT/pp, non-phosphorylated and bis-phosphorylated wild-type cTnI, respectively; cTnI-G203S and cTnI-G203S/pp, non-phosphorylated and bis-phosphorylated cTnI-G203S, respectively; cTnI-K206Q and cTnI-K206Q/pp, non-phosphorylated and bis-phosphorylated cTnI-K206Q, respectively; DTT, dithiotreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; fHCM, familial hypertrophic cardiomyopathy; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid]; PKA, cAMP-dependent protein kinase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; S1, myosin subfragment-1; TCA, trichloroacetic acid; TRITC, tetramethylrhodamine-5-(and-6)-isothiocyanate.

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[1] Amino acids are given either in one or three-letter code.

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residues (Ser 22 and 23 in the human sequence) of cTnI [5]. This phosphorylation is known to cause two effects:

(i) mono- or bis-phosphorylation reduces the maximum Ca^{2+}-dependent thin filament-activated myosin S1-ATPase (actoS1-ATPase) activity as well as the maximum Ca^{2+}-controlled mean sliding velocity of thin filaments driven by myosin heads [6];

(ii) bis-phosphorylation additionally causes a rightward shift of the Ca^{2+} activation curves of actoS1-ATPase, filament sliding velocity, force generation [6–8], and myosin S1-binding rate [9].

Seven mutations in the gene of cTnI were associated with familial hypertrophic cardiomyopathy (fHCM) [10,11], which is one of the most frequently occurring autosomal-dominant inherited heart diseases involving mutations affecting sarcomeric proteins [12]. The mutations found in the gene of cTnI are missense mutations (R145G/Q, R162W, S199N, G203S, and K206Q) [10,11] or single amino acid deletion (ΔK183) [10] and might affect the role of cTnI as a signal integrator. A study on cTnI G203S and cTnI K206Q by Takahashi-Yanaga et al. [13] indicated that these mutations cause alteration in Ca^{2+}-dependent processes on the myofilament level (Ca^{2+}-activated myofibrillar ATPase, skinned fibre force generation, and Ca^{2+} binding to cTnC). However, the physiologically important modulatory effects of cTnI phosphorylation by PKA have not yet been addressed in the study of these mutants. Previously we have shown that the mutation R145G leads to a loss of functional properties associated with phosphorylation/dephosphorylation of wild-type cTnI [6]. Here we study in vitro regulatory properties of two other fHCM-linked cTnI mutants (G203S and K206Q) in both, the non- and the bis-phosphorylated states, as well as effects of the amino acid exchange and of bis-phosphorylation on the secondary structure of cTnI using circular dichroism (CD) spectroscopy. It is shown that these cTnI mutations affect the backbone structure of cTnI and the regulatory function of the cTn complex in different ways: cTnI G203S behaves like wild-type cTnI and cTnI K206Q leads to increased maximum levels of actoS1-ATPase activity and thin filament sliding velocity in both phosphorylation states but phosphorylation/dephosphorylation does no longer affect the Ca^{2+} sensitivity of these processes or the secondary structure of cTnI.

2. Materials and methods

2.1. Construction of human cTnI mutants G203S and K206Q

The mutations at codon 203 of cTnI changing from GGC (Gly) to AGC (Ser), and at codon 206 changing from AAA (Lys) to CAG (Gln) were constructed using the QuickChange™ site-directed mutagenesis kit (Stratagene). Briefly, a long-distance PCR was carried out to amplify the whole vector (pET3c) containing the cDNA sequence of wild-type cTnI with the following oligonucleotides: G203S sense, 5'-AGT GGA ATG GAG AGC CGC AAG AAA AAG-3'; G203S antisense, 5'-CTT TTT CTT GCG GCT CTC CAT TCC ACT-3'; K206Q sense, 5'-GAG GGC CGC AAG CAA AAG TTT GAG AGC-3'; and K206Q antisense, 5'-GCT CTC AAA CTT TTG CTT GCG GCC CTC-3'. *Pfu Turbo* DNA polymerase was used in the PCR. After removal of template by *Dpn*I digestion, PCR products were directly transformed into *Escherichia coli* strain XL1-Blue supercompentent cells. Positive clones were sequenced to confirm the mutations.

2.2. Expression, purification, and characterization of cTnI-G203S and cTnI-K206Q, and wild-type cTnI and cTnC

The mutants G203S and K206Q of human cTnI were overexpressed and purified as described previously for human wild-type cTnI [14]. About 10–20 mg of the recombinant proteins were obtained from 1 l of cell culture. The mass of mutant human cTnI, G203S 23 905 ± 7 Da and K206Q 23 777 ± 2 Da, was determined by electro-spray ionization mass spectrometry and was identical to the masses calculated from the amino acid sequences. The human wild-type cTnT and cTnC were overexpressed in *E. coli* BL21 (DE3); they were purified and characterized as described previously [6,15].

2.3. CD spectroscopy

Spectra of human wild-type, G203S and K206Q, cTnI were recorded at 20 °C on a Jasco 710 Spectropolarimeter, UK (software: spectra management V1.51.00) in the range of 190–250 nm (path length: 0.05 cm) with a bandwidth of 1 nm and a speed of 50 nm/min with 10 accumulations. A protein concentration of 0.25 mg/ml in 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 200 mM KCl, and 0.2 mM dithiotreitol (DTT) was used. Spectra were smoothed using the Savitsky–Golay function (Prism, GraphPad Software, Inc., US). The percentual amounts of secondary structure elements were calculated with the SOMCD method based on an unsupervised learning neural network [16,17].

2.4. Reconstitution of human cTn

The human cTn complex containing cTnT, cTnC, and either wild-type or mutant (G203S or K206Q) cTnI was reconstituted as described previously [6]. The composition of the complex was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel scanning using Whole Band Analyser Software (Bioimage, UK). A native bovine cTn holocomplex was employed as standard.

2.5. Phosphorylation of human cTnI in the cTn complex

Wild-type or mutant (G203S or K206Q) cTnI was phosphorylated at both serine residues, Ser 22 and 23, by the catalytic subunit of PKA as described previously [6] employ-

ing the following PKA activities: 120 mU/mg cTnI for wild-type cTnI and cTnI-G203S, 60 mU/mg cTnI for cTnI-K206Q to avoid unspecific phosphorylation. Bis-phosphorylation of cTnI was monitored by isoelectric focusing (IEF) [18]. cTn containing exclusively bis-phosphorylated wild-type or mutant cTnI was employed for reconstitution of thin filaments.

2.6. Thin filament reconstitution

Regulated thin filaments were reconstituted from its components [6] by overnight incubation of isolated skeletal muscle actin [19] (8 μM polymerized G-actin) with tropomyosin [20] (2 μM) and reconstituted cTn (2 μM) containing either bis-phosphorylated wild-type cTnI or bis-phosphorylated mutant (G203S or K206Q) cTnI. The reconstitution buffer contained 20 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid] (HEPES)/KOH (pH 7.5), 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 2 mM DTT. Reconstituted thin filaments containing bis-phosphorylated wild-type or mutant cTnI were subsequently dephosphorylated with protein phosphatase 2A as described previously [9]. Dephosphorylation of cTnI was verified by ELISA. The different types of reconstituted thin filaments were bound to the surface of a 96-well micro-titre plate by stepwise dilutions. Each well was incubated with either anti-phospho-cTnI (1G11, 1:10 000) or anti-non-phospho-cTnI (22B11, 1:5000) [21] mouse-anti-human antibody. Peroxidase-conjugated rabbit-anti-mouse antibody (1:20 000) was employed as the secondary antibody.

2.7. Generation and purification of the antibodies

cTnI was isolated from human heart according to Katrukha et al. [22]. Phosphorylation of cTnI (15 μM) was carried out in 40 mM Tris–HCl (pH 7.4), 127 mM NaCl, 1.3 mM EGTA, 0.5 mM DTT, 150 μM ATP, 100 mM KCl, 3.2 mM $CaCl_2$, and 45 μCi/ml [γ-^{32}P]-ATP. The reaction was started by addition of catalytic subunit of PKA (Sigma P-2645) at 0.25 U/μg of cTnI. Phosphorylation was allowed for 4 h at room temperature with shaking and was stopped by drop freezing in liquid nitrogen. The molar ratio of ^{32}P incorporated into cTnI was determined by liquid scintillation counting of aliquots of the reaction medium applied onto Whatman 3 MM paper, washed sequentially (12% trichloroacetic acid (TCA), and 6% TCA and acetone), and dried on air. Reaction medium without catalytic subunit of PKA served as control and reaction mixture without cTnI and PKA was used as blank.

Dephosphorylation of cTnI was carried out by incubating cTnI (2.4 μM) with 5 U of alkaline phosphatase from *E. coli* (Sigma P-4252) in 20 mM Tris–HCl (pH 7.4), 0.5 mM EGTA, 0.5 mM DTT, and 15% glycerol at room temperature for 4 h. The extent of dephosphorylation was checked using previously phosphorylated [^{32}P] cTnI (1.4 mol of phosphate/mol of cTnI) as substrate.

Monoclonal antibodies against phosphorylated and non-phosphorylated cTnI were obtained using a standard protocol [23]. Briefly, Balb/c mice were immunized intraperitoneally with 0.1 mg of either phosphorylated or dephosphorylated cTnI, emulgated in complete Freund's adjuvant. Two additional intraperitoneal immunizations were carried out on days 31 and 61 using incomplete Freund's adjuvant. The final boosts were administered on days 91 and 93 with 0.05 mg of the antigen delivered intraperitoneally and intravenously. Spleenocytes from mice were fused with myeloma cells (line Sp 2/0). Hybridoma cells were tested by means of ELISA using phosphorylated and dephosphorylated cTnI as antigens. Selected hybridomas were tested for cross reaction with skeletal muscle TnI (HyTest, Finland). Hybridomas chosen on the basis of specificity were cloned by two rounds of limiting dilution into aminopterin-free medium. Stable hybridoma clones were cultured as ascite tumours in Balb/c mice. Monoclonal antibodies were purified from ascitic fluid by protein A-Sepharose affinity chromatography. Antibody concentrations were determined by the Lowry's method [24] with mouse serum IgG (Calbiochem, La Jolla, CA) as calibrator. The monoclonal antibodies 1G11 and 22B11 against phosphorylated and non-phosphorylated cTnI, respectively, were used.

2.8. Assays

Rabbit skeletal muscle myosin and myosin S1 were prepared by standard methods [25]. Myosin S1-ATPase activity and actoS1-ATPase activity activated by reconstituted thin filaments were determined in the range from pCa 9.8 to 5.0 as described previously [6]. The actoS1-ATPase activity of unregulated thin filaments (devoid of tropomyosin and troponin) determined under the same experimental conditions served as reference. The in vitro mean sliding velocity of phalloidin–tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-labelled reconstituted thin filaments driven by surface-bound myosin molecules was determined under similar experimental conditions as the actoS1-ATPase assay [6]. Unregulated thin filaments were employed as reference.

2.9. Data analysis

Data analysis was carried out as described previously [6]. In brief, both the actoS1-ATPase activity and the thin filament mean sliding velocity were normalized to the corresponding reference (100%) obtained in the presence of unregulated actin filaments under the same experimental conditions. Normalized data sets were analysed to give mean ± S.E.M. which were in turn fit to the Hill equation:

$$y(\mathrm{pCa}) = a + b/(1 + 10^{n(\mathrm{pCa} - \mathrm{pCa}_{50})}) \quad (1)$$

to obtain the pCa at half-maximum activation (pCa_{50}), the Hill coefficient (n), the basal level at pCa >> pCa_{50} (a), and the amplitude of the Ca^{2+}-dependent response (b). Relative values obtained by normalizing the maximum normalized

value of a data set to 1 and the minimum one to zero were fit to the function:

$$Y(\mathrm{pCa}) = 1/(1 + 10^{m(\mathrm{pCa} - \mathrm{pCa_H})}) \quad (2)$$

to obtain the midpoint pCa (pCa_H) and the steepness (m) of the Ca^{2+} activation curve. Both fitting procedures were performed using SigmaPlot 2001 (SPSS Inc., US). A two-way unpaired Student's t-test was used to compare mean ± S.E.M.

3. Results

3.1. States of reconstituted thin filaments

The human cTn complexes reconstituted from purified recombinant human cTnT, cTnC, and either wild-type or mutant (G203S or K206Q) cTnI were comparable in subunit composition to native bovine cTn used as reference (Fig. 1A). Within standard deviation of the method employed the subunit composition determined by SDS-PAGE gel densitometry is identical for cTn containing either wild-type cTnI or cTnI-G203S or cTnI-K206Q. PKA-dependent phosphorylation of the various cTnI species was performed on the reconstituted cTn complexes. cTnI is the only PKA-phosphorylatable subunit of cTn and Ser 22 and 23 are commonly agreed to be the only PKA-dependent phosphorylation sites of cTnI in vivo. cTnI isolated from these complexes after phosphorylation was present exclusively in the bis-phosphorylated form as shown by IEF (Fig. 1B). Neither non- nor mono- nor higher-phosphorylated species can be detected. The cTn complex containing bis-phosphorylated cTnI species was used for reconstitution of thin filaments because cTn containing non-phosphorylated cTnI forms seems to assemble poorly into thin filaments. ELISA of the reconstituted thin filaments revealed that the bis-phosphorylated form of wild-type or mutant cTnI is present exclusively at this stage (Fig. 2B). Dephosphorylation of these reconstituted thin filaments with protein phosphatase

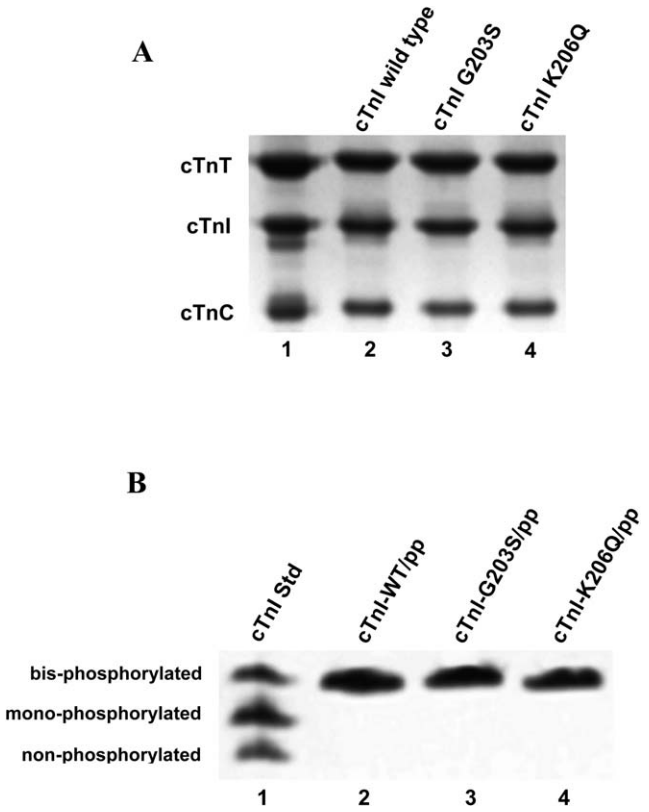

Fig. 1. (A) SDS-PAGE of the reconstituted human cTn complex. Subunits pattern of native bovine cTn (lane 1) and reconstituted cTn containing either wild-type or mutant cTnI species as indicated (lanes 2–4) revealed the following subunit compositions (cTnT/cTnI/cTnC): bovine cTn 1:1:1, reconstituted cTn containing wild-type cTnI 1:0.97:0.98, cTnI G203S 1:0.98:1.05, or cTnI K206Q 1:1.01:1.03. (B) IEF of cTnI after phosphorylation by PKA. A mixture of bovine cTnI containing non-, mono-, and bis-phosphorylated forms served as standard (lane 1). Wild-type cTnI (lane 2) and the mutant cTnI species indicated (lanes 3–4) were bis-phosphorylated in the reconstituted cTn complexes as described in Section 2.

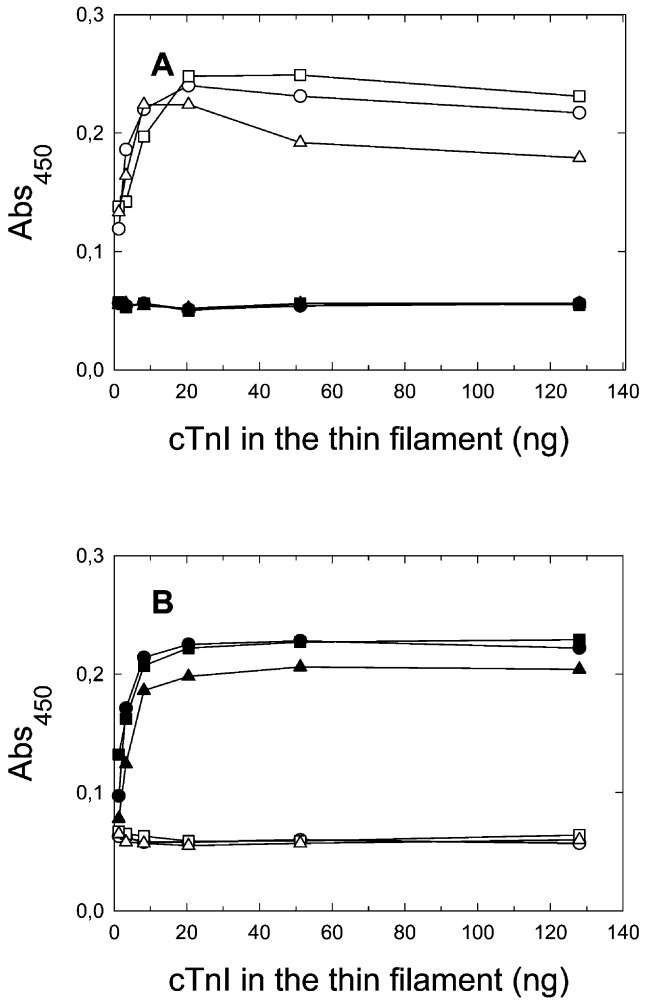

Fig. 2. Detection of the non-phosphorylated (A) and the phosphorylated form (B) of cTnI (wild type, mutant G203S and K206Q) by ELISA. Anti-non-phosphorylated cTnI antibody (22B11, 1:5000) (A) and anti-phosphorylated cTnI antibody (1G11, 1:10 000) (B) were used to detect cTnI in the reconstituted thin filaments. Peroxidase-conjugated rabbit-anti-mouse antibody (1:20 000) was employed as the secondary antibody. Titration of reconstituted thin filaments before (■, ●, ▲; for wild type, G203S, and K206Q, respectively) and after (□, ○, △; for wild type, G203S, and K206Q, respectively) protein phosphatase 2A-treatment.

2A yielded corresponding thin filaments with non-phosphorylated cTnI as demonstrated by means of monoclonal antibodies, which specifically recognize non-phosphorylated cTnI (Fig. 2A). The results prove the experimental concept of creating thin filaments with either non-phosphorylated wild-type cTnI or cTnI G203S or cTnI K206Q and the corresponding bis-phosphorylated cTnI species.

3.2. Effects of the amino acid exchanges in cTnI on the secondary structure

The amino acid exchange at position 203 or 206 of cTnI leads to slightly different CD spectra in comparison to that of wild-type cTnI (Fig. 3) suggesting a decrease in helicity of the secondary structure of cTnI by about 5% due to these mutations. The spectra of cTnI G203S and cTnI K206Q are identical. Phosphorylation of Ser 22 and 23 of either wild-type cTnI or cTnI G203S leads to lower minima at 210 and 222 nm by about 2 U, respectively (Fig. 3A). The changed spectra indicate an increase in helicity of the backbone of these two cTnI species by about 8–14% as estimated with the SOMCD method. In contrast, phosphorylation of cTnI K206Q does not alter the CD spectrum significantly (Fig. 3B).

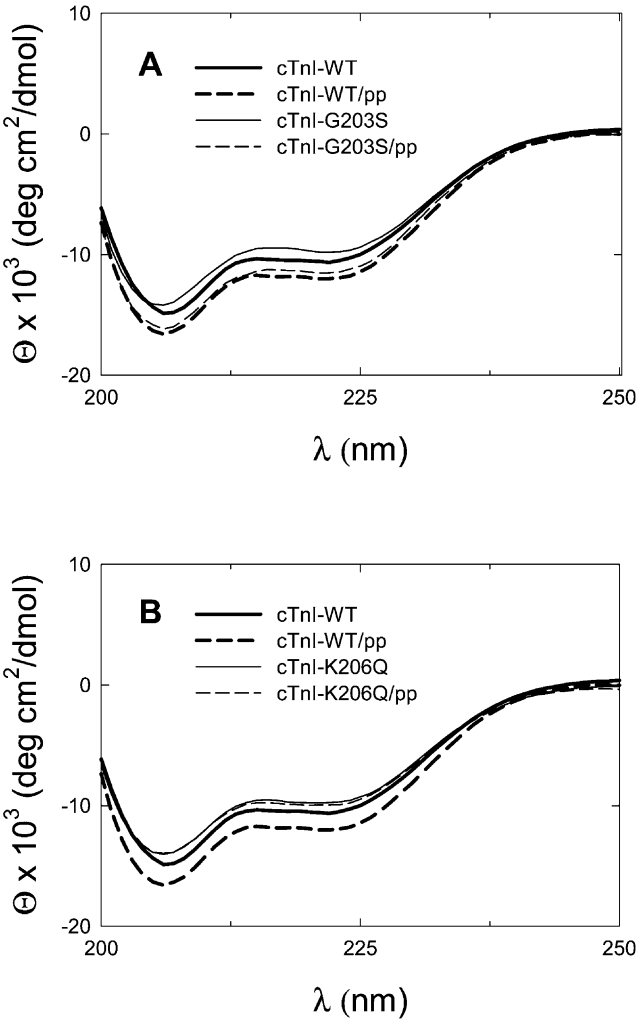

Fig. 3. CD spectra of wild-type cTnI (bold lines in A,B), cTnI G203S (A), and cTnI K206Q (B) in either the non-phosphorylated (continuous lines) or the phosphorylated (dashed lines) state. Samples were prepared and spectra were recorded as described in Section 2. Wild-type cTnI was completely bis-phosphorylated whereas cTnI G203S and cTnI K206Q contained about 30% and 50% mono-phosphorylated and 70% and 50% bis-phosphorylated forms, respectively, as revealed by IEF.

3.3. Effects of cTnI mutants G203S and K206Q on Ca^{2+}-dependent actoS1-ATPase activities

Ca^{2+}-dependent actoS1-ATPase activation was used to assess regulatory properties of the defined cTnI in the reconstituted thin filaments. Fig. 4A–C illustrate the Ca^{2+}-dependent sigmoidal rise in normalized actoS1-ATPase activities under control of cTn complexes containing either wild-type or mutant (G203S or K206Q) cTnI in the non- (cTnI-WT, cTnI-G203S, and cTnI-K206Q) or the bis-phosphorylated state (cTnI-WT/pp, cTnI-G203S/pp, and cTnI-K206Q/pp). cTnI G203S in the non- or the bis-phosphorylated state yields a similar maximum activity as wild-type cTnI (compare Fig. 4B with A, Table 1). The suppressing effect (42 ± 15%, $P < 0.05$; Fig. 4B) resulting from bis-phosphorylation of this mutant cTnI is nearly identical to that obtained with bis-phosphorylation of wild-type cTnI (43 ± 7%, $P < 0.05$; Fig. 4A). In contrast, mutation K206Q significantly increases the maximum activity to 164 ± 17% and 110 ± 17% for non- and bis-phosphorylated cTnI forms, respectively, compared to wild-type cTnI ($P < 0.05$; compare Fig. 4C with A, Table 1). Despite this enhancing effect of the mutation K206Q, the suppressing effect due to bis-phosphorylation is still observed with this mutant cTnI (Fig. 4C). It appears even stronger than that obtained with wild-type cTnI or cTnI G203S (maximum activity reduced by 54 ± 19%). The basic actoS1-ATPase activity exhibited at pCa 9.8 with either cTnI mutant is similar to wild-type cTnI independent of the cTnI phosphorylation state (Fig. 4A–C). Fig. 4D–F summarize the Ca^{2+} sensitivity and steepness of the Ca^{2+} activation of actoS1-ATPase activity. Compared to wild-type cTnI, the mutation G203S does not change the Ca^{2+} sensitivity of actoS1-ATPase activity independent of the phosphorylation state of cTnI (compare Fig. 4E with D, Table 1). Bis-phosphorylation of this mutant cTnI causes a slight decrease in the Ca^{2+} sensitivity by 0.11 ± 0.04 pCa units (Fig. 4E, Table 1), as does bis-phosphorylation of wild-type cTnI (0.21 ± 0.02 pCa units, $P < 0.05$; Fig. 4D, Table 1). Whether this is reflecting a true change of the activation properties remains doubtful because the shape of the activation curve is also affected. The mutation K206Q does not affect the Ca^{2+} sensitivity in the non-phosphorylated state (compare Fig. 4F with D, Table 1) but seems to abolish the desensitizing effect of bis-phosphorylation of cTnI (Fig. 4F).

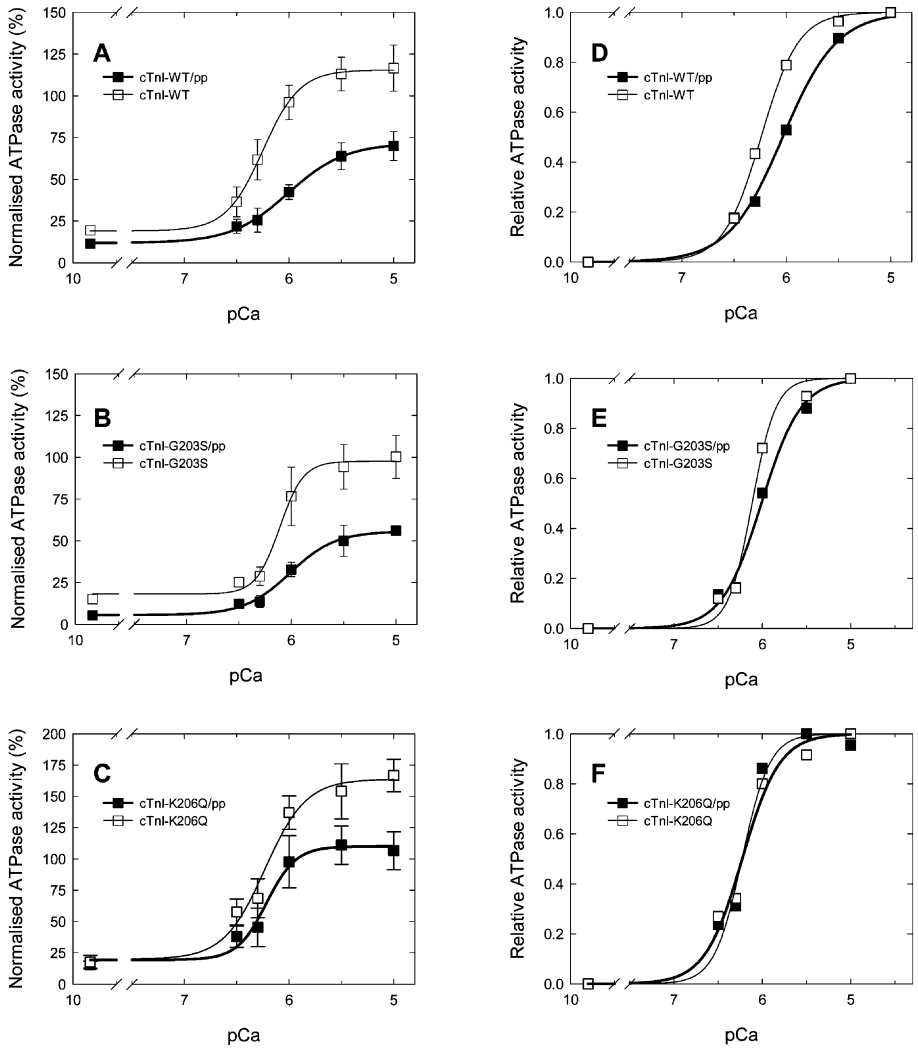

Fig. 4. Ca^{2+}-dependent activation of actoS1-ATPase by thin filaments containing wild-type cTnI (A,D), or G203S (B,E) or K206Q (C,F) mutant cTnI in either the non- or the bis-phosphorylated state. (A–C) The actoS1-ATPase activities (mean ± S.E.M.) normalized to the F-actin-activated myosin S1-ATPase activities measured under the corresponding conditions (50.6 ± 7.1 pmol/s, taken as 100%). The curves represent non-linear least-squares fits to the data employing Eq. (1) (see Section 2) with the fit parameters, respectively, for, *a, b*, pCa_{50}, and *n*: 0.19 ± 0.02, 0.96 ± 0.02, 6.25 ± 0.01, and 2.47 ± 0.17 (cTnI-WT); 0.12 ± 0.02, 0.60 ± 0.03, 6.00 ± 0.04, and 1.60 ± 0.18 (cTnI-WT/pp); 0.18 ± 0.04, 0.80 ± 0.06, 6.11 ± 0.04, and 3.84 ± 0.97 (cTnI-G203S); 0.06 ± 0.03, 0.50 ± 0.04, 6.01 ± 0.06, and 2.00 ± 0.46 (cTnI-G203S/pp); 0.20 ± 0.12, 1.44 ± 0.16, 6.23 ± 0.07, and 2.22 ± 0.70 (cTnI-K206Q); and 0.19 ± 0.02, 0.91 ± 0.10, 6.22 ± 0.06, and 3.10 ± 1.07 (cTnI-K206Q/pp). (D–F) ActoS1-ATPase activities normalized to the maximum difference of the corresponding data in A–C (see Section 2, Eq. *N* (2)). The values of the fit parameter pCa_H are compiled in Table 1.

Table 1
Normalized maximum levels (V_{max}) and Ca^{2+} sensitivity (pCa_H) of actoS1-ATPase activity and thin filament in vitro motility in the presence of cTnI G203S and cTnI K206Q in the non- and bis-phosphorylated states

	The actoS1-ATPase activity						Filament mean sliding velocity					
	cTnI-WT	cTnI-WT/pp	cTnI-G203S	CTnI-G203S/pp	cTnI-K206Q	cTnI-K206Q/pp	cTnI-WT	cTnI-WT/pp	cTnI-G203S	cTnI-G203S/pp	cTnI-K206Q	cTnI-K206Q/pp
V_{max}	1.15 ± 0.03	0.72 ± 0.07	0.98 ± 0.10	0.56 ± 0.11	1.64 ± 0.17	1.10 ± 0.11	1.05 ± 0.01	0.91 ± 0.03	1.13 ± 0.07	0.99 ± 0.01	1.40 ± 0.09	1.11± 0.07
pCa_H	6.24 ± 0.05	6.03 ± 0.02	6.12 ± 0.03	6.01 ± 0.03	6.23 ± 0.04	6.23 ± 0.03	6.62 ± 0.01	6.40 ± 0.02	6.58 ± 0.05	6.39 ± 0.01	6.53 ± 0.05	6.60 ± 0.04
N	9	6	4	5	6	8	5	5	5	5	5	5

Data corresponding to wild-type cTnI obtained in parallel are included for comparison. The data are given as mean ± S.E.M. of *N* experiments. $V_{max} = a + b$ (see Eq. (1)) is the maximum actoS1-ATPase activity and the mean filament sliding velocity, respectively. For details see Figs. 4 and 5.

3.4. Effects of cTnI mutants G203S and K206Q on Ca^{2+}-dependent thin filament in vitro motility

To study the relationship between actoS1-ATPase activity and the mechanical output powered by this molecular interactions, reconstituted thin filaments from the same batches which have been subjected to the actoS1-ATPase assays were subsequently applied to an in vitro motility assay under similar experimental conditions (Fig. 5). The sliding paths of up to 700 fluorescence-labelled reconstituted thin filaments

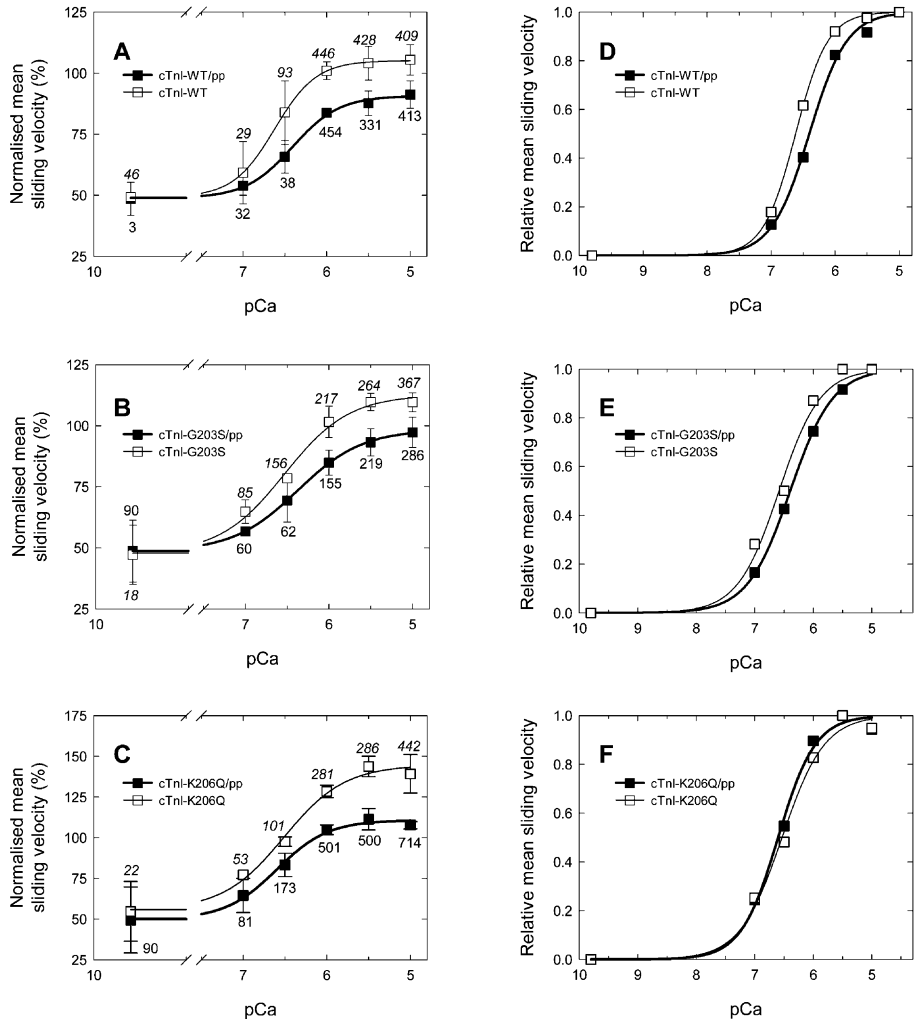

Fig. 5. Ca^{2+}-dependent in vitro motility of regulated thin filaments containing wild-type cTnI (A,D), or mutant cTnI G203S (B,E) or K206Q (C,F) in either the non- or the bis-phosphorylated state. (A–C) Mean sliding velocities of thin filaments (mean ± S.E.M.) normalized to that of phalloidin–TRITC-labelled F-actin under the corresponding conditions (1.65 ± 0.04 μm/s corresponds to 100%). The number of analysed filament paths of a data set is given by the number close to the corresponding error bar (italic for non-phosphorylated cTnI). The curves represent non-linear least-squares fits to the data employing Eq. (1) (see Section 2) with the fit parameters, respectively, for, *a, b*, pCa_{50}, and *n*: 0.49 ± 0.01, 0.56 ± 0.01, 6.62 ± 0.01, and 1.74 ± 0.05 (cTnI-WT); 0.49 ± 0.01, 0.42 ± 0.02, 6.41 ± 0.05, and 1.55 ± 0.23 (cTnI-WT/pp); 0.48 ± 0.03, 0.65 ± 0.01, 6.53 ± 0.09, and 1.13 ± 0.25 (cTnI-G203S); 0.49 ± 0.01, 0.50 ± 0.01, 6.37 ± 0.02, and 1.13 ± 0.04 (cTnI-G203S/pp); 0.52 ± 0.05, 0.89 ± 0.08, 6.51 ± 0.10, and 1.20 ± 0.30 (cTnI-K206Q); and 0.50 ± 0.03, 0.61 ± 0.05, 6.60 ± 0.08, and 1.41 ± 0.30 (cTn-K206Q/pp). (D–F) Relative values in mean sliding velocity normalized to the maximum difference of the corresponding data in A–C. The curves represent non-linear least-squares fits to the data employing Eq. (2) (Section 2). The values of the fit parameter pCa_H are compiled in Table 1.

were tracked at a given pCa and analysed. The data were normalized to those obtained with unregulated thin filaments (see Section 2). As found in the actoS1-ATPase activity assays, mutant cTnI G203S behaves like wild-type cTnI. The maximum velocities are not significantly affected by this mutation independent of the phosphorylation state of cTnI (compare Fig. 5A with B, Table 1). Bis-phosphorylation of cTnI G203S causes a significant decrease in the maximum velocity by about 14% ($P < 0.05$; Fig. 5B, Table 1), which is similar to the decrease observed with bis-phosphorylation of wild-type cTnI (14%, $P < 0.05$; Fig. 5A, Table 1). The mutation K206Q profoundly increases the maximum velocity compared to the wild type. The maximum velocities for cTnI-K206Q and cTnI-K206Q/pp are approximately 35% and 20% higher, respectively, than those for wild-type cTnI in the corresponding phosphorylation state (compare Fig. 5C with A, Table 1). Nevertheless, bis-phosphorylation of this mutant cTnI significantly decreased the maximum velocity by 30% ($P < 0.05$; Fig. 5C, Table 1). At pCa 9.8 the mean sliding velocities of 48–52% of reference were similar for all types of reconstituted thin filaments independent of mutation or phosphorylation of cTnI (Fig. 5A–C, the differences being statistically not significant). It should be noted that this basal level is due to a small portion of not completely reconstituted thin filaments, which are moving at this pCa (5.8–7.3% of all filaments) [6]. The in vitro mean sliding velocities as function of pCa normalized to the difference between pCa 9.8 and 5.0 exhibit indistinguishable Hill coefficients for wild-type and mutant cTnI (Fig. 5D–F). There are, however, effects on the midpoint pCa due to bis-phosphorylation of the cTnI

mutants or the mutations themselves. Bis-phosphorylation of cTnI-G203S causes a shift of the Ca^{2+} activation curves towards higher [Ca^{2+}] (0.19 ± 0.05 pCa unit, $P < 0.005$; Fig. 5E, Table 1) as does bis-phosphorylation of wild-type cTnI (0.22 ± 0.02 pCa unit, $P < 0.005$; Fig. 5D, Table 1). This mutation itself does not have a significant effect on the Ca^{2+} sensitivity of the filament sliding velocity compared to the wild type (compare Fig. 5E with D, Table 1). In contrast, bis-phosphorylated cTnI-K206Q is significantly more sensitive to Ca^{2+} than bis-phosphorylated wild-type cTnI, whereas the non-phosphorylated form is not (compare Fig. 5F with D, Table 1). The desensitizing effect due to bis-phosphorylation of cTnI is abolished by the mutation K206Q (Fig. 5F, Table 1).

4. Discussion

It has previously been shown by fluorescence resonance energy transfer that phosphorylation of the Ser 22 and 23 in the cardiac-specific N-terminal extension of cTnI causes a decrease in the mean distance between the N- and C-terminal regions of cTnI [26]. Here we demonstrate that phosphorylation of these two residues in wild-type cTnI leads to an increase in the helicity of the backbone structure of the protein. As far as the mutations themselves are concerned, both of them (G203S and K206Q) tend to slightly decrease the helicity (Fig. 3) whereas an arginine to glycine exchange at position 145 in the inhibitory region of cTnI causes a larger change of the cTnI backbone towards higher helicity [27]. Nevertheless, neither the latter amino acid exchange [27] nor the two mutations investigated here seem to strongly affect the overall structure of cTnI. Phosphorylation of cTnI-G203S induces a small but significant increase in the helicity of the backbone structure by about the same extent, as does phosphorylation of wild-type cTnI. In contrast, no alteration in the secondary structure has been observed upon phosphorylation of cTnI-K206Q. This is surprising as both mutations are located at a narrow spot in the same region close to the C-terminus of cTnI and suggest that the lysine at position 206 is especially important for the structural responsiveness of cTnI towards phosphorylation.

The difference in response to phosphorylation of the two mutants of cTnI is also reflected by the functional behaviour of reconstituted thin filaments. In reconstituted cTn complexes, which contain either cTnI G203S or cTnI K206Q linked to fHCM, the cardiac-specific serine residues of cTnI are readily accessible to PKA. The complete phosphorylation of Ser 22 and 23 of cTnI in the cTn complex and in the reconstituted thin filaments is demonstrated by IEF and by ELISA using specific anti-phospho- or -non-phospho-cTnI antibodies, respectively. These results clearly show that in the experiments discussed below we are dealing with reconstituted thin filaments, which contain either non-phosphorylated or bis-phosphorylated wild-type or mutant cTnI only. Functional consequences of the two fHCM-causing cTnI mutants were measured with the same preparation of reconstituted thin filaments using actoS1-ATPase activity and in vitro motility assays. This approach allows a high degree of control over the proteins constituting a contractile unit as well as the phosphorylation state of cTnI. The latter aspect was not included in a previous study of these mutant cTnI [13,28]. The reconstituted thin filaments containing cTnI G203S or cTnI K206Q are well under control of the regulatory system and exhibit a general Ca^{2+}-responsiveness known from wild-type cTnI and cTnI R145G [6,7]. At low free-Ca^{2+} concentrations (pCa 9.8), the normalized mean in vitro sliding velocities of about 50% of reference level observed with all types of filaments are due to less than 10% of all filaments. At maximum Ca^{2+} activation (pCa 5.0), more than 80% of all filaments contribute to the observed filament motility. The results of the actoS1-ATPase activity and thin filament in vitro motility measurements reveal a practically identical behaviour of wild-type cTnI and cTnI G203S. The mutation G203S itself does not cause any specific effects and bis-phosphorylation of this mutant cTnI alters actoS1-ATPase activity and in vitro thin filament motility in the same way as does bis-phosphorylation of wild-type cTnI. This is in agreement with Burton et al. [28] who have reported that thin filaments containing cTnI G203S were indistinguishable from those containing wild-type cTnI in in vitro motility and ATPase assays, although the phosphorylation state of cTnI was not shown. Bis-phosphorylation of cTnI K206Q also reduces the Ca^{2+}-dependent maximum actoS1-ATPase activity and filament sliding velocity but the suppressed response levels are similar to those obtained with non-phosphorylated wild-type cTnI. This is due to the fact that the mutation K206Q itself considerably increases the maximum response levels of these processes. Furthermore, the shift in the Ca^{2+} sensitivity of both processes obtained upon bis-phosphorylation of wild-type cTnI has not been observed with cTnI K206Q.

The two cTnI mutants behave rather differently in the experiments carried out here. The mutation G203S, which is involved in the development of hypertrophy only at the cardiac apex [10], does not seem to differ from wild-type cTnI, which indicates the involvement of multiple factors in the development of this disease. In contrast, the mutation K206Q that causes a characteristic ventricular fHCM [10] leads to an increased Ca^{2+}-activated actoS1-ATPase activity, which implies an altered actin–myosin interaction dynamics. An increased actomyosin ATPase rate can be explained by an increased cross-bridge detachment rate [29]. This interpretation is supported by the observation that the mutation K206Q also leads to an increased maximum unloaded in vitro sliding speed of the thin filaments which can be attributed to an increased cross-bridge detachment rate [30], i.e. a shorter duty time during the cross-bridge cycle. Thus, this mutation may be expected to lead to a reduced myocardial force development. The only available force measurements with this cTnI mutant in partially cTnI-replaced skinned rabbit cardiac muscle fibres does not report absolute force levels and finds no effect of this mutation on the relative steady-

state force [13]. Whether or not cTnI K206Q might represent a direct hypertrophy-inducing signal is still an open question. Interestingly, the cTnT mutation I79N found to be responsible for a severe form of fHCM with the highest risk of sudden cardiac death in young adults also leads to increased filament sliding speed as well as decreased force [31,32].

The mutation G203S does not significantly increase the Ca^{2+} sensitivity of the myofibrilar ATPase activity and force generation while the mutation K206Q does [13]. With respect to the mutation G203S, this is in agreement with the results obtained with reconstituted thin filaments presented here and elsewhere [28]. An increase in the Ca^{2+} sensitivity of actoS1-ATPase activity and in vitro filament motility due to the mutation K206Q have been observed by us only with bis-phosphorylated cTnI K206Q. There is no information about the phosphorylation state of cTnI in the experiments carried out with partially replaced porcine cardiac myofibrils [13].

Studies with cardiac myofibrils and reconstituted thin filaments have shown that phosphorylation/dephosphorylation of cTnI modulates the regulatory system of the cardiac contractile mechanism [6–9,34,35]. There is, however, some dissent about the major physiological effects of this post-translational modification of cTnI. A study with skinned rat cardiac muscle reports an increase in myofibrillar ATPase activity and unloaded shortening velocity due to PKA treatment [34]. This is opposite to our finding of a decreased maximum actoS1-ATPase activity and in vitro sliding velocity of reconstituted thin filaments resulting from bis-phosphorylation of human cTnI. A more specific study using wild-type and transgenic mice overexpressing non-phosphorylatable slow skeletal TnI in the myocardium suggests an increased cross-bridge cycling rate due to phosphorylation of mouse cTnI by PKA [35]. It is not known whether a hybrid system introducing slow skeletal TnI [35] or human cTn together with skeletal actin, tropomyosin, and myosin as used here is adding to this disagreement.

We have previously shown that the fHCM-related mutation R145G, located in the inhibitory region of cTnI, renders the actomyosin system insensitive to phosphorylation of cTnI by PKA [6]. It seems that the degree of phosphorylation/dephosphorylation of Ser 22 and 23 of cTnI reflects the functional state of the myocardium as in the normal human heart the phosphorylated forms of cTnI prevail (our unpublished observations) whereas in the failing heart the non-phosphorylated form of cTnI becomes dominant [33]. In terms of function, the mutation K206Q of cTnI seems to represent some dephosphorylated state-enhancing signal.

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References

[1] Solaro RJ, Rarick HM. Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. Circ Res 1998;83:471–80.

[2] Van Eyk JE, Kay CM, Hodges RS. A comparative study of the interactions of synthetic peptides of the skeletal and cardiac troponin I inhibitory region with skeletal and cardiac troponin C. Biochemistry 1991;30:9974–81.

[3] Tripet B, Van Eyk JE, Hodges RS. Mapping of a second actin–tropomyosin and a second troponin C binding site within the C terminus of troponin I, and their importance in the Ca^{2+}-dependent regulation of muscle contraction. J Mol Biol 1997;271:728–50.

[4] Gordon AM, Homsher E, Regnier M. Skeletal and cardiac muscle contractile activation: tropomyosin 'rocks and rolls'. New Physiol Sci 2001;16:49–55.

[5] Jaquet K, Thieleczek R, Heilmeyer Jr LMG. Pattern formation on cardiac troponin I by consecutive phosphorylation and dephosphorylation. Eur J Biochem 1995;231:486–90.

[6] Deng Y, Schmidtmann A, Redlich A, Westerdorf B, Jaquet K, Thieleczek R. Effects of phosphorylation and mutation R145G on human cardiac troponin I function. Biochemistry 2001;41:14593–602.

[7] Robertson SP, Johnson JD, Holroyde MJ, Kranias EG, Potter JD, Solaro RJ. The effect of troponin I phosphorylation on the Ca^{2+}-binding properties of the Ca^{2+}-regulatory site of bovine cardiac troponin. J Biol Chem 1982;257:260–3.

[8] Zhang R, Zhao J, Potter JD. Phosphorylation of both serine residues in cardiac troponin I is required to decrease the Ca^{2+} affinity of cardiac troponin C. J Biol Chem 1995;270:30773–80.

[9] Reiffert SU, Jaquet K, Heilmeyer Jr LMG, Ritchie MD, Geeves MA. Bisphosphorylation of cardiac troponin I modulates the Ca^{2+}-dependent binding of myosin subfragment S1 to reconstituted thin filaments. FEBS Lett 1996;384:43–7.

[10] Kimuara A, Harada H, Park J-E, Nishi H, Satoh M, Takahashi M, et al. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. Nat Genet 1997;16:379–82.

[11] Mogensen J, Klausen IC, Egeblad H, Baandrup U. Sudden cardiac death in familial hypertrophic cardiomyopathy is associated with a noval mutation in the troponin I. Circulation 1999;100:I–618.

[12] Bonne G, Carrier L, Richard P, Hainque B, Schwartz K. Familial hypertrophic cardiomyopathy: from mutations to functional defects. Circ Res 1998;83:580–93.

[13] Takahashi-Yanaga F, Morimoto S, Harada K, Minakamin R, Shiraishi F, Liu Q-W, et al. Functional consequences of the mutations in human cardiac troponin I gene found in familial hypertrophic cardiomyopathy. J Mol Cell Cardiol 2001;32:2095–107.

[14] Reiffert SU, Jaquet K, Heilmeyer Jr LMG, Herberg FW. Stepwise subunit interaction changes by mono- and bisphosphorylation of cardiac troponin I. Biochemistry 1998;39:13516–25.

[15] Babu A, Su H, Ryu Y, Gulati J. Determination of residue specificity in the EF-hand of troponin C for Ca^{2+} coordination by genetic engineering. J Biol Chem 1992;267:15469–74.

[16] Andrade MA, Chacón P, Merelo JJ, Morán F. Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. Protein Eng 1993;6:383–90.

[17] Unneberg P, Merelo JJ, Chacón P, Morán F. SOMCD: method for evaluating protein secondary structure from UV circular dichroism spectra. Proteins—structure, function, and genetics 2001;42:460–72.

[18] Ardelt P, Dorka P, Jaquet K, Heilmeyer Jr LMG, Körtke H, Körfer R, et al. Microanalysis and distribution of cardiac troponin I phospho species in heart areas. Biol Chem 1998;379:341–7.

[19] Teubner A, Wegner A. Kinetic evidence for a readily exchangeable nucleotide at the terminal subunit of the barbed ends of actin filaments. Biochemistry 1998;37:7532–8.

[20] Smillie LB. Preparation and identification of alpha- and beta-tropomyosins. Meth Enzymol 1982;85:234–41.

[21] Katrukha A, Bereznikova A, Filatov V, Esakova T. Biochemical factors influencing measurement of cardiac troponin I in serum. Clin Chem Lab Med 1999;37:1091–5.

[22] Katrukha AG, Bereznikova AV, Esakova TV, Filatov VL, Bulargina TV, Gusev NB. A new method of human cardiac troponin I and troponin T purification. Biochem Mol Biol Int 1995;36:195–202.

[23] Katrukha AG, Bereznikova AV, Esakova TV, Pettersson K, Lovgren T, Severina ME, et al. Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex. Clin Chem 1997;43:1379–85.

[24] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951;193:265–75.

[25] Margossian SS, Lowey S. Preparation of myosin and its subfragments from rabbit skeletal muscle. Meth Enzymol 1982;85:55–71.

[26] Dong WJ, Chandra M, Xing J, She M, Solaro RJ, Cheung HC. Phosphorylation-induced distance change in a cardiac muscle troponin I mutant. Biochemistry 1997;36:6754–61.

[27] Lang R, Gomes AV, Zhao J, Housmans PR, Miller T, Potter JD. Functional analysis of a troponin I (R145G) mutation associated with familial hypertrophic cardiomyopathy. J Biol Chem 2002;277:11670–8.

[28] Burton D, Abdulrazzak H, Knott A, Elliott K, Redwood C, Watkins H, et al. Two mutations in troponin I that cause hypertrophic cardiomyopathy have contrasting effects on cardiac muscle contractility. Biochem J 2002;362:443–51.

[29] Homsher E, Lee DM, Morris C, Pavlov D, Tobacman LS. Regulation of force and unloaded sliding speed in single thin filaments: effects of regulatory proteins and calcium. J Physiol 2000;524:233–43.

[30] Spudich JA. How molecular motors work. Nature (London) 1994;372:515–8.

[31] Lin D, Bobkova A, Homsher E, Tobacman LS. Altered cardiac troponin T in vitro function in the presence of a mutation implicated in familial hypertrophic cardiomyopathy. J Clin Invest 1996;97:2842–8.

[32] Sweeney HL, Feng HS, Yang Z, Watkins H. Functional analyses of troponin T mutations that cause hypertrophic cardiomyopathy: insights into disease pathogenesis and troponin function. Proc Natl Acad Sci USA 1998;95:14406–10.

[33] Bodor GS, Oakeley AE, Allen PD, Crimmins DL, Ladenson JH, Anderson AAW. Troponin I phosphorylation in the normal and failing adult human heart. Circulation 1997;96:1495–500.

[34] Saeki Y, Kobayashi T, Minamisawa S, Sugi H. Protein kinase A increases the tension cost and unloaded shortening velocity in skinned rat cardiac muscle. J Mol Cell Cardiol 1997;29:1655–63.

[35] Kentish JC, Mccloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, et al. Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. Circ Res 2001;88:1059–65.