Bisphosphorylation of cardiac troponin I modulates the Ca^{2+}-dependent binding of myosin subfragment S1 to reconstituted thin filaments

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Abstract **We have reconstituted thin filaments comprising pyrene-labelled actin (pyr-actin), tropomyosin (Tm) and cardiac troponin (cTn). cTn was isolated in two defined phosphorylation states; completely dephosphorylated on all subunits and with only the cTnI subunit bisphosphorylated. The thin filament was saturated with cTn at a pyr-actin/Tm/cTn ratio of 7:1:1. The calcium-dependent binding of S1 to thin filaments was measured in a stopped-flow spectrophotometer and the dependence of the observed rate constant on [Ca^{2+}] fitted to the Hill equation. The only significant difference between the two phosphorylation states of the filaments was a 0.36 decrease in the pCa_{50} on bisphosphorylation.**

Key words: Cardiac troponin; Phosphorylation; Calcium regulation; Thin filament; Myosin S1 binding

1. Introduction

Cardiac troponin (cTn) is composed of the three subunits cTnT (tropomyosin binding subunit), cTnI (inhibitory subunit) and cTnC (Ca^{2+}-binding subunit). The cAMP-dependent protein kinase (PKA) phosphorylates two adjacent serine residues in the heart specific N-terminal sequence of cTnI in position 23, 24 in bovine and 22, 23 in rabbit and human heart [1,2]. Serine-24 is phosphorylated ~12-fold faster than serine-23 [3]. Similarly, protein phosphatase 2A (PP-2A) removes phosphate from phosphoserine-24 approx. 2-fold faster than from phosphoserine-23 [4]. Thus, four species are generated by the combined action of PKA and PP-2A on cTnI, namely two monophosphorylated, one bis-and one dephosphorylated species [4]. All these forms are found in troponin isolated from heart [5,2].

Specific ^{31}P-NMR signals are obtained for each phosphate group in the two monophospho forms and one signal for the phosphates in the cTnI bisphospho form present in the holotroponin complex [6]. Only when bisphosphorylated can the phosphates of cTnI interact with acidic groups within another cTn subunit, most probably cTnC.

It has been shown by several groups [7] that phosphorylation of cTnI in skinned fibers decreases the Ca^{2+} sensitivity of the myofibrils. However, in these experiments the actual phosphorylation state of cTnI was not determined.

cTnC and cTnI can be extracted from skinned fibers [8,9] and the thin filament can be reconstituted with native or mutagenized cTn components to form Ca^{2+}-regulated myofibrils. Results obtained with these methods indicate that the bisphospho form of cTnI might cause the decrease in Ca^{2+} sensitivity [7,10]. However, in these reconstituted systems the influence of cTnT phosphorylation is not considered. cTnT is phosphorylated by at least two different protein kinases at multiple serine and/or threonine residues [5,11–13].

To address the question of the relative roles of phosphorylation of cTnI and cTnT, we have used thin filaments reconstituted in vitro with defined phosphorylation states of the cTnT and cTnI subunits. In addition, we introduced pyrene-labelled actin (pyr-actin) which responds to myosin subfragment 1 (S1) binding with a 70% decrease in the intensity of pyrene fluorescence. Employing the skeletal muscle proteins it has been shown that the observed rate constant (k_{obs}) of S1 binding to an excess of pyr-actin·Tm·sTn (Tm, tropomyosin; sTn, skeletal troponin) is a function of the Ca^{2+} concentration. A plot of k_{obs} vs pCa can be analysed using the Hill equation and shows a pCa_{50} of 5.6 and a Hill coefficient of 1.8 [14]. We have used the same approach here, but reconstituting thin filaments with cTn completely dephosphorylated or phosphorylated only in the cTnI subunit, i.e. no phosphate in the cTnT subunit. The work presented here shows that cTn is similar to sTn except that bisphosphorylation correlates with a change in calcium sensitivity.

2. Materials and methods

2.1. Proteins

S1 was obtained by chymotryptic cleavage of rabbit skeletal muscle myosin and isolated according to the method of Weeds and Taylor [15]. F-Actin was isolated from skeletal muscle by the method of Lehrer and Kewar [16] and then labelled at Cys-374 with *N*-(1-pyrene)iodoacetamide (pyr-actin) to a degree of approx. 80% according to the procedure of Criddle et al. [17]. Tm was prepared as described by Smillie [18]. cTn was isolated from bovine heart according to the method of Tsukui and Ebashi [19] and as modified by Beier et al. [20]. The phosphate content of the cTn holocomplex and of isolated cTnI and cTnT subunits was determined by the method of Stull and Buss [21]. cTn subunits were obtained by separation of the complex using reversed phase chromatography as described by Swiderek et al. [1]. The catalytic subunit of PKA was isolated according to Herberg et al. [22]. PP-2A was isolated from bovine heart as described by Mumby et al. [23] (for nomenclature see [24]).

2.2. Phosphoforms of cardiac troponin

cTnI was phosphorylated by incubating 20 μM cTn with 200–250 μU/ml recombinant catalytic subunit of PKA for 1.5 h at 30°C in 20 mM MOPS, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTE, 2 mM ATP, pH 7.0.

cTn containing phosphate exclusively in cTnI was obtained by specific dephosphorylation of cTnT with alkaline phosphatase according to Villar-Palasi and Kumon [25].

Thin filaments reconstituted with fully dephosphorylated cTn ($cTnTP_0IP_0$, for nomenclature see Table 1) gave very variable results in the assays described below. This was thought to be due to poor

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assembly of cTnTP$_0$IP$_0$ into the filament. We therefore dephosphorylated the cTnI after it was reconstituted in either Tm·cTn or the complete thin filament by treating the proteins in a solution of 50 mM Tris-HCl, 100 mM KCl, 2 mM $MnCl_2$, 1 mM DTE, pH 7.4 with PP-2A (enzyme : Tn = 1 : 300 by mass).

2.3. Reconstitution of thin filaments

Thin filaments were reconstituted by mixing pyr-actin, Tm and cTn-TP$_0$IP$_2$ (description see below) in a ratio of 7 : 2 : 2 in 20 mM MOPS, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTE, pH 7.0. The phosphate content of the thin filaments following dephosphorylation of cTnI were determined according to Stull and Buss [21].

2.4. Stopped-flow experiments

Experiments were carried out in 20 mM MOPS, 0.14 M KCl and 5 mM $MgCl_2$, pH 7.0 at 20°C, unless stated otherwise. In stopped-flow experiments the reaction was initiated by mixing equal volumes of two solutions (thin filaments and S1). The protein concentrations after mixing are quoted.

Fluorescence stopped-flow experiments were carried out on a Hi-Tech Scientific SF-61 or SF-61MX spectrophotometer equipped with a 100 W mercury/xenon lamp. Excitation light of 365 nm was obtained using a monochromator, and emission was through a KV 389 cut-off filter. Data were collected as 500 12-bit data points and analysed using a non-linear least-squares fitting program as supplied by Hi-Tech.

3. Results

Freshly isolated cTn contained a total of 1.5 mol phosphate per mol protein (Table 1). The cTnI present in this cTn was fully phosphorylated by PKA and subsequently cTnT was dephosphorylated by alkaline phosphatase resulting in a cTn-TP$_0$IP$_2$ holocomplex (for nomenclature see Table 1). Some of this complex was dephosphorylated by PP-2A either directly or after reconstitution with Tm and pyr-actin. Thus, cTn complexes were obtained containing no phosphate at the cTnT subunit and either two phosphates at cTnI (cTn-TP$_0$IP$_2$) or no phosphate at cTnI (cTn-TP$_0$IP$_0$; Table 1). In the following studies the properties of these two cTn forms were compared.

Upon binding of S1 to pyr-actin·Tm·sTn (all from skeletal muscle) the fluorescence intensity of the pyrene label decreases by 70%. This signal can be used to measure the rate of S1 binding to pyr-actin. Under pseudo-first-order conditions (actin present in excess) the reaction is a single exponential ($F_t = F_0 \exp(-k_{obs}t)$) in both the presence and absence of calcium, but the exponential rate constant (k_{obs}) is slower in the absence of calcium. This was interpreted in terms of a calcium-sensitive equilibrium between two forms of the pyr-actin·Tm·sTn complex one of which (blocked) was unable to bind S1. The exponential rate constant is given by:

$$k_{obs} = k_{+1}[\text{actin}]K_B/(1 + K_B) \quad (1)$$

where K_B defines the equilibrium between the two conformations ('blocked' and 'closed+open') of the thin filament (for more detail see [14,26]). In the presence of calcium $K_B >> 1$ and $k_{obs} = k_{+1}[\text{actin}]$. In the absence of calcium k_{obs} is reduced and the ratio $k_{obs}(+Ca)/k_{obs}(-Ca)$ defines $(1+K_B)/K_B$.

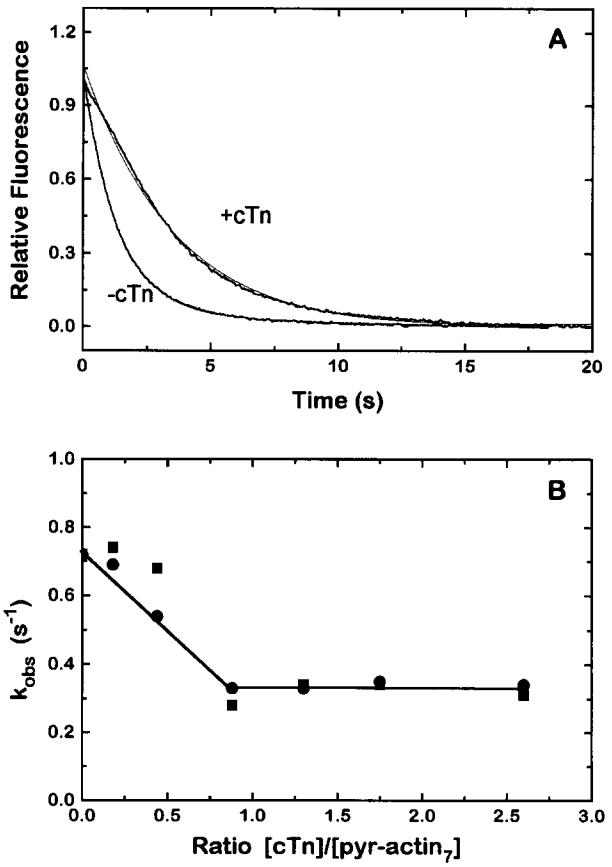

Fig. 1. The cTn dependence of the observed rate of S1 binding to pyr-actin·Tm in the absence of calcium. (A) The fluorescence transient was observed on mixing 0.5 μM pyr-actin and 0.15 μM Tm with 0.1 μM S1 in the presence of increasing concentrations of dephosphorylated cTn and 1 mM EGTA. The data were fitted to a single exponential function and the observed rate constants are shown superimposed on the data. For clarity only 2 transient curves are shown at cTn concentrations of 0 and 0.4 μM with k_{obs} of 0.72 and 0.33 s^{-1}, respectively. (B) The k_{obs} values were plotted against the ratio of cTn:pyr-actin$_7$ for both cTn-TP$_0$IP$_0$ (■) and cTn-TP$_0$IP$_2$ (●).

A similar calcium-dependent reaction was seen for thin filaments reassembled with cTn. Pyr-actin was decorated with an excess of Tm employing a ratio of 7 pyr-actin to 2 Tm. Adding increasing amounts of cTn to this mixture, in the presence of calcium, had little effect on k_{obs} (maximal decrease of 20%; data not shown). In the absence of calcium k_{obs} decreased as the cTn concentration increased (Fig. 1). A saturation point

Table 1
Phosphate content of cTnI and cTnT in freshly isolated cardiac troponin and after incubation with protein kinase A and protein phosphatases

Treatment	mol P/mol cTnT	mol P/mol cTnI	Name of the complex
Freshly isolated cTn	0.4 ± 0.2	1.1 ± 0.1	cTn-TP$_{0.4}$IP$_{1.1}$
cTn phosphorylated by PKA	0.4 ± 0.2	1.8 ± 0.2	cTn-TP$_{0.4}$IP$_2$
cTn dephosphorylated by alk. phosphatase	0.04 ± 0.03	1.8 ± 0.2	cTn-TP$_0$IP$_2$
cTn dephosphorylated by PP-2A	0.04 ± 0.03	0.1 ± 0.07	cTn-TP$_0$IP$_0$

In the first three lines the mean and standard deviation (S.D.) of the phosphate content of three cTn preparations is shown and in the last line the mean and standard deviation of the phosphate content of six reconstitutions of thin filaments. cTn-TP$_{0.4}$IP$_{1.1}$, cardiac troponin with 0.4 mol phosphate per mol cTnT subunit and 1.1 mol phosphate per mol cTnI subunit.

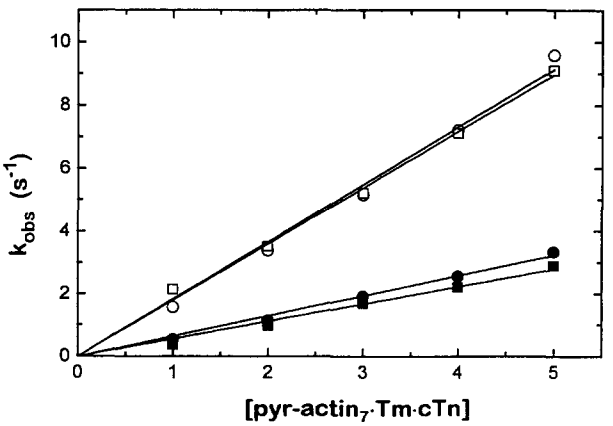

Fig. 2. The observed rate constants for the binding of S1 to increasing concentrations of pyr-actin·Tm·cTn with phosphorylated cTn (●,○) or dephosphorylated cTn (■,□). The Tm·cTn concentration was in a ratio of 2:7 with the pyr-actin and the S1 concentration was 1/5th of the pyr-actin concentration. The buffer was composed as described in section 2 with the addition of either 1 mM EGTA (filled symbols) or 1 mM $CaCl_2$ (unfilled symbols). All data sets were fitted with a linear regression with the intercepts not significantly different from zero. The slopes of the lines are −Ca, 0.65 M^{-1} s^{-1} ($cTnTP_0IP_2$), 0.56 M^{-1} s^{-1} ($cTnTP_0IP_0$); +Ca, 1.82 M^{-1} s^{-1} ($cTnTP_0IP_2$), 1.79 M^{-1} s^{-1} ($cTnTP_0IP_0$).

was attained at a ratio of 7 pyr-actin to 1 cTn. Adding more cTn, up to a maximum ratio of 3:7, did not result in any further decrease in k_{obs} (Fig. 1B). This demonstrates that a pyr-actin$_7$·Tm$_1$·cTn$_1$ complex was formed and that cTn binds to pyr-actin·Tm before binding to free Tm. In this respect, there was no apparent difference in the regulatory thin filament reconstituted with cTn-TP_0IP_0 or $cTnTP_0IP_2$ (Fig. 1B). As saturation was achieved with both cTn complexes at a ratio of 7 pyr-actin to 1 cTn this suggests that the dissociation constant of both forms of cTn for pyr-actin·Tm is less than the 0.15 μM of cTn used in this measurement. In all subsequent experiments the filaments were assembled by mixing pyr-actin:Tm:cTn in the ratio 7:2:2 to ensure full saturation of the pyr-actin with Tm and cTn.

The k_{obs} obtained in both the presence and absence of Ca^{2+} is linearly dependent upon the concentration of pyr-actin over the range of 1–5 μM for both $cTnTP_0IP_0$ and $cTnTP_0IP_2$ (Fig. 2). No significant difference was observed between the thin filaments reconstituted with $cTnTP_0IP_0$ and $cTnTP_0IP_2$. The slope of the fitted line in the presence of Ca^{2+} was similar to that in the absence of cTn (and to that obtained from sTn in presence of calcium). This is consistent with little occupancy of the 'blocked' state and therefore $K_B >> 1$. Thus, the slope of the line in the presence of calcium (Fig. 1A) defines k_{+1}. In the absence of calcium a straight line was also observed and, if the model used for sTn is correct, then the slope is defined by $k_{+1}\ K_B/(1+K_B)$ (Eq. 1). Thus, the removal of calcium reduces the amount of pyr-actin available for the S1 to bind and therefore k_{obs} is reduced. In this experiment the ratio $k_{obs}(+Ca)/k_{obs}(-Ca)$ was about 3 for filaments containing either $cTnTP_0IP_2$ or $cTnTP_0IP_0$. In six experiments with different preparations of proteins the ratio of $k_{obs}(+Ca)/k_{obs}(-Ca)$ varied between 1.9 and 3.0 ($K_B = 1.1$–0.5). In four of the experiments phosphorylation of cTnI increased the ratio by less than 5%, which is at the limit of the accuracy of the measurement. We therefore conclude that phosphorylation of cTnI has no detectable influence on the extent to which the filament can be switched off by cTn. The ratio is sensitive to the quality of the reconstituted filaments; the better the filament the more completely the filament will be switched off by the removal of calcium. Thus, the ratio of 3 can be considered the lower limit for a filament reconstituted with fully active Tm and cTn. This ratio is similar to that seen for sTn.

The calcium dependence of binding of S1 to the two species of thin filaments pyr-actin·Tm·$cTnTP_0IP_0$ and pyr-actin·Tm·$cTnTP_0IP_2$ was measured and the data for one experiment with pyr-actin·Tm·$cTnTP_0IP_2$ are shown exemplarily in Fig. 3. A reduction in calcium concentration leads to a decrease in k_{obs} (Fig. 3A). At pCa 4 the observed rate constant for both species is maximal. In Fig. 3B the calcium dependence of k_{obs} is fitted to the Hill equation. The pCa_{50} value of the pyr-actin·Tm·$cTnTP_0IP_0$ thin filament is 5.6 and that of the pyr-actin·Tm·$cTnTP_0IP_2$ is 5.28. The phosphorylation results in a rightward shift of 0.32 pCa units. The mean of six experiments reveals a difference of 0.36 ± 0.14 pCa units (Table 2). The statistical analysis with the paired Student's *t*-test shows that the rightward shift of the pCa_{50} observed with the phosphorylated form is 'extremely significant'.

High variations in the Hill coefficient ($n_H = 0.65$–1.74) were obtained for the six experiments. However, for a given preparation the Hill coefficient was the same for both forms of cTn and thus phosphorylation does not affect n_H. This parameter may be more sensitive to the precise assembly of the proteins in the thin filament than the pCa_{50} and therefore like K_B shows more variation between preparations than the pCa_{50}.

4. Discussion

We showed clearly that bisphosphorylation of cTnI in the absence of cTnT phospho forms alters the Ca^{2+} sensitivity of S1 binding to the thin filament. The rightward shift of the pCa_{50} value observed here (0.36 ± 0.14 pCa units) is a little larger than that obtained by Zhang et al. [7] (0.27 ± 0.03 pCa units). This difference is small given the different experimental protocols, but the difference could be the result of a lower level of phosphorylation and hence a mixture of different cTnI phosphorylation states in the skinned fibres. The 0.36 pCa shift occurs with completely dephosphorylated cTnT.

Table 2
pCa_{50} values obtained by measuring the Ca^{2+} dependence of S1 binding to the thin filaments with different phosphospecies of cardiac troponin ($cTnTP_0IP_0$ or $cTnTP_0IP_2$)

	pCa_{50}	
pyr-actin·Tm·$cTnTP_0IP_0$	pyr-actin·Tm·$cTnTP_0IP_2$	ΔpCa_{50}
5.76 ± 0.14	5.40 ± 0.16	0.36 ± 0.14

The mean and S.D. is given from six experiments in which the pCa_{50} are the midpoints determined by the Hill equation of the Ca titration. $P = 0.0009$ vs. ΔpCa_{50} values analysed by paired Student's *t*-test represents 'extremely significant'.

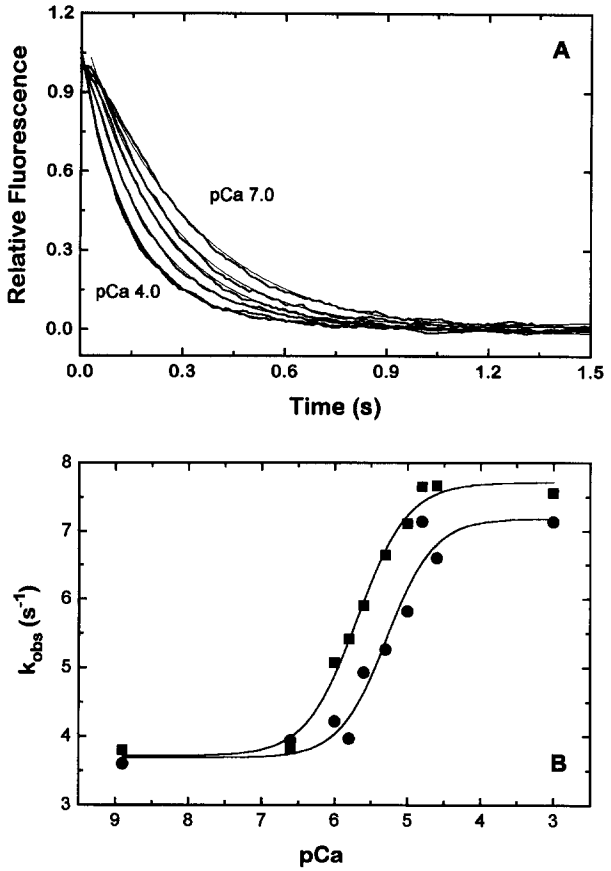

Fig. 3. The influence of cTn phosphorylation on the calcium-dependent binding of S1 to pyr-actin·Tm·cTn. (A) The fluorescence transient was observed on mixing 5 μM pyr-actin, 1.45 μM Tm and 1.45 μM $cTnTP_0IP_2$ with 1 μM S1 at different calcium concentrations. For clarity only 5 curves are shown and the best-fit single exponential is superimposed on each data set. The pCa (k_{obs}) values are 4.0 (6.9 s^{-1}), 4.5 (7.3 s^{-1}),5.0 (4.4 s^{-1}), 6.0 (3.8 s^{-1}), 7.0 (2.7 s^{-1}). The calcium concentrations were obtained by mixing 2 mM EGTA and 2 mM CaEGTA in appropriate proportions. (B) k_{obs} was plotted as a function of pCa for both $cTnTP_0IP_2$ (●) and $cTnTP_0IP_0$ (■). The data were fitted to the Hill equation. The best-fit parameters are: pCa midpoint 5.28 and 5.60 and the Hill coefficients 1.35 and 1.37 for $cTnTP_0IP_2$ and $cTnTP_0IP_0$, respectively. Free Ca^{2+} concentrations were calculated based on the dissociation constants of Sillen and Martell [29].

Thus any effect of cTnT phosphorylation must be minor on this parameter.

Bisphosphorylation of cTn has no effect on K_B and n_H. However, in the experiments described here both parameters were sensitive to the reconstitution of the thin filament. Variations in n_H (0.65–1.74) have been observed suggesting that the reconstituted filament is not always identical to the native complex. This is not due to the phosphorylation state of cTnI and suggests that the variations in n_H are caused by variations in one or more of the thin filament protein preparations. Furthermore, a $n_H > 1$ was often obtained, although there is only one calcium specific regulatory binding site in heart cTnC. A $n_H > 1$ must mean that there is cooperativity between adjacent Tm·cTn complexes [27].

The scatter in maximal k_{obs} observed at both high and low calcium when fully dephosphorylated protein·$cTnTP_0IP_0$ is reconstituted with pyr-actin and Tm can be eliminated by reconstituting the thin filament or at least the Tm·cTn complex before dephosphorylation of the cTnI in cTn. In this case the k_{obs} at pCa 3 and pCa 8.9 did not differ significantly. Thus complete dephosphorylation of cTn seems to lead to conformational changes in the holotroponin complex which makes reconstitution of a regulated thin filament more difficult.

In the experiment performed here cTn behaves in a similar way to sTn, the removal of calcium leads to occupancy of the blocked state of the thin filament and the value of K_B is similar for both proteins [13]. Differences are due to the additional phosphorylation sites in cTnI (bisphosphorylation leads to a rightward shift of pCa_{50} vs. k_{obs}) and to the functionless calcium-binding site I in cTnC, which might explain the smaller n_H observed for cTn compared to sTn [13].

The effect of cTnI bisphosphorylation is the decrease in the calcium sensitivity of S1 binding to the thin filament. ^{31}P-NMR spectra showed that only phosphate groups present in the bisphosphorylared species interact with another cTn subunit [6]. Recent studies have shown that bisphosphorylation decreases the affinity of cTnI for cTnC and for actin [28]. However, there exist three further cTnI states, namely the two monophosphorylated ones and the dephosphorylated one as well as multiple cTnT states whose functions are still not known to date. According to ^{31}P-NMR measurements the phosphates in the monophospho forms of cTnI do not interact with other cTn subunits and thus an influence on calcium sensitivity as observed for the bisphospho cTnI seems unlikely.

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