Calcium- and myosin-dependent changes in troponin structure during activation of heart muscle

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Running title: Changes in troponin structure in heart muscle

Key words: cardiac muscle, troponin, myosin

Total number of words (excluding references and figure legends): 4696

(TOC category: Cardiovascular**)**

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Abstract

Each heartbeat is triggered by a pulse of intracellular calcium ions which bind to troponin on the actin-containing thin filaments of heart muscle cells, initiating a change in filament structure that allows myosin to bind and generate force. We investigated the molecular mechanism of calcium regulation in demembranated trabeculae from rat ventricle using polarised fluorescence from probes on troponin C (TnC). Native TnC was replaced by double-cysteine mutants of human cardiac TnC with bifunctional rhodamine attached along either the C helix, adjacent to the regulatory Ca^{2+}-binding site, or the E helix in the IT arm of the troponin complex. Changes in the orientation of both troponin helices had the same steep Ca^{2+}-dependence as active force production, with a Hill coefficient (n_H) close to 3, consistent with a single co-operative transition controlled by Ca^{2+} binding. Complete inhibition of active force by 25 μM blebbistatin had very little effect on the Ca^{2+}-dependent structural changes and in particular did not significantly reduce the value of n_H. Binding of rigor myosin heads to thin filaments following MgATP depletion in the absence of Ca^{2+} also changed the orientation of the C and E helices, and addition of Ca^{2+} in rigor produced further changes characterized by increased Ca^{2+} affinity but n_H close to 1. These results show that, although myosin binding can switch on thin filaments in rigor conditions, it does not contribute significantly under physiological conditions. The physiological mechanism of co-operative Ca^{2+}-regulation of cardiac contractility must therefore be intrinsic to the thin filaments.

Contraction of heart muscle is driven by an interaction between myosin, actin and MgATP that is controlled on a beat-to-beat basis by transient binding of Ca^{2+} ions to the troponin/tropomyosin complex in the actin-containing thin filaments (Tobacman, 1996; Gordon *et al*. 2000; Kobayashi & Solaro, 2005). Although the structures of most of the major protein components of the regulatory complex have been elucidated, the physiological mechanism of the control of cardiac contractility is not yet understood at the molecular level. Activation and relaxation of heart muscle cells occur over a narrow range of Ca^{2+} concentrations, and the steep Ca^{2+}-dependence of the regulatory mechanism indicates a high degree of co-operativity. This might be due to coupling between adjacent actin/tropomyosin/troponin units in the thin filament (Tobacman, 1996; Tobacman & Sawyer, 1990; Galinska-Rakoczy *et al*. 2008) or to activation of the thin filament by myosin binding (Bremel & Weber, 1972; Rosenfeld & Taylor, 1985; Robinson *et al*. 2004). Both these mechanisms can operate under specific conditions *in vitro*, but the nature of the structural changes and co-operative interactions that occur during physiological activation of the heart cannot be reliably extrapolated from *in vitro* studies.

Here we have investigated the molecular mechanism of Ca^{2+} regulation of cardiac contractility in demembranated ventricular trabeculae, in which the native structural relationships between the full complement of proteins in the regulatory system are preserved. We attached bifunctional fluorescent probes to pairs of sites on specific α-helices of troponin, and the polarisation of the fluorescence reports the orientation of each helix with respect to the actin filament or muscle cell axis. The probe attachment sites were chosen using the high resolution structures of isolated troponin components, and engineered by expressing mutants of troponin C (TnC, the component of troponin containing the regulatory Ca^{2+} site) with cysteines at the chosen sites (Fig. 1*A*; Takeda et al., 2003). One TnC mutant had cysteines at

residues 55 and 62 (*yellow* spheres) on the C helix in the N-terminal lobe (*red*) of TnC, which contains the regulatory Ca^{2+} site (*black* sphere). Another TnC mutant had cysteines at positions 95 and 102 along the E helix in the IT arm, which is composed of the C-terminal half of TnC (*green*) containing two Ca^{2+}/Mg^{2+} binding sites (*grey*), and parts of troponin I (TnI, *blue*) and troponin T (TnT, *gold*).

The pair of cysteines in each of these TnC mutants was cross-linked with bifunctional rhodamine (BR) (Corrie *et al*. 1999; Ferguson *et al*. 2003; Sun *et al*. 2006), and each BR-TnC was separately exchanged for the native TnC of demembranated trabeculae from rat ventricle. Thus the two probes used here report changes in the orientation of the two major domains of the troponin core complex, the regulatory lobe of TnC and the IT arm, during Ca^{2+}-dependent activation in the cellular environment. The relative orientation of these two domains differs substantially between published crystal structures of the core complex (Takeda *et al*. 2003; Vinogradova *et al*. 2005), and a change in this relative orientation may play a key role in the regulatory mechanism (Sun *et al*. 2006). We also investigated the role of myosin in the regulatory mechanism by inhibiting active force with blebbistatin (Straight *et al*, 2003; Dou *et al*. 2007; Farman *et al*. 2008), and by inducing rigor binding of myosin heads by MgATP depletion.

Methods

Animals

Wistar rats (200–250 g) were stunned and killed by cervical dislocation (Schedule 1 procedure in accordance with UK Animal (Scientific Procedures) Act 1986). The hearts were removed and rinsed free of blood in Krebs solution containing (mM): NaCl, 118; $NaHCO_3$, 24.8; Na_2HPO_4, 1.18; $MgSO_4$, 1.18; KCl, 4.75; $CaCl_2$, 2.54; glucose, 10; bubbled with 95%

O_2–5% CO_2; pH 7.4 at 20 °C. Suitable trabeculae (free running, unbranched, diameter < 250 µm) were dissected from the right ventricle in Krebs solution containing 25 mM 2,3-butanedione-monoxime, permeabilised in relaxing solution (see below) containing 1% Triton X-100 for 30 min, and stored in relaxing solution containing 50% (vol/vol) glycerol at -20 °C for experiments, normally within 2 days of dissection.

Preparation of BR labelled troponin C

Double cysteine mutants E55C/D62C and E95C/R102C of human cardiac TnC in the Pet3a expression vector were obtained by site-directed mutagenesis and expressed in *E. coli.* C35 and C84 were replaced by serine. TnC was expressed, purified and labelled as described previously for the skeletal isoform (Ferguson *et al*. 2003). The cysteines were cross-linked with bifunctional rhodamine (BR) to form 1:1 BR:TnC conjugates (Corrie *et al*. 1998), and purified to >95% homogeneity by reverse-phase HPLC. Stoichiometry and specificity of BR-labeling were confirmed by electrospray mass spectrometry. The measured mass of TnC with BR crosslinking residues 55 and 62 (TnC-BR_{55-62}) was 18,858.5 ± 0.9 Da (calculated mass 18,858.6 Da), and that of TnC-BR_{95-102} was 18,817.9 ± 0.8 Da (calculated mass 18,817.3 Da).

Reconstitution of TnC into ventricular trabeculae

Demembranated trabeculae were mounted, via aluminium T-clips, at sarcomere length 2.1–2.2 µm between a force transducer (AE 801) and a fixed hook in a 60 µl glass trough containing relaxing solution. The experimental temperature was 20–22 °C.

Experimental solutions contained 25 mM imidazole, 5 mM MgATP (except rigor solutions), 1 mM free Mg^{2+}, 10 mM EGTA (except pre-activating solution), 0 – 10 mM total calcium, 1 mM dithiothreitol and 0.1% (vol/vol) protease inhibitor cocktail (P8340, Sigma). Ionic strength was adjusted to 200 mM with potassium propionate; pH was 7.1 at 20 °C. The

purity of EGTA (E4378, Sigma) was 98.7% according to the manufacturer's analysis. The concentration of free Ca^{2+} was calculated using the program WinMAXC V2.5 (http://www.stanford.edu/~cpatton/maxc.html). The calculated free [Ca^{2+}] was in the range 1 nM to 63 μM in the presence of MgATP and 1 nM to 158 μM in its absence. In pre-activating solution, [EGTA] was 0.2 mM and no calcium was added. When required, 25 μM blebbistatin (B0560, Sigma) was added from a 10 mM stock solution in DMSO.

Native TnC was extracted from trabeculae by incubation in 0.5 mM trifluoperazine (Fluka 91665), 20 mM MOPS, 5 mM EDTA and 130 mM potassium propionate, pH 7.0, 20°C for 30 s, followed by 30 s in relaxing solution. After 20–25 such cycles, the residual force at maximal Ca^{2+} activation at [Ca^{2+}] = 63 μM was 30 ± 3% (n=14) of its pre-extraction value, indicating that about two-thirds of the native TnC had been removed. Trifluoperazine as used in this TnC extraction protocol has no other effect on the structure or function of skeletal muscle fibres (Ferguson *et al*. 2003; Sun *et al*. 2006). Trabeculae were then incubated in relaxing solution containing 1.8 mg/ml TnC-BR_{55-62} or TnC-BR_{95-102} for 30 min at 20 °C, and the extent of TnC reconstitution was estimated from force at maximal Ca^{2+} activation. No further increase in force was produced by longer incubation in TnC.

Measurement of TnC helix orientation by polarised fluorescence

Polarised fluorescence intensities were measured as described previously for skeletal muscle fibres (Sun *et al*. 2006). A central 0.7-mm segment of a trabecula was briefly illuminated from below with 532 nm light polarised either parallel or perpendicular to the trabecular axis. BR fluorescence at 610 nm was collected both in line with the illuminating beam and at 90° to both the illuminating beam and the trabecular axis. Each emitted beam was separated into parallel and perpendicular components, and three independent orientation order parameters were calculated from the measured intensities: $<P_{2d}>$, describing the amplitude of rapid (sub-

nanosecond) probe motion and $<P_2>$ and $<P_4>$, describing the distribution of angles θ between BR fluorescence dipole and thin filament axis averaged over slower timescales (Dale *et al*. 1999). A mean θ was estimated for a Gaussian orientation distribution (standard deviation σ_g) and reported as θ_f, the mean axial angle of the distribution in the trabecular co-ordinate frame formed by folding the tail of the molecular Gaussian distribution into the region of positive θ (Julien *et al*. 2007).

Each trabecular activation was preceded by a 1-min incubation in pre-activating solution. Isometric force and fluorescence intensities were measured after steady-state force had been established in each activation. Maximum force was recorded before and after each series of activations at sub-maximal $[Ca^{2+}]$, and was 37.9 ± 1.6 mN mm^{-2} (cross sectional area 18724 ± 2242 μm^2, n=14) before TnC extraction. If the maximum force decreased by >15%, the trabecula was discarded. The dependence of force and θ_f on $[Ca^{2+}]$ was fitted to data from individual trabeculae using nonlinear least-squares regression to the Hill equation:

$$Y = [Ca^{2+}]^{n_H} / (EC_{50}{}^{n_H} + [Ca^{2+}]^{n_H})$$

where EC_{50} is the $[Ca^{2+}]$ corresponding to half-maximal change in Y, and n_H is the Hill coefficient. All values are given as mean ± standard error except where noted, with *n* representing the number of trabeculae.

Results

Incorporation of labelled TnC into cardiac muscle cells

The *in situ* orientation of the C helix of TnC in the N-terminal regulatory lobe (Fig. 1*A*) was determined by replacing about two-thirds of the native TnC of demembranated ventricular trabeculae by TnC-BR_{55-62}, in which residues 55 and 62 were cross-linked with bifunctional

rhodamine (BR). Maximum Ca^{2+}-activated isometric force after TnC exchange was 82 ± 3% (n=5) of that before exchange (Table 1). Incorporation of unlabelled wild-type TnC by the same protocol produced the same small reduction of maximum force, consistent with a non-specific effect of the TnC exchange protocol. The dependence of force after TnC exchange on free [Ca^{2+}] was fitted by the Hill equation (Fig. 1*B*, triangles). The [Ca^{2+}] giving half-maximum force (EC_{50}) was 5.40 ± 0.57 μM (n=5) and the Hill coefficient (n_H), describing the steepness of the Ca^{2+}-dependence, was 3.10 ± 0.24 (Table 1). EC_{50} clearly increased following TnC exchange, but n_H did not change significantly (P > 0.05, paired comparison).

The orientation of the E helix in the C-terminal lobe of TnC, part of the IT arm in the troponin ternary complex (Fig. 1*A*), was determined using a TnC mutant with bifunctional rhodamine cross-linking residues 95 and 102 (TnC-BR_{95-102}). Active isometric force after replacement of native TnC by TnC-BR_{95-102} was 84 ± 2 % (n=5) of that before TnC exchange (Table 1). EC_{50} for force increased after the introduction of TnC-BR_{95-102}, but there was no significant change in n_H. Thus, as in case of the C helix probe, introduction of the BR probe on the E helix of TnC reduced the Ca^{2+} affinity of the regulatory site of TnC with no significant effect on the steepness of the force-Ca^{2+} relationship. The reduced Ca^{2+} affinity appears to be related to the presence of the BR probe on either the C or E helix, since there was no significant change in EC_{50} following exchange of wild-type TnC (Table 1).

In situ orientation of the C and E helices of troponin C

The orientation of the BR fluorescence dipole, and thus of the C or E helix of TnC to which it was attached, was determined from the polarization of fluorescence from trabeculae containing BR-TnC (Corrie *et al*. 1999; Ferguson *et al*. 2003; Sun *et al*. 2006). For a cylindrically symmetrical muscle cell, the results can be expressed in terms of a set of order parameters that describe the orientation distribution of the BR dipoles with respect to the

trabecular axis (Dale *et al*. 1999), and these order parameters are reported in Supplementary Table 1. We used a Gaussian model of these dipole orientation distributions with peak angle θ_f and standard deviation σ_g (Julien *et al*. 2007), to describe the main features of the orientation changes. θ_f increased by about 5° on Ca^{2+}-activation of the trabeculae, for both the C helix (Fig. 1*B*, circles) and the E helix (Fig. 1*C*, circles; Table 2). Although these orientation changes are small, they were highly reproducible. There was no significant change in probe dispersion σ_g on Ca^{2+}-activation.

The Ca^{2+}-dependence of the θ_f changes was described using the Hill equation, which gave a good fit to the data for the C helix probe (Fig. 1*B*, dashed line), with EC_{50} 6.01 ± 0.54 μM and n_H 3.0 ± 0.1 (n=5). These values were not significantly different from those for force in the same trabeculae (Fig. 1*B*, triangles, continuous line). θ_f for the E helix probe (Fig. 1*C*, circles) showed a small additional component in the sub-threshold range for activation, as observed previously for monofunctional probes on Cys^{84} of TnC (Bell *et al*. 2006). This component, which may be associated with Ca^{2+} binding to the Ca^{2+}/Mg^{2+} sites near the E helix (Fig. 1*A*), could be partly responsible for the offset between the Ca^{2+}-dependence of θ_f for this probe and force (Fig. 1*C*). The major component of the E helix orientation change for $[Ca^{2+}] > 1$ μM was fitted to the Hill equation (dashed line), with EC_{50} = 4.60 ± 0.50 μM. n_H was close to 3, similar to that for force in these trabeculae, and to the values reported above for the C helix (Table 2).

The EC_{50} values for the major component of the changes in orientation of the C and E helices are consistent with the Ca^{2+}-affinity of the regulatory site of troponin (Holroyde *et al*. 1980; Pan & Solaro, 1987). The structural changes in both regions of troponin have a Ca^{2+}-dependence similar to that of force. Thus, as $[Ca^{2+}]$ is varied, force is linearly related to the structural change in troponin, as expected for a simple two-state model in which troponin molecules are in either a Ca^{2+}-free OFF or a Ca^{2+}-bound ON state, with force proportional to

the fraction ON. Ca^{2+}-control of both the structural change and force are highly co-operative, however, as indicated by n_H values close to 3.

Effect of Force-Generating Myosin Heads on Troponin Orientation

To determine the effect of force-generating myosin heads on these changes in troponin structure, we reduced active force to $1.6 \pm 0.6\%$ (n=8) of the control value using 25 μM blebbistatin. This is a small molecule which binds specifically with high affinity to the actin-binding cleft of cardiac myosin, preventing strong binding to actin (Straight *et al.* 2003; Allingham *et al.* 2005), and inhibiting actomyosin ATPase activity (Kovacs *et al.* 2004; Farman *et al.* 2008) and active force generation by cardiac muscle (Dou *et al.* 2007; Farman *et al.* 2008). The Ca^{2+}-dependence of the orientation of the C helix of TnC after inhibition of active force by blebbistatin (Fig. 2*A*, circles) was almost identical to that in control conditions (dashed line). EC_{50} increased slightly ($P < 0.05$, paired comparison), but there was no significant change in n_H, which remained close to 3 (Table 2). These results provide strong evidence that the co-operativity of the Ca^{2+}-dependent change in troponin structure is not due to force-generating myosin heads. Similar results were obtained for the E helix probe (Fig. 2*B*), although here the quantitative interpretation is complicated by the biphasic orientation change noted above. Unexpectedly, blebbistatin reduced θ_f for the E helix by about 1° over the whole range of $[Ca^{2+}]$. The Hill equation fit to θ_f for $[Ca^{2+}] > 1$ μM (continuous line) again had n_H close to 3 (Table 2), as in control conditions (dashed line), and EC_{50} was larger than in control conditions ($P < 0.05$), as observed for the C helix probe.

Effect of Myosin Head Binding in Rigor on Troponin Orientation

The results described in the previous section show that the steep Ca^{2+}-dependence of active force generation is not due to binding of force-generating myosin heads to the thin filaments.

However it is well established that myosin head binding can activate the thin filaments at very low [MgATP], when myosin makes a rigor bond with actin (Bremel & Weber, 1972). Myosin binding in rigor conditions also increases the Ca^{2+} affinity of TnC (Rosenfeld & Taylor, 1985; Robinson *et al*. 2004). To clarify the mechanisms operating in the two sets of conditions, we measured the structural changes in TnC using the C- and E-helix TnC probes in ventricular trabeculae in rigor conditions.

Binding of myosin heads to actin in rigor conditions produced changes in the orientation of both helices even at 1 nM [Ca^{2+}] (Table 2). θ_f for the C helix increased by 2.5° and θ_f for the E helix by ca 8°. The change in the orientation of the E helix was larger than that produced by Ca^{2+} binding during physiological activation at 5 mM MgATP. Binding of rigor heads also increased the dispersion of the probe orientations (σ_g); for example, σ_g for the E-helix probe increased from 22.9 ± 0.4° at 5mM MgATP to 30.2 ± 2.2° in rigor at 1 nM [Ca^{2+}]. This broadening of the orientation distributions in rigor contrasts with the absence of any significant change in σ_g when trabeculae were activated at 5mM MgATP (Table 2).

Addition of Ca^{2+} in rigor produced further increases in θ_f (Fig. 3*A*,*B*, circles; Table 2), but these were spread over a much wider range of [Ca^{2+}] than during physiological activation at 5 mM MgATP (dashed lines). For quantitative analysis of the Ca^{2+} dependence in rigor, we took into account that only about two-thirds of the troponin molecules in trabeculae at sarcomere length 2.15 μm are located in the overlap region of the thin filament where myosins are available for binding (Fig 3, inset). The θ_f data (Fig. 3, circles) were therefore fitted by the sum of two Hill equations (continuous lines), with the relative amplitude of the two components as a free parameter. The best fit was obtained when two-thirds of the total angle change was accounted for by a component with EC_{50} 0.25–0.28 μM and n_H 0.7–1.1 for both the C and E helix probes (Table 2) that we associate with the overlap region (black arrow). These values of EC_{50} and n_H are similar to those measured for conformational

changes of troponin in the rigor complex in solution (Robinson *et al*. 2004). The remaining one-third was accounted for by a component with EC_{50} 4.4–4.8 μM for both the C and E helices, close to that for physiological activation at 5 mM MgATP, associated with the non-overlap region (grey arrow). n_H for this component could not be determined precisely, but was 2.2 ± 0.3 and 2.2 ± 0.6 for the C and E helices respectively, consistent with a degree of co-operativity that is similar to or somewhat lower than that measured during physiological activation. These results suggest that, even in rigor conditions, the non-overlap region of the thin filament exhibits a co-operative structural change on binding Ca^{2+} that is independent of local myosin binding.

Discussion

Co-operative Ca^{2+} regulation of cardiac contractility is intrinsic to the thin Filament

The results presented above can be explained by a simple two-state model for Ca^{2+} regulation of contraction in heart muscle. The Ca^{2+}-driven transition between the two states is co-operative, in the sense that the Ca^{2+}-dependence of changes in troponin structure and force both have a Hill coefficient (n_H) close to 3. However this co-operativity is not due to force-generating myosin heads, because it is not affected by specific inhibition of active force by blebbistatin. We conclude that the mechanism of co-operativity must be intrinsic to the thin filaments. Such a mechanism has been inferred previously from solution studies, and is likely to involve tropomyosin-mediated coupling between troponin monomers along the thin filament and/or between its two strands (Tobacman, 1996; Tobacman & Sawyer, 1990; Galinska-Rakoczy *et al*. 2008). The present conclusion is at variance with the alternative hypothesis that myosin binding plays a dominant role in switching on the thin filaments

during activation of heart muscle, and three-state model of regulation associated with this concept (McKillop & Geeves, 1993; Gordon *et al*. 2000; Kobayashi & Solaro, 2005). The next three sections reconsider the evidence for the myosin activation hypothesis in the light of the present results.

Rigor and active force-generating myosin heads have distinct effects on troponin structure

Activation of thin filaments by rigor binding of myosin heads, accompanied by an order of magnitude increase in the affinity of troponin for Ca^{2+}, is a well-established phenomenon (Bremel & Weber, 1972; Rosenfeld & Taylor, 1985; Robinson *et al*. 2004), and the rigor state has been widely used as a convenient model for the force-generating myosin heads during active contraction at physiological [MgATP] in both molecular- and cell-level studies. The present results, however, show that rigor and force-generating myosin heads have distinct effects on Ca^{2+}-dependent changes in troponin structure. In rigor, the change in troponin structure is characterized by a Ca^{2+} affinity an order of magnitude greater than in physiological conditions, but there is no co-operativity (n_H close to 1), in contrast with physiological conditions (n_H close to 3). Ca^{2+} titrations in rigor indicated the presence of two populations of troponin molecules, corresponding to those in the overlap region of the sarcomere where rigor heads have an effect, and the non-overlap region where they do not. The larger Gaussian width (σ_g) of the orientation distributions in rigor also suggests the presence of two populations of troponin molecules. At physiological [MgATP], in contrast, only one population of troponin molecules is present, implying that troponins in the overlap and non-overlap regions of the sarcomere have the same Ca^{2+}-dependence and providing further evidence for the absence of an effect of active force-generating myosin heads on troponin structure. In summary, the present results show that the effect of myosin binding to

actin in rigor conditions is not a reliable model for its effect on troponin structure and thin filament activation under physiological conditions.

Previous studies of troponin structure and Ca^{2+}-binding in muscle cells

The present work differs from previous studies that used extrinsic probes to investigate changes in troponin structure in heart muscle at physiological [MgATP] in two key respects. First, we used bifunctional probes to measure the orientation of specific troponin helices; previous studies used monofunctional probes in which the orientation and motion of the probe with respect to the protein was unknown, and different results were obtained with different probe sites (e.g. Putkey *et al*. 1997; Martyn *et al*. 2001). Second, none of the previous studies used blebbistatin to inhibit active force generation and thereby determine the role of force-generating myosin heads on thin filament activation. Blebbistatin is a more potent and specific inhibitor than the millimolar vanadate used in previous studies of structural changes in TnC reported by fluorescent probes (Martyn *et al*. 2001; Bell *et al*. 2006) and of direct Ca^{2+} binding (Hofmann & Fuchs, 1987; Wang & Fuchs, 1994). TnC can be extracted from skeletal muscle fibers in solutions containing millimolar vanadate (Allhouse *et al*. 1999; Agianian *et al*. 2004), and both TnC and TnI are extracted from cardiac muscle in these conditions (Strauss *et al*. 1992). Thus the effects of vanadate reported in previous studies of troponin structure and Ca^{2+} binding may not have been solely due to its inhibition of myosin.

Shortening -dependence of Ca^{2+} activation

Shortening of heart muscle cells during activation produces a transient increase in intracellular free [Ca^{2+}] (Allen & Kurihara, 1982; Gordon & Ridgway; 1987; Allen & Kentish, 1988), indicating a decrease in the Ca^{2+} affinity of troponin. Since fewer myosin

heads are bound to thin filaments during shortening, these transient [Ca^{2+}] increases suggest that myosin head binding can affect the Ca^{2+} affinity of troponin under physiological conditions, in apparent contradiction with the present results. However the affinity change implied by these [Ca^{2+}] transients is small. For example, with n_H = 3 and free [Ca^{2+}] = 6 μM an increase in EC_{50} from 6 to 7 μM, similar to that observed for the C helix probe when force was inhibited by blebbistatin (Table 1), would release about 8 μM total Ca^{2+} from the ca 70μM troponin in a heart muscle cell. This could account for the observed changes in free [Ca^{2+}] given reasonable estimates of intracellular Ca^{2+} buffering (Berlin *et al.* 1994). The small increase in EC_{50} associated with force inhibition suggests that the troponin ON state is slightly favoured by binding of force-generating myosin heads, as expected from a regulatory mechanism in which both processes are coupled to tropomyosin movement. This small effect of force-generating myosin heads on Ca^{2+} affinity (an order of magnitude smaller than that of rigor myosin heads) might be accompanied by a similarly small effect of force inhibition by blebbistatin on the Hill coefficient n_H (Table 1), although no such change was detectable at the 5% significance level in the present experiments. The finding that almost normal co-operativity of Ca^{2+}-regulation was maintained in the presence of blebbistatin argues strongly against a dominant role for myosin-dependent affinity changes in the co-operative response to Ca^{2+} binding.

Changes in troponin structure during Ca^{2+} activation

The change in troponin structure during activation of ventricular trabeculae reported by probes on the C and E helices of TnC corresponds to a change in the angle between each helix and the actin filament axis of only about 5°. In isolated TnC in solution, the angle between the C and D helices of TnC changes by 12° on binding Ca^{2+}, and by 34° on binding both Ca^{2+} and the switch peptide (residues 147-163) of TnI (Sia *et al.* 1997; Li *et al*. 1999).

These C/D angle changes are associated with opening of the N-lobe of TnC, exposing a hydrophobic binding site for the TnI switch peptide, and are thought to represent an essential step in the signalling pathway that leads to muscle activation. The present results do not exclude the possibility that the N-lobe opens in this way during physiological activation of heart muscle, but if so the motion of the C helix must be predominantly azimuthal, i.e. around the filament axis. Further experiments with probes at other sites on TnC will be required to characterise this motion and measure the extent of N-lobe opening *in situ*. However the comparison of the ca 5° C-helix tilt observed here with the ca 27° tilt of the same helix in skeletal muscle (Sun *et al*. 2006) already shows that the underlying conformational changes in the N-lobe of TnC on activation are different in skeletal and cardiac muscle. In skeletal muscle, the whole N-lobe seems to tilt substantially with respect to the C-lobe, IT arm, and thin filament during activation (Sun *et al*. 2006). The present results suggest that this motion does not occur in cardiac muscle, but the structural basis and physiological significance of this difference remain to be elucidated.

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Acknowledgments

We are grateful to Dr J. E. T. Corrie for bifunctional rhodamine, Professor B. D. Sykes for the DNA for cardiac TnC, Dr R. Thorogate for help with mass spectrometry, Professor J. C. Kentish for help and advice on the trabecular preparation and solutions, and comments on the manuscript. This work was supported by the British Heart Foundation (FS/04/083).

Figure legends

Fig. 1. Orientation changes of the C and E helices of troponin C on binding Ca^{2+}. (*A*) Structure of core complex of cardiac troponin in the Ca^{2+}-saturated form (ref 4; PDB 1J1D); containing troponin C (TnC; red, green) and parts of troponin I (TnI; blue, cyan) and troponin T (TnT; gold). Bifunctional rhodamine probes cross-linked either cysteines 55 and 62 (yellow spheres) along the C helix or 95 and 102 along the E helix of TnC. (*B*) and (*C*) Orientation (θ_f, circles) of the C and E helices of TnC respectively and isometric force (triangles) in the same sets of trabeculae in the presence of 5 mM MgATP. The dashed and continuous lines are fits of the Hill equation to θ_f and force respectively. Error bars denote SEM for n=5 trabeculae.

Fig. 2. Effect of force inhibition by 25μM blebbistatin on the orientation of (*A*) the C and (*B*) the E helix of TnC in the presence of 5 mM MgATP. Circles denote θ_f in the presence of blebbistatin, continous lines denote Hill fits to these data, and dashed lines are from the Hill fits in Fig. 1, in the absence of blebbistatin.

Fig. 3. The orientation of (*A*) the C and (*B*) the E helix of TnC in the absence of MgATP. Circles denote θ_f, continous lines denote double Hill equation fits described in the text, and dashed lines are from the Hill fits in Fig. 1 for 5mM MgATP. The inset shows the degree of overlap between the myosin and thin filaments at the sarcomere length used in these experiments.

Table 1. Ca^{2+}-dependence of isometric force (mean ± SEM).

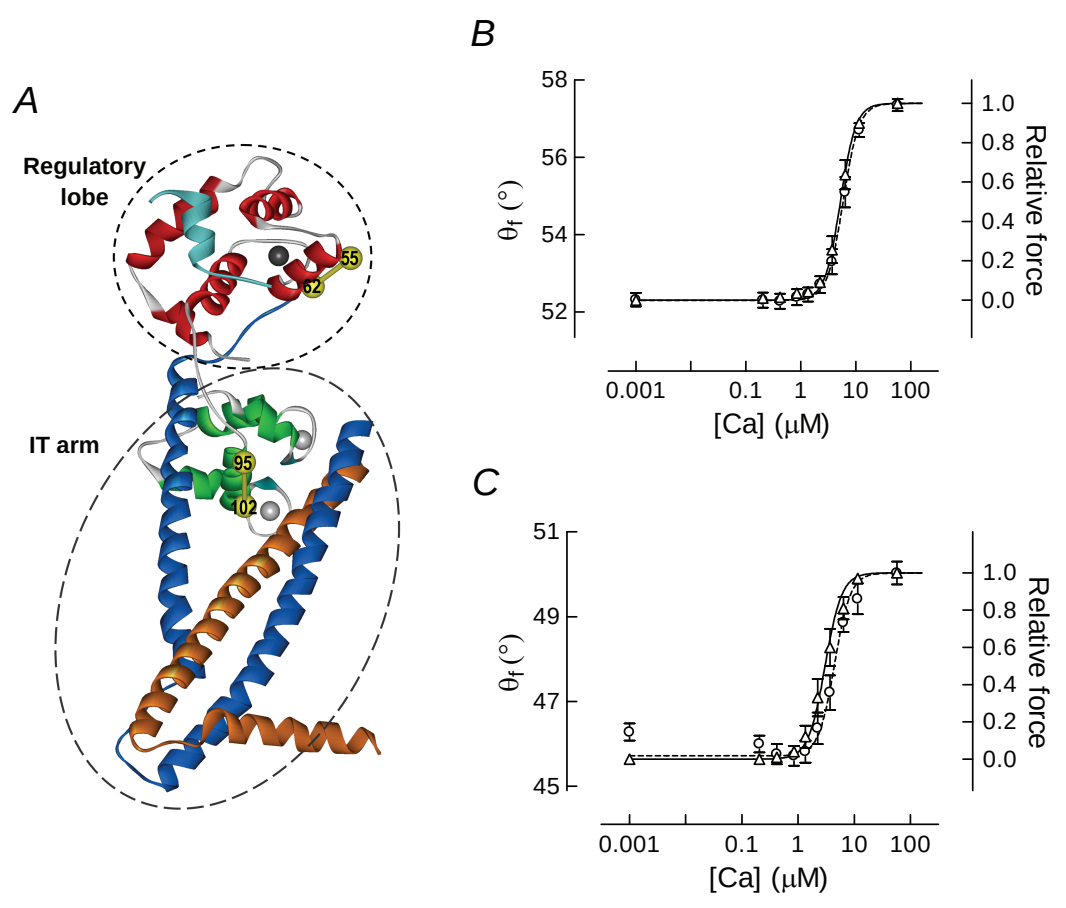
	C helix; TnC-BR_{55-62} (n=5)	**E helix**; TnC-BR_{95-102} (n=5)	**Wild-type TnC** (n=4)
Before TnC reconstitution			
EC_{50} (μM)	2.53 ± 0.21	1.82 ± 0.33	1.29 ± 0.25
n_H	3.51 ± 0.48	3.59 ± 0.15	3.57 ± 0.45
After TnC reconstitution			
Maximum force	0.82 ± 0.03	0.84 ± 0.02	0.82 ± 0.01
EC_{50} (μM)	5.40 ± 0.57	3.17 ± 0.49	1.54 ± 0.3
n_H	3.10 ± 0.24	2.92 ± 0.25	3.13 ± 0.43

EC_{50} and n_H are fitted parameters of the Hill equation (see *Materials and Methods*). Maximum Ca^{2+}-saturated force was measured after TnC exchange as a fraction of that in the same trabeculae before exchange.

Table 2. Ca^{2+}- dependence of troponin C orientation parameters

	C helix; TnC-BR_{55-62}			**E helix**; TnC-BR_{95-102}		
	5 mM MgATP (n=5)	5 mM MgATP + 25 µM Blebbistatin (n=4)	0 mM MgATP, Rigor (n=5)	5 mM MgATP (n=5)	5 mM MgATP + 25 µM Blebbistatin (n=4)	0 mM MgATP, Rigor (n=4)
θ_f (°)						
1 nM [Ca^{2+}]	52.3 ± 0.2	52.2 ± 0.2 ^{ns}	54.8 ± 0.1 *	46.3 ± 0.2	45.5 ± 0.1	54.4 ± 0.4
Maximal [Ca^{2+}]	57.4 ± 0.2	57.0 ± 0.2 *	57.9 ± 0.1 *	50.0 ± 0.3	48.4 ± 0.2	56.9 ± 0.4
First Hill fit						
Amplitude (°)	5.04 ± 0.12	4.81 ± 0.05	1.12 ± 0.27	4.29 ± 0.34	3.70 ± 0.26	0.95 ± 0.20
EC_{50} (µM)	6.01 ± 0.54	7.29 ± 0.71 *	4.83 ± 0.44	4.60 ± 0.50	7.39 ± 1.00	4.37 ± 0.45
n_H	3.01 ± 0.13	2.86 ± 0.27 ^{ns}	2.24 ± 0.33	3.06 ± 0.32	2.85 ± 0.13	2.21 ± 0.64
Second Hill fit						
Amplitude (°)			1.97 ± 0.20			1.59 ± 0.18
EC_{50} (µM)			0.28 ± 0.08			0.25 ± 0.05
n_H			1.05 ± 0.12			0.73 ± 0.04
σ_g (°)						
1 nM [Ca^{2+}]	24.3 ± 0.4	24.9 ± 0.2	27.4 ± 1.3	22.9 ± 0.4	23.4 ± 0.5	30.2 ± 2.2
Maximal [Ca^{2+}]	24.8 ± 0.6	25.1 ± 0.3	26.0 ± 1.1	22.6 ± 0.6	22.5 ± 0.5	30.8 ± 2.8

Mean ± SEM. Comparison: paired t-test, two-tailed; ^{ns}, P>0.05; *, P<0.05.


A
Regulatory lobe
55
52
IT arm
95
102
B
58
56
54
52
θf (°)
1.0
0.8
0.6
0.4
0.2
0.0
Relative force
0.001
0.1
1
10
100
[Ca] (μM)
C
51
49
47
45
θf (°)
1.0
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Relative force
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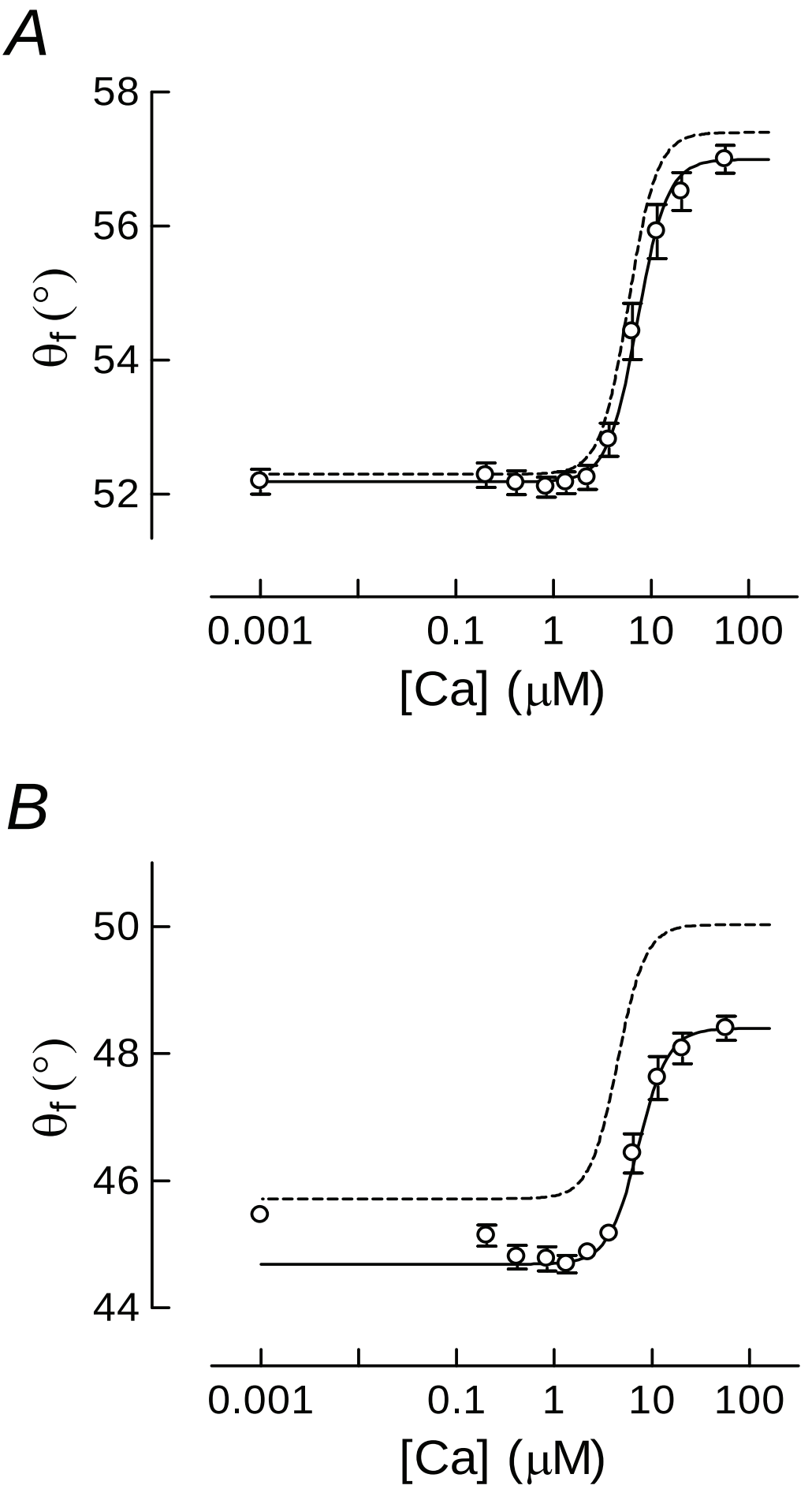
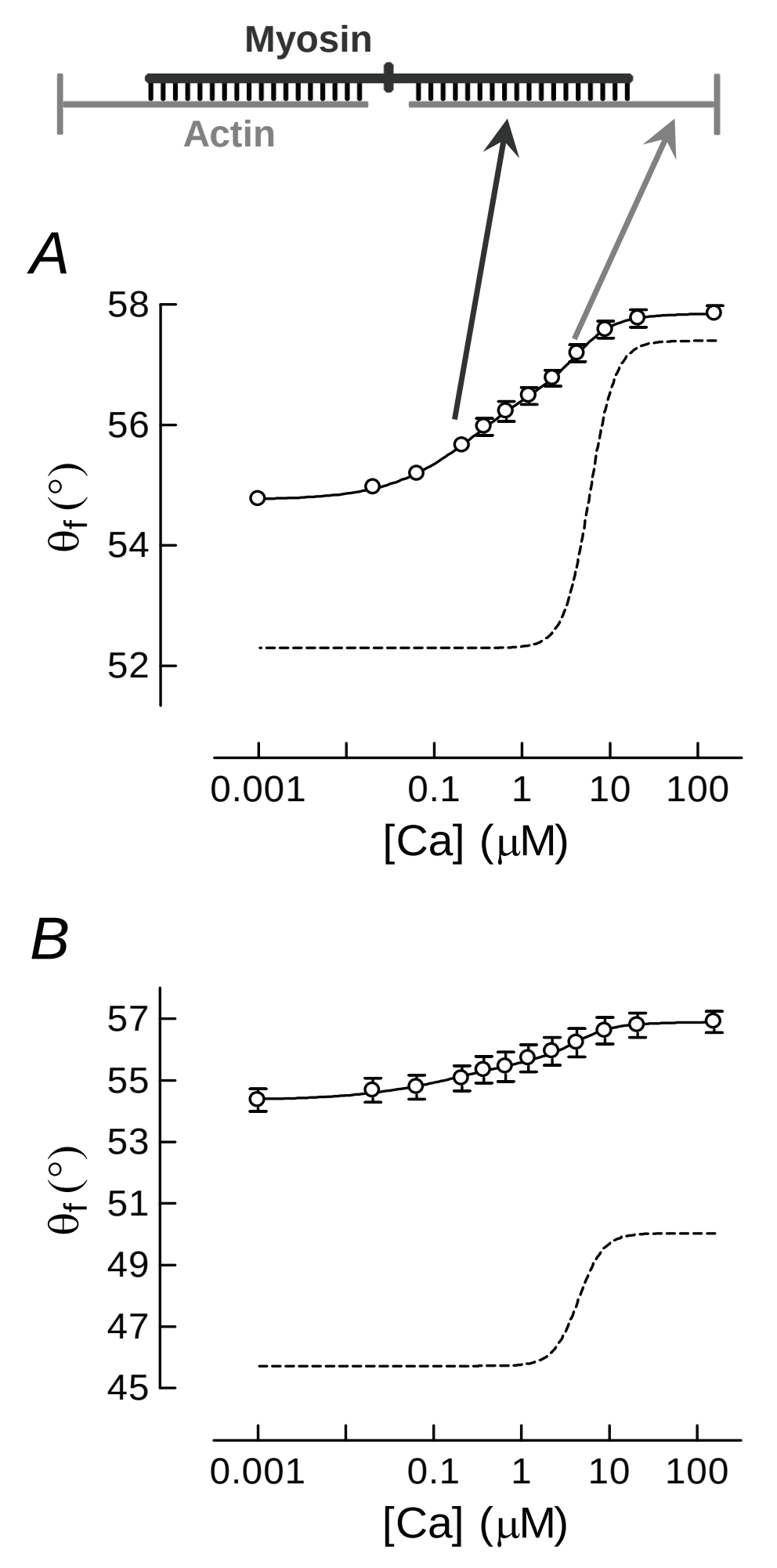
[Ca] (μM)


A
58
56
54
52
θf (°)
0.001
0.1
1
10
100
[Ca] (μM)
B
50
48
46
44
θf (°)
0.001
0.1
1
10
100
[Ca] (μM)


Myosin
Actin
A
58
56
54
52
θf (°)
0.001
0.1
1
10
100
[Ca] (μM)
B
57
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47
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[Ca] (μM)