Influence of Length on Force and Activation-Dependent Changes in Troponin C Structure in Skinned Cardiac and Fast Skeletal Muscle

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ABSTRACT Linear dichroism of 5′ tetramethyl-rhodamine (5′ATR) was measured to monitor the effect of sarcomere length (SL) on troponin C (TnC) structure during Ca^{2+} activation in single glycerinated rabbit psoas fibers and skinned right ventricular trabeculae from rats. Endogenous TnC was extracted, and the preparations were reconstituted with TnC fluorescently labeled with 5′ATR. In skinned psoas fibers reconstituted with sTnC labeled at Cys 98 with 5′ATR, dichroism was maximal during relaxation (pCa 9.2) and was minimal at pCa 4.0. In skinned cardiac trabeculae reconstituted with a mono-cysteine mutant cTnC (cTnC(C84)), dichroism of the 5′ATR probe attached to Cys 84 increased during Ca^{2+} activation of force. Force and dichroism-[Ca^{2+}] relations were fit with the Hill equation to determine the pCa_{50} and slope (*n*). Increasing SL increased the Ca^{2+} sensitivity of force in both skinned psoas fibers and trabeculae. However, in skinned psoas fibers, neither SL changes or force inhibition had an effect on the Ca^{2+} sensitivity of dichroism. In contrast, increasing SL increased the Ca^{2+} sensitivity of both force and dichroism in skinned trabeculae. Furthermore, inhibition of force caused decreased Ca^{2+} sensitivity of dichroism, decreased dichroism at saturating [Ca^{2+}], and loss of the influence of SL in cardiac muscle. The data indicate that in skeletal fibers SL-dependent shifts in the Ca^{2+} sensitivity of force are not caused by corresponding changes in Ca^{2+} binding to TnC and that strong cross-bridge binding has little effect on TnC structure at any SL or level of activation. On the other hand, in cardiac muscle, both force and activation-dependent changes in cTnC structure were influenced by SL. Additionally, the effect of SL on cardiac muscle activation was itself dependent on active, cycling cross-bridges.

INTRODUCTION

It has been well documented for both fast (Moss et al., 1983; Martyn and Gordon, 1988; Fuchs and Wang, 1991) and slow (Stephenson and Wendt, 1984; Wang and Fuchs, 1994) skeletal muscle and cardiac muscle (Hibberd and Jewell, 1982; Kentish et al., 1986) that an increase in sarcomere length (SL) causes an increase in the Ca^{2+} sensitivity of contractile force, shifting force-[Ca^{2+}] relations toward lower [Ca^{2+}]. This occurs over a wide range of increasing SL in skinned skeletal fibers, even over the range of SLs where maximum force varies little (Martyn and Gordon, 1988). In contrast, both the Ca^{2+} sensitivity of force and maximum Ca^{2+}-activated force increase over the same SL range in cardiac muscle (Kentish et al., 1986). This strong SL dependence of force activation in cardiac muscle is the physiologic basis of Starling's Law. Even though the influence of SL on the Ca^{2+} dependence of force is common to all vertebrate striated muscle fiber types, the underlying mechanism is not completely understood.

An increase in the Ca^{2+} sensitivity of force with increasing SL could indicate that the apparent affinity of the thin filament regulatory protein troponin C (TnC) for Ca^{2+} has increased. This may be especially true in skinned cardiac muscle because increasing SL increases Ca^{2+} bound to thin filaments during activation (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994). However, in skinned skeletal fibers neither direct steady-state measurements of Ca^{2+} binding to thin filaments of fast (Fuchs and Wang, 1991) or slow skeletal fibers (Wang and Fuchs, 1994) nor indirect measurements of the Ca^{2+} affinity of skeletal TnC (sTnC) (Patel et al., 1997) appear to be influenced by SL. However, transient changes in SL and force appear to influence Ca^{2+}-binding to thin filaments in intact barnacle (Gordon and Ridgway, 1987) and fast skeletal fibers (Vandenboom et al., 1998), implying that changes in SL or force do alter thin filament Ca^{2+} binding and thus the Ca^{2+} affinity of TnC. Thus, steady-state measurements indicate that SL-dependent shifts in the Ca^{2+} sensitivity of force occur without alteration of Ca^{2+} binding to TnC, whereas transient changes in SL and force cause an apparent change in thin filament Ca^{2+} binding in skeletal fibers. Therefore, the question remains unresolved; do changes in SL alter the Ca^{2+}-binding properties or structure of TnC in skinned skeletal fibers?

In cardiac muscle, increasing SL causes increased force and thin filament Ca^{2+} binding (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994). As for skeletal fibers (McDonald et al., 1997), the SL dependence of thin filament activation appears to result from accompanying changes in myofilament lattice spacing (LS) (McDonald et al., 1995; Fuchs and Wang, 1996, 1997). Furthermore, the importance of strong, cycling cross-bridge binding in the mechanism responsible for the SL dependence of force-[Ca^{2+}] relations is emphasized because both the amount of Ca^{2+} bound to thin filaments is decreased and the SL dependence of Ca^{2+} binding eliminated by force inhibition (Hofmann and Fuchs,

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1987a). Thus, if strong, cycling cross-bridge binding contributes to the SL dependence of thin filament activation in both skeletal and cardiac muscle, the mechanism in each appears to be different.

To determine the relative contributions of Ca^{2+} binding and strong cross-bridge attachment to the mechanism of SL-dependent activation in skeletal and cardiac muscle we monitored Ca^{2+}- and cross-bridge-induced changes in the structure of TnC. Skinned fast skeletal fibers were reconstituted with native sTnC labeled at Cys 98 with the fluorescent probe 5′-tetramethyl-rhodamine (5′ATR), and 5′ATR dichroism was measured to monitor changes in sTnC structure. Likewise, in skinned cardiac muscle, TnC structure was monitored after reconstitution with monocysteine mutant cardiac TnC (cTnC(C84)) labeled with 5′ATR at Cys 84. We have shown that the 5′ATR probe located at Cys 98 on sTnC is sensitive to both Ca^{2+} binding at sites I and II and strong (rigor) cross-bridge attachment (Martyn et al., 1999). The response was similar with other probes at Cys 98 or at Met 25 near site I at the N-terminus of sTnC (Martyn et al., 1999). The 5′ATR probe located at Cys 84 in cTnC is sensitive to Ca^{2+} binding to site II in cTnC and thin filament activation by either cycling or rigor cross-bridges (Martyn et al., 2001). To test for a role of cycling cross-bridges, force was inhibited with 2,3-butanedione monoxime (BDM) in skeletal fibers, enabling comparison with our previous studies (Martyn et al., 1999). In cardiac muscle force was inhibited with 1.0 mM sodium vanadate (Vi) to facilitate comparison with Ca^{2+}-binding studies (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994). If changes in SL influence steady-state force-$[Ca^{2+}]$ relations by altering Ca^{2+} binding to TnC in skinned skeletal or cardiac muscle, either directly or through altered cross-bridge binding, the structural response to Ca^{2+} of either sTnC-5′ATR in skinned skeletal fibers or cTnC(C84)-5′ATR in skinned cardiac muscle should be influenced by changes in SL or inhibition of force.

MATERIALS AND METHODS

Segments of single muscle fibers were prepared from glycerinated rabbit psoas as described previously (Martyn et al., 1999). Isolated fiber segments were treated with 1% Triton X-100 in pCa 9.2 solution for 10 min to remove membranous elements. The ends of fiber segments were fixed with glutaraldehyde to reduce end compliance (Chase and Kushmerick, 1988) and were attached via aluminum foil T-clips to small wire hooks on the mechanical apparatus. At a relaxed SL of 2.5 μm, fiber length (L_F) averaged 2.3 ± 0.30 mm (mean ± SEM; $n = 5$) and the diameter averaged 52.2 ± 0.40 μm (mean ± SEM.; $n = 5$). To minimize activation-dependent alterations in myofilament lattice spacing (Brenner and Yu, 1985), 4% w/v Dextran T-500 was added to all solutions (Matsubara et al., 1985; Kawai et al., 1993).

Right ventricular trabeculae were isolated from rat hearts and chemically skinned in 1% Triton X-100, as previously described (Martyn et al., 2001). As above, the ends of trabeculae were fixed with glutaraldehyde (Chase and Kushmerick, 1988) and attached to the length changer and force transducer using aluminum T-clips. No Dextran T-500 was added to solutions bathing skinned cardiac muscle. At a relaxed length of 2.4 μm, the average diameter of trabeculae was 124 ± 12 μm and average length was 1.75 ± 0.13 mm (means ± SEM; $n = 6$ preparations).

Mechanical and optical apparatus

The mechanical and optical apparatus used in this study are described in more detail in Martyn et al. (1999). Briefly, preparations were mounted in a 100-μl chamber set on a moveable temperature-controlled platform attached to an inverted epi-fluorescence microscope (Zeiss Axiovert 35). Force was measured with a Cambridge Technology model 400A force transducer, and fiber length (L_F) was controlled with a linear scanning motor (General Scanning GP-120). In some experiments, stiffness was measured by low-amplitude (0.15%) sinusoidal oscillations (500 Hz) of preparation length. Stiffness was determined by Fourier analysis of the resulting oscillations in force. SL was monitored by helium-neon laser diffraction. Skeletal fibers were rejected if they did not exhibit a clearly defined diffraction pattern at maximum Ca^{2+}-activated force (pCa 4.0). With skinned cardiac trabeculae, diffraction could be measured at lower levels of activation but became diffuse and difficult to measure at maximal force. For cardiac experiments, the initial SL was varied from 2.4 to 2.0 μm.

During dichroism measurements the fiber was illuminated by a Hg vapor lamp (HBO 50/3, OSRAM GmbH, Munich, Germany) filtered at 540 ± 5 nm and then passed through a 12% neutral density and polarizing filter oriented perpendicular to the fiber axis for excitation of 5′ATR-labeled TnC. The polarization angle of the exciting light was sinusoidally (42 kHz) alternated parallel and perpendicular to the fiber axis by a photo-elastic modulator (PEM; model 80, Hinds International, Portland, OR) with the peak-to-peak retardation set to 413 nm. The light emitted following absorption by 5′ATR-labeled TnC (590–630 nm) was collected by the objective (NA = 0.32) and focused onto a photomultiplier tube (Hamamatsu type R938HA, Hamamatsu City, Japan). The output of the photomultiplier was monitored with a wide bandpass current-to-voltage converter (−3 db at 450 kHz). To determine the root mean squared amplitude of the sinusoidally varying difference in fluorescence intensity parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the fiber axis, the output of the amplifier was directed to a lock-in amplifier (Ithaco model 3961B, Ithaca, NY). Dichroism or polarization anisotropy (r) was measured and expressed using the equation given by Tanner et al. (1992):

$$r = (-2D/L)/6J_2(\pi) + (1 - 3J_0(\pi)D/L),$$

where D is the root mean squared value of $I_\parallel - I_\perp$ (from the output of the lock-in amplifier), L is the total fluorescence ($I_\parallel + 2I_\perp$) and J_n is the nth-order Bessel function. The retardation of the PEM was set so that the zero-order Bessel function (J_0) was zero. Further details of the optical measurements are described in Martyn et al. (1999).

The magnitude of dichroism depends on the average orientation of the population of probes with respect to the magic angle, at which dichroism would be zero. Thus, an increase in dichroism could indicate either an increase in the degree of order of the population of probes or a change in average orientation of the probes, so that they become more oriented parallel to the fiber axis. Likewise, decreasing dichroism would indicate either that the average angle of the probes approached the magic angle or that the probes became more disordered. Measurements of linear dichroism do not allow distinction between these possible mechanisms, although recent EPR measurements of maleimide-labeled TnC indicate that Ca^{2+} binding to TnC causes both disorder and angle changes (Li and Fajer, 1994).

Data acquisition and control

Data were acquired during continuous, steady-state Ca^{2+} activations at submaximal and maximal (pCa 4.0) levels. Periodic cycles of shortening/

restretch were applied to skeletal fibers to maintain mechanical properties and structure during activation (see Fig. 1) as previously described (Chase et al., 1994). Measurements of isometric force and fluorescence were made during the steady-state period between the cycles of unloading/restretch. The force baseline for each condition was determined unambiguously during a large-amplitude slack release. Fiber force was normalized to cross-sectional area.

Multiple measurements of dichroism were made in each solution (see Figs. 1 and 3). To minimize the contributions of non-fiber fluorescence the field of view was constrained with an adjustable field mask made slightly larger than the fiber. Measurements were made both with the preparation in the field of view and removed from the field for background measurements. Background values were subtracted from those made with the preparation in view. All values of dichroism were normalized to the average of the values obtained at pCa 9.2 taken before and after a measurement, and values of force were normalized to the initial pCa 4.0 activation following reconstitution with fluorescently labeled TnC. The digitized data were analyzed using custom software. Data were further analyzed by linear least-squares regression (Excel version 4.0 for Windows, Microsoft Corp., Redmond, WA) or by nonlinear least-squares regression (Sigma Plot version 4.1, Jandel Scientific, San Rafael, CA). The relation between force, fluorescence, and $[Ca^{2+}]$ was fitted by a nonlinear least-squares regression to the Hill equation:

$$Y = 1/(1 + 10^{n(\mathrm{pCa}-\mathrm{pCa}_{50})})$$

where pCa_{50} is the negative log of the $[Ca^{2+}]$ that produces half-maximal force and n determines the slope of the Ca^{2+} dependence. Statistical analyses were performed using Excel (version 4.0 for Windows, Microsoft). Student's t-test was used to compare the means of data.

Solutions

Relaxing and activating solutions were prepared as described previously (Martyn and Gordon, 1988) and contained 5 mM Mg^{2+}-adenosine 5′-triphosphate (MgATP), 15 mM phosphocreatine (PCr), 15 mM EGTA, at least 40 mM MOPS, 135 mM $Na^+ + K^+$, 1 mM Mg^{2+}, pH 7.0, 250 U/ml creatine phosphokinase (CK), and Dextran T-500 (4% w/v; Pharmacia, Piscataway, NJ). Propionate (P) was the major anion. To alter solution $[Ca^{2+}]$, varying amounts of CaP_2 were added as determined with a computer program taking into account the desired free $[Ca^{2+}]$ and the binding constants of all solution constituents for Ca^{2+}; ionic strength was 170 mM. During experiments in which force and dichroism are compared the temperature was 10°C for skeletal fibers and 20°C for skinned cardiac trabeculae and varied by <1°C during an experiment.

Preparation of labeled proteins

sTnC was isolated and purified according to the method described by Greaser and Gergely (1971). sTnC was then labeled with 5′ATR as previously described (Martyn et al., 1999). Mono-cysteine mutant cTnC(C84), with Cys 35 mutated to Ser, was prepared and labeled with 5′ATR as previously described (Martyn et al., 2001).

Skeletal TnC extraction and reconstitution

sTnC was extracted from single skinned psoas fibers by bathing fibers in a solution containing 5.0 mM EDTA, 10 mM imidazole, pH 6.7, at 10°C (Moss, 1992). Fibers were extracted for 10 min, followed by determination of the residual Ca^{2+}-sensitive force at maximal activation $[Ca^{2+}]$ (pCa 4.0). The level of residual force averaged 52.0 ± 4.0 (mean ± SEM; n = 5 fibers) of pre-extraction controls. This level of post-extraction force probably corresponds to ~50% extraction of endogenous TnC (Moss et al., 1985).

Skeletal fibers were reconstituted with labeled TnC (~1.0 mg/ml) in pCa 9.2 relaxing solution containing 1.0 mM dithiothreitol (DTT) for 20–30 min at 10°C. Following reconstitution with sTnC-5′ATR, isometric force at pCa 4.0 was 90.0 ± 1.6% (mean ± SEM; n = 5 fibers) of pre-extraction controls. As previously reported (Martyn et al., 1999), exchange of 5′ATR labeled sTnC into skinned psoas fibers did not alter force-$[Ca^{2+}]$ relations when compared with either unextracted controls or fibers that were extracted and reconstituted with unlabeled native sTnC.

Cardiac TnC extraction and reconstitution

Native cTnC was extracted from the skinned cardiac trabeculae by exposing them to a low-ionic-strength solution that contains 5 mM K_2EDTA, 20 mM TRIS 20, pH 7.2 (Gulati et al., 1991). To begin cTnC extraction the fiber was first placed in rigor solution (zero [ATP]; zero $[Ca^{2+}]$) solution at 5–8°C and then extraction solution at 5–8°C for 5 min, at which time the temperature was raised to 30°C for 30–50 min. After extraction, the fiber was placed in relaxing solution at 20°C, and Ca^{2+}-activated force was determined in pCa 4.0 activating solution. Post-extraction force was 24.2 ± 11.3% (mean ± SEM; n = 6 preparations) of pre-extraction levels (pCa 4.0). Trabeculae were then reconstituted by incubation for 30 min in a relaxing solution (zero added Ca^{2+}), containing 10–50 μM fluorescently labeled cTnC. Post-reconstitution force was 83.3 ± 3.4% of pre-extraction maximum values (mean ± SEM; n = 6 preparations). As we previously reported, substitution of 5′ATR-labeled cTnC(C84) caused a small increase in the Ca^{2+} sensitivity of force (Martyn et al., 2001). Following reconstitution of either skinned skeletal or cardiac preparations with 5′ATR-labeled TnC they were exposed for 5 min to relaxing solution (pCa 9.2) containing 2 mg/ml BSA to remove nonspecifically bound labeled TnC. This procedure eliminated the large background fluorescence observed without the treatment.

RESULTS

Skeletal fibers

The $[Ca^{2+}]$ dependence of force and dichroism was determined in skinned rabbit psoas muscle fibers that had been reconstituted with native sTnC labeled at Cys 98 with 5′ATR. SL was set at either 2.56 ± 0.01 or 2.15 ± 0.01 μm (mean ± SEM; n = 5 fibers) and adjusted during activation to hold SL isometric at the selected value. At the longer SL and maximum activating $[Ca^{2+}]$ (pCa 4.0) force averaged 328 ± 59 mN mm^2 (mean ± SEM; n = 5 fibers); relaxed force (pCa 9.2) averaged 3.0 ± 0.10% (mean ± SEM; n = 5 fibers) of the maximum Ca^{2+}-activated force. Representative traces of force and changes in fluorescence at long and short SL are illustrated in Fig. 1 for data acquired at maximal (Fig. 1 *A*; pCa 4.0) or sub-maximal Ca^{2+} activation of force (Fig. 1 *B*; pCa 6.1). At pCa 4.0, force was only slightly decreased at the shorter SL, whereas at sub-maximal pCa, isometric force was significantly less at short SL than at long SL. Ca^{2+} activation caused dichroism in fibers reconstituted with 5′ATR-labeled sTnC to decrease from the level in relaxing solution (pCa 9.2), as we previously described (Martyn et al., 1999). The demodulated fluorescence signal ($I_\parallel - I_\perp$) (Fig. 1, *A* and *B*; *middle traces*) decreased to near background levels at pCa 4.0 (Fig. 1, *A* and *B*; *lower trace*). There was a small but statistically significant ($p < 0.05$) increase in total fluorescence ($I_\parallel$ +

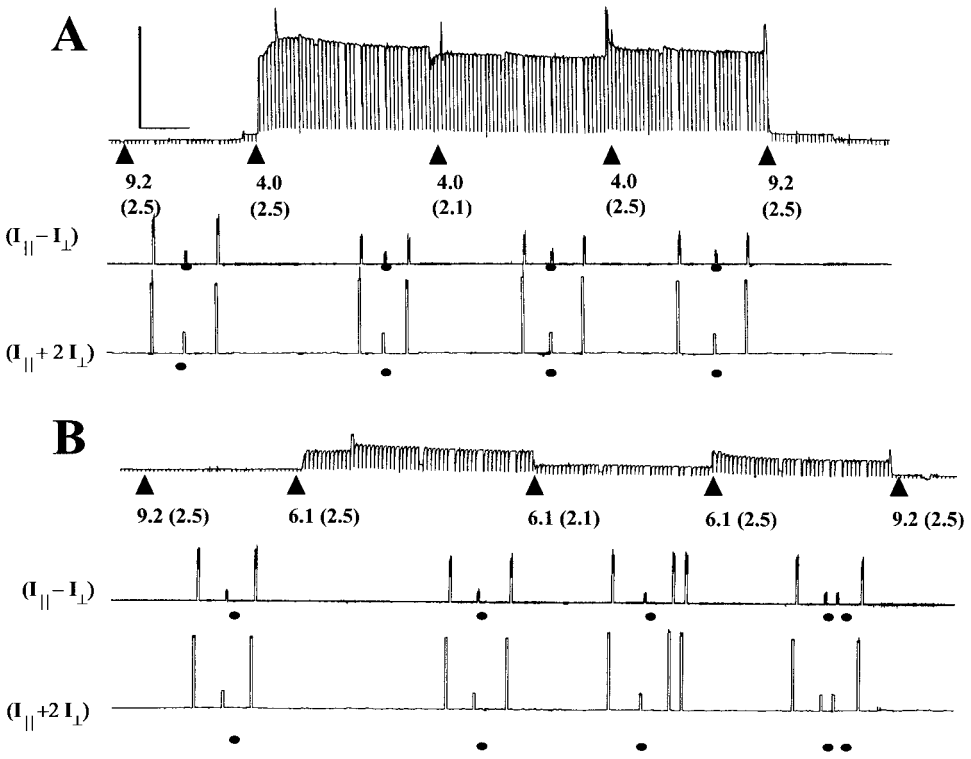

FIGURE 1 Representative traces of force (*top*), demodulated 84-kHz sinusoidal variation of the fluorescence signal ($I_{\parallel} - I_{\perp}$; *middle trace*) and total fluorescence ($I_{\parallel} + 2I_{\perp}$; *bottom trace*) are shown for maximal Ca^{2+} activation of force at pCa 4.0 (*A*) and at sub-maximal activation at pCa 6.1 (*B*) for a single rabbit skinned psoas fiber that was reconstituted with native sTnC labeled with 5′ATR at Cys 98. For each condition, force and fluorescence were measured at either 2.5 or 2.1 μm SL. Changes in solution pCa are indicated below the force traces by arrows, with the corresponding SLs indicated in parentheses next to each pCa. The filled dots below the fluorescence traces indicate measurements made with the fiber out of the field of view. These values were subtracted from the corresponding bracketing measurements made with the fiber in view. The transients in the force traces result from the periodic (0.2 Hz) Brenner cycles (Brenner; 1983). The vertical calibration bar indicates 250 mN mm^{-2} and the horizontal bar 1 min.

$2I_{\perp}$) when SL was decreased, presumably reflecting the presence of more sarcomeres and labeled protein in the optical field.

Effects of SL on the Ca^{2+} dependence of force and dichroism in skeletal fibers

Force-[Ca^{2+}] relations were found to be shifted to greater [Ca^{2+}] by decreasing SL from 2.56 (●; *solid lines*) to 2.15 (○; *dashed lines*) μm, as indicated in Fig. 2 *A*. Maximum Ca^{2+}-acivated force (pCa 4.0) decreased ~15% at the shorter SL. Force-[Ca^{2+}] relations were determined from five fibers, and data from each fiber was fit with the Hill equation; the average (± SEM) values of pCa_{50} and n_H are included in Table 1, along with the values of maximum force. Decreasing SL decreased the pCa_{50} by 0.2 pCa unit, with no significant change in the slope (n_H) of force-[Ca^{2+}] relations (Table 1).

As illustrated in Fig. 1, measurements of the dichroism of 5′ATR-labeled sTnC in skinned psoas fibers were made during Ca^{2+} activation of force at both 2.5 and 2.15 μm SL. The Ca^{2+} dependence of dichroism is illustrated in Fig. 2 *B*. Absolute dichroism was 0.07 ± 0.002 in relaxing solution and decreased upon Ca^{2+} activation of force to 0.01 ± 0.001 (mean ± SEM; $n = 5$ fibers). These values were unaffected by changes in SL. To facilitate comparison with force data, relative dichroism is expressed as one minus the ratio of the value in activating over relaxing solution ($1 - dic/dic_{9.2}$). Data from individual fibers were fit with the Hill equation, and the averaged values of pCa_{50} and n_H were used to generate the fitted curves in Fig. 2, *A* and *B*, and are included for comparison with corresponding values for force-[Ca^{2+}] relations in Table 1. The curve fit to force-[Ca^{2+}] relations at 2.5 μm SL is included (*dashed curve*) for reference. In striking comparison to force-[Ca^{2+}] data (Fig. 2 *A*), the Ca^{2+} dependence of dichroism and structural changes in sTnC were insensitive to changes in SL in controls (Fig. 2 *B*).

In a subset of fibers, isometric force was inhibited with 30 mM BDM and the Ca^{2+} dependence of dichroism measured. BDM at 30 mM caused force at pCa 4.0 to decrease to 0.23 ± 0.01 at 2.4 μm SL and to 0.06 ± 0.01 (mean ± SEM; $n = 3$ fibers) at 2.1 μm SL, both referenced to maximum force at the longer length. Force inhibition with BDM caused no significant change in the Ca^{2+} sensitivity of dichroism at either SL. This is consistent with our previous observations at a single SL (Martyn et al., 1999). The Hill fit parameters for dichroism-[Ca^{2+}] relations in the presence of 30 mM BDM are included in Table 1.

Ca^{2+} dependence of force and 5′ATR-labeled cTnC(C84) dichroism in skinned cardiac muscle

Force-[Ca^{2+}] and dichroism-[Ca^{2+}] relations were measured at two initial SLs in skinned right ventricular trabeculae from rats. Fig. 3 illustrates representative force and fluorescence traces obtained at 2.4 μm SL with maximal (pCa 4.0; Fig. 3 *A*) and sub-maximal levels (pCa 6.4; Fig.

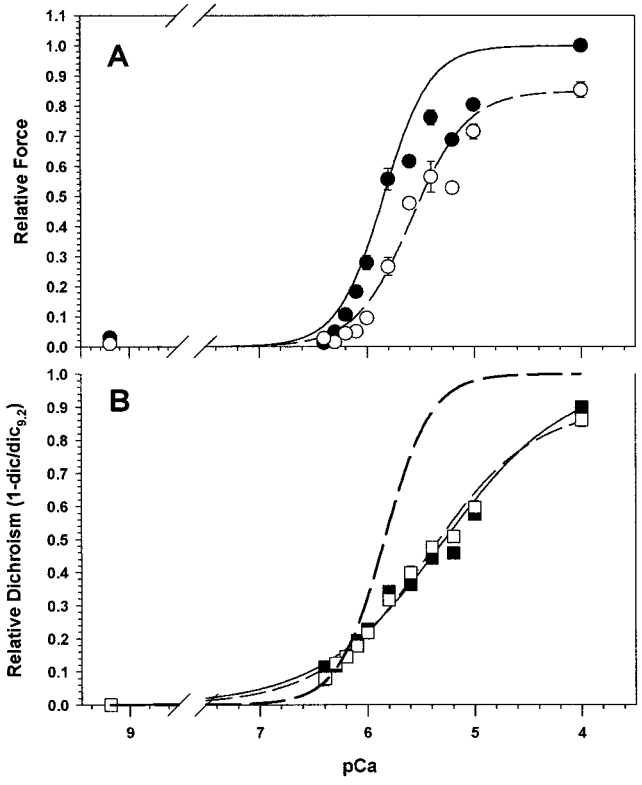

FIGURE 2 Force-$[Ca^{2+}]$ (*A*; ●, ○) and dichroism-$[Ca^{2+}]$ (*B*; ■, □) relations were determined at 2.56 ± 0.01 (●, ■) and 2.15 ± 0.01 (○, □) μm SL. Data were obtained from five fibers (means ± SEM). Force and dichroism-$[Ca^{2+}]$ relations from individual fibers were fit with the Hill equation, and the average values of pCa_{50} and n_H (Table 1) were used to generate the curves through the data points at long (——) and shorter (— — —) SL. In *B*, the fit to the control force-Ca^{2+} data at 2.5 μm SL in *A* are shown for reference (---).

3B) of Ca^{2+} activation. As for Fig. 1, the middle traces indicate the changes in the 84-kHz component of fluorescence ($I_{\|} - I_{\perp}$), whereas the bottom trace is the total fluorescence ($I_{\|} + 2I_{\perp}$). At an initial SL of 2.4 μm the absolute value of dichroism of 5′ATR-labeled cTnC(C84) reconstituted into skinned trabeculae was 0.065 ± 0.0004 at pCa 9.2, increasing to 0.156 ± 0.003 at pCa 4.0 (means ± SEM; $n = 6$ preparations). Decreasing initial SL to 2.0 μm had no effect on dichroism at pCa 9.2 whereas at pCa 4.0 there was a small but significant decrease ($p < 0.05$) in dichroism to 0.146 ± 0.005 (means ± SEM; $n = 6$ preparations). Maximum Ca^{2+}-activated force at 2.4 μm SL was 164.2 ± 24.1 mN/mm^2 (mean ± SEM; $n = 6$ preparations).

The Ca^{2+} dependence of force (Fig. 4 *A*) and dichroism (Fig. 4 *B*) were measured at initials SLs of 2.4 (*filled symbols*) and 2.0 (*open symbols*) μm, as in Fig. 2, *A* and *B*. Force is expressed in Fig. 4 *A* as a fraction of the value at pCa 4.0 and 2.4 μm SL, and dichroism is expressed as a fraction of the value at 2.4 μm SL and pCa 9.2. The Hill fit parameters for the data shown in Fig. 4 are summarized in Table 2. Decreasing SL caused a 0.34 ± 0.09 pCa unit decrease in pCa_{50} of force-$[Ca^{2+}]$ relations and a corresponding decrease of 0.22 ± 0.03 pCa units in dichroism-$[Ca^{2+}]$ relations (mean ± SEM; 6 trabeculae). Maximum force at 2.0 μm SL was 58% ± 4% of the value at 2.4 μm (mean ± SEM; 6 trabeculae), and the decline of relative stiffness at the short SL was not different than for force ($p > 0.05$). Although decreasing SL decreased maximum force to 58%, the corresponding decrease in dichroism at pCa 4.0, although significant ($p < 0.05$), was only 6% of the value at 2.4 μm SL.

To determine whether the effect of SL on force and dichroism in skinned cardiac muscle (Fig. 4, *A* and *B*; Table 1) was dependent on strong cycling cross-bridges, dichroism-$[Ca^{2+}]$ relations were determined when force was inhibited with 1.0 mM Vi in all bathing solutions. Inhibition of force in skinned cardiac muscle with Vi has been shown to decrease the Ca^{2+} sensitivity of dichroism of 5′ATR-labeled cTnC (Martyn et al., 2001) and decrease Ca^{2+} binding to cardiac thin filaments (Hofmann and Fuchs, 1987a; Wang and Fuchs, 1994). At pCa 4.0 with 1.0 mM Vi, force was only 0.05 ± 0.02 of the value for non-inhibited controls (means ± SEM; $n = 5$ trabeculae). The data in Fig. 5 illustrate that inhibition of force with 1.0 mM Vi caused decreased Ca^{2+} sensitivity of dichroism at both 2.4 (*filled symbols*) and 2.0 (*open symbols*) μm SL and a significantly ($p < 0.05$) decreased SL dependence of the pCa_{50} of dichroism-$[Ca^{2+}]$ relations (Fig. 4 *B*). For comparison with control data, the Hill fit curves from Fig. 4 *B* are included in Fig. 5. The Hill equation parameters for the data in Fig. 5 are included in Table 2. Additionally, force inhibition caused dichroism at each SL to decrease ~20% ($p < 0.05$) from control values (Fig. 4 *B*; Table 2). These data indicate that at saturating $[Ca^{2+}]$ a significant portion of the structural changes occurring in cTnC during activation of cardiac muscle results from the effects of cycling, force-producing cross-bridges. Furthermore, when this component of thin filament activation is eliminated by force inhibition there is a loss of the effect of SL on activation.

DISCUSSION

We have compared the effects of SL on force-$[Ca^{2+}]$ relations and the structural responses of skeletal and cardiac TnC to Ca^{2+} binding and the binding of cycling cross-bridges to the thin filament. Although decreasing SL causes a decrease in the Ca^{2+} sensitivity of force in skeletal fibers, there is no corresponding alteration of dichroism-$[Ca^{2+}]$ relations. In contrast, decreasing SL causes the Ca^{2+} sensitivities of both force and structural changes in cTnC to decrease in skinned cardiac muscle. Inhibition of force in skinned cardiac muscle results in perturbation of cTnC structure and greatly reduces the influence of SL on the response of cTnC to Ca^{2+} activation.

TABLE 1 Summary of Hill equation fits to the data shown in Fig. 2

Measurement	SL (μm)	[BDM] (mM)	pCa_{50}	n_H	Maximum Value
Force*	2.5	0	5.84 ± 0.06	2.0 ± 0.15	1.0
Force*	2.1	0	5.58 ± 0.06†	1.66 ± 0.27†	0.85 ± 0.03†
Dichroism§	2.5	0	5.26 ± 0.12	0.68 ± 0.03	0.90 ± 0.05
Dichroism§	2.1	0	5.40 ± 0.06‡	0.84 ± 0.04	0.86 ± 0.06‡
Dichroism§	2.5	30	5.42 ± 0.14	0.77 ± 0.13	0.97 ± 0.04
Dichroism§	2.1	30	5.41 ± 0.14‡	0.70 ± 0.10‡	0.97 ± 0.07‡

Data (means ± SEM) were obtained from five skeletal fibers in the absence of BDM and three fibers for force inhibition with 30 mM BDM.
*Expressed as a fraction of the value obtained at pCa 4.0 and long SL.
†Difference is significant ($p > 0.05$).
‡Difference not significant ($p > 0.05$).
§Dichroism is expressed as ($1 - dic/dic_{9.2}$).

Contributions of dichroism from non-overlap and overlap regions of thin filaments

Incomplete extraction of TnC in skinned skeletal and cardiac preparations and the contributions of populations of probes along the thin filaments should be taken into account when interpreting the results. We have argued for skeletal fibers that the insensitivity of sTnC to cycling cross-bridges could not be completely explained by a preferential distribution of 5′ATR-labeled sTnC in the non-overlap region (I-band) of the sarcomere (Martyn et al., 1999). This same argument applies to this study. If a preferential distribution of 5′ATR-labeled sTnC in the I-band were a factor, at short SL the dichroism signal should have exhibited an increased sensitivity to cross-bridges because relatively more probe would be exposed to the effects of both Ca^{2+} and cross-bridges. Our observation that the dichroism-[Ca^{2+}] relation was unaltered by either SL (Fig. 2 *B*) or force inhibition (Table 1) argues that this is not the case.

These same concerns apply to skinned cardiac muscle because we could not completely extract cTnC, as residual force following extraction was 24% of maximum pre-extraction levels. Also, TnC may be preferentially extracted from the I-band (Yates et al., 1993) resulting in higher occupancy of 5′ATR-labeled cTnC in the non-overlap than overlap zones. During extraction of cardiac trabeculae, SL was typically 2.1 μm, leaving ~0.25 μm of thin filament in the I-band of each half-sarcomere. Thus, the dichroism signal consisted of components from the overlap (D_{ov}) and I-band (D_I) regions of the thin filaments ($D_{total} = D_{ov} + D_I$). Each component would be proportional to both the fractional length of the thin filament (frac$L = L_{zone}/1.05$) and the relative occupancy of the region with 5′ATR. If we assume 100% extraction/reconstitution with 5′ATR-labeled cTnC in the non-overlap thin filament and 75% in the overlap region, at 2.4 μm D_I is 47% (0.4/(0.4 + (0.6)(0.75)) and D_{ov} is 53% of D_{total}. As we proposed for skeletal fibers

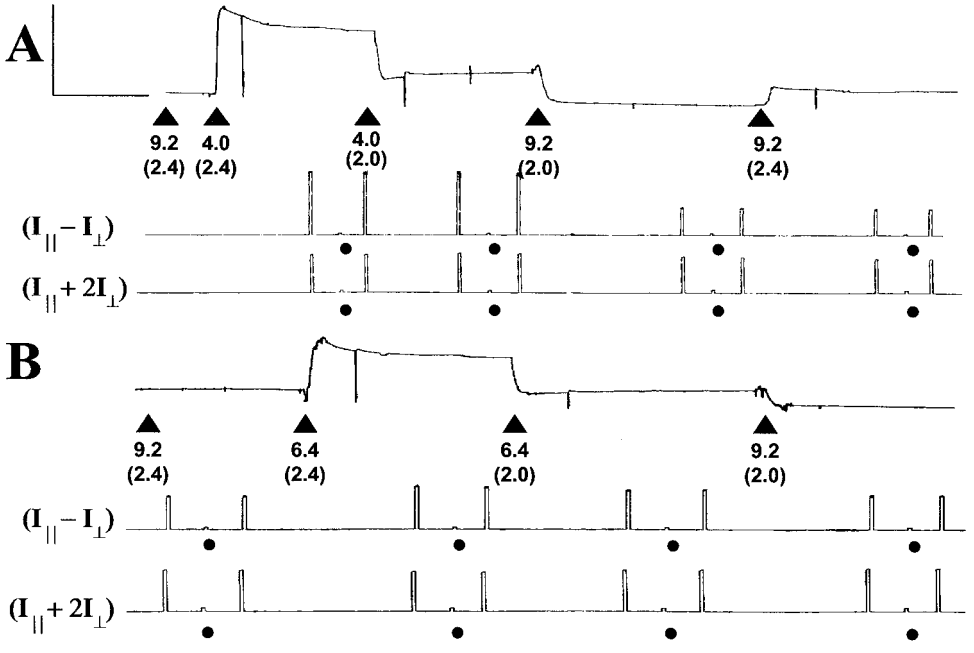

FIGURE 3 Representative traces of force (*top*), the demodulated 84-kHz sinusoidal variation of the fluorescence signal ($I_{\parallel} - I_{\perp}$; *middle trace*) and total fluorescence ($I_{\parallel} + 2I_{\perp}$; *bottom trace*) are shown for maximal Ca^{2+} activation at pCa 4.0 (*A*) and at sub-maximal activation (*B*; pCa 6.4) for a skinned right ventricular trabeculae that had been reconstituted with 5′ATR labeled cTnC(C84). For each condition, force and fluorescence were measured at either 2.4 or 2.0 μm SL. Changes in solution pCa are indicated below the force traces by arrows, with the corresponding SLs indicated in parentheses below each pCa. The filled dots below the fluorescence traces indicate measurements made with the trabeculae out of the field of view. The vertical calibration bar indicates 200 mN mm^{-2} and the horizontal bar 1 min.

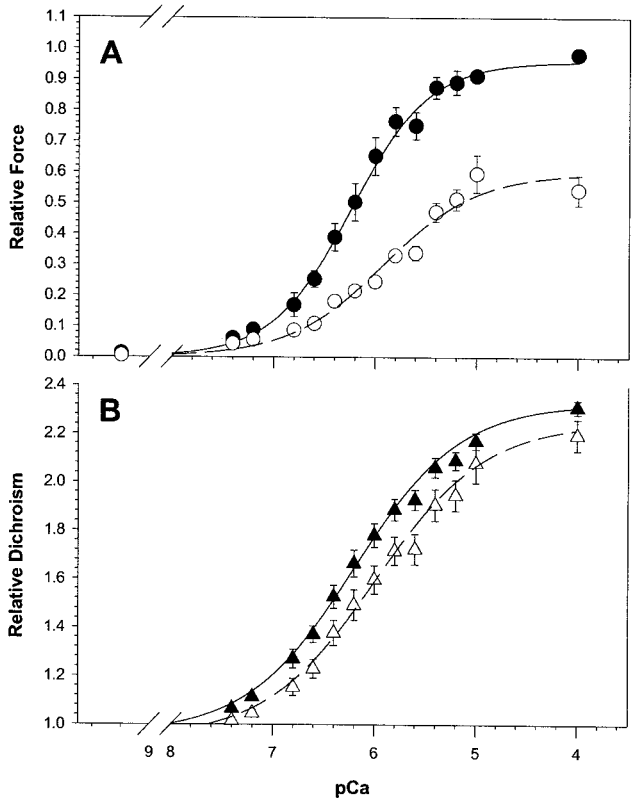

FIGURE 4 Force-[Ca^{2+}] (*A*; ●, ○) and dichroism-[Ca^{2+}] (*B*; ▲, △) relations were determined at 2.4 (●, ■) and 2.0 (○, □) μm initial SL. Data were obtained from six skinned trabeculae (means ± SEM). Force and dichroism-[Ca^{2+}] relations from individual trabeculae were fit with the Hill equation, and the average values of pCa_{50} and n_H (Table 2) were used to generate the curves through the data points at long (——) and shorter (— — —) SL.

(Martyn et al., 1999), in cardiac muscle the dichroism signal would be made of the response of probes to Ca^{2+} alone in the I-band and to a combination of Ca^{2+} and cross-bridges in the overlap zone (Martyn et al., 2001). The decrease in dichroism at pCa 4.0 resulting from force inhibition (Fig. 5 *B* and Table 2) enables us to estimate that the relative contribution of cycling cross-bridge attachment in the overlap zone is ~20% of D_{total}. Given these assumptions, D_I (due to Ca^{2+} binding to cTnC alone) is 38% (0.4/(0.4 + (0.6)(0.75)) × 80) and D_{ov} (due to Ca^{2+} binding and cross-bridges) is 42% of D_{total}. Thus, in the overlap region cross-bridge attachment contributes 32% to D_{ov} (0.20/(0.20 + 0.42), compared with the uncorrected value of 20% (Fig. 5; Table 2).

These estimates further imply that the SL-dependent shift of dichroism-[Ca^{2+}] relations in Fig. 4 *B* may represent a lower estimation of the effects of SL. This is because at the long SL the dichroism signal consisted of portions sensitive only to Ca^{2+} in the I-band and to both Ca^{2+} and cross-bridges in the overlap zone, whereas at the short SL virtually all of the thin filament would have been in the overlap zone. Changes in cTnC structure in response to Ca^{2+} alone in the I-band would be expected to have a lower sensitivity to Ca^{2+}, as we demonstrated when cycling cross-bridge binding is inhibited at either SL (Fig. 5 *B*). Therefore, at the longer SL the measured Ca^{2+} sensitivity of dichroism probably represented a lower estimate, whereas at the shorter SL the measured Ca^{2+} sensitivity was more representative of the overlap region alone. However, for simplicity we have not taken into account the potential spread of activation from strong cross-bridge binding in the overlap zone into the I-band that has been demonstrated in Ca^{2+}- and rigor cross-bridge-activated skeletal thin filaments (Cantino et al., 1993). If cycling cross-bridges induced a spread of activation into the I-band at longer SL in cardiac muscle, the observed Ca^{2+} sensitivity of dichroism would be closer to that of the overlap region alone. According to these simple corrections, non-homogeneous extraction/reconstitution of skinned cardiac muscle with 5′ATR-labeled cTnC(C84) and distribution of probes in the overlap and I-band thin filament regions may lead to an underestimation of both the effects of SL on the Ca^{2+} sensitivity of dichroism (Fig. 4 *B*; Table 2) and the contributions of cross-bridge to the structural changes in cTnC (Fig. 5 *B*; Table 2).

Effects of SL on force- and dichroism-[Ca^{2+}] relations in skinned fast skeletal fibers

The data indicate that the Ca^{2+} sensitivity of force in skinned psoas fibers from rabbit increases with increasing SL, whereas Ca^{2+}-induced structural changes in the N-terminus of 5′ATR-labeled sTnC are unaffected by altered SL (Fig. 2 *B*). This is consistent with and extends the observations that radioactive Ca^{2+} binding to skeletal muscle thin filaments (Fuchs and Wang, 1991) and indirect measurements of the affinity of sTnC for Ca^{2+} in skinned fibers (Patel et al., 1997) are insensitive to SL. Thus, changes in SL and accompanying changes in myo-filament lattice spacing (LS) do not alter myoplasmic [Ca^{2+}] and Ca^{2+} bound to the thin filament, contrary to our previous suggestion that they might (Martyn and Gordon, 1988).

Although dichroism of 5′ATR labeled sTnC is sensitive to Ca^{2+}-binding to the N-terminal Ca^{2+}-specific site of sTnC (Martyn et al., 1999), it is quite insensitive to partial force inhibition with BDM (Table 1). This supports the observations that nearly complete inhibition of maximum Ca^{2+}-sensitive force with the phosphate analog aluminofluoride or sodium vanadate (Vi) did not alter either sTnC structure (Martyn et al., 1999) or Ca^{2+} bound to skeletal thin filaments (Fuchs and Wang, 1991; Wang and Fuchs, 1994). However, whereas Ca^{2+} binding to sTnC appears to be insensitive to either changes in SL (Fig. 2 *B*) or partial inhibition of active force (Table 1), sTnC structure has been shown to be sensitive to rigor cross-bridges. Rigor cross-bridge binding increases Ca^{2+} binding to skeletal thin filaments (Fuchs, 1977, 1978) and alters sTnC structure in

TABLE 2 Summary of Hill equation fits to the data shown in Figs. 4 and 5

Measurement	SL (μm)	[Vi] (mM)	pCa_{50}	n_H	Maximum value
Force*	2.4	0	6.24 ± 0.03	1.12 ± 0.09	0.98 ± 0.03
Force*	2.0	0	5.92 ± 0.08†	0.93 ± 0.06‡	0.58 ± 0.04†
Dichroism§	2.4	0	6.20 ± 0.04	0.83 ± 0.06	2.33 ± 0.03
Dichroism§	2.0	0	5.99 ± 0.06†	0.80 ± 0.09‡	2.25 ± 0.08†
Dichroism§	2.4	1.0	5.58 ± 0.15	0.98 ± 0.08	2.08 ± 0.03
Dichroism§	2.0	1.0	5.52 ± 0.03‡	1.06 ± 0.06‡	1.97 ± 0.06‡

Data (means ± SEM) were obtained from six cardiac trabeculae without Vi and five trabeculae for force inhibition by 1.0 mM
*Expressed as a fraction of the value obtained at pCa 4.0 and long SL.
†Difference is significant ($p > 0.05$).
‡Difference not significant ($p > 0.05$).
§Dichroism is expressed as a fraction of the value in pCa 9.2 (relaxing) solution.

skinned psoas fibers reconstituted with 5′ATR-labeled sTnC (Martyn et al., 1999). Therefore, the lack of an effect of force inhibition on sTnC structure could result from either an uncoupling sTnC structure from the state of Tm on the thin filaments or from low fractional attachment of strong, cycling cross-bridges during force generation, as we previously suggested (Martyn et al., 1999). However, observations in barnacle (Gordon and Ridgway, 1987) and fast skeletal muscle fibers (Vandenboom et al., 1998) suggest that a small effect of cross-bridges on TnC Ca^{2+} affinity in skeletal fibers cannot be ruled out. In both studies, measurements were made of the effects of altered SL on free myoplasmic [Ca^{2+}], which may be a more sensitive detector of small changes in Ca^{2+} bound to the thin filaments than changes in TnC structure using fluorescent probes.

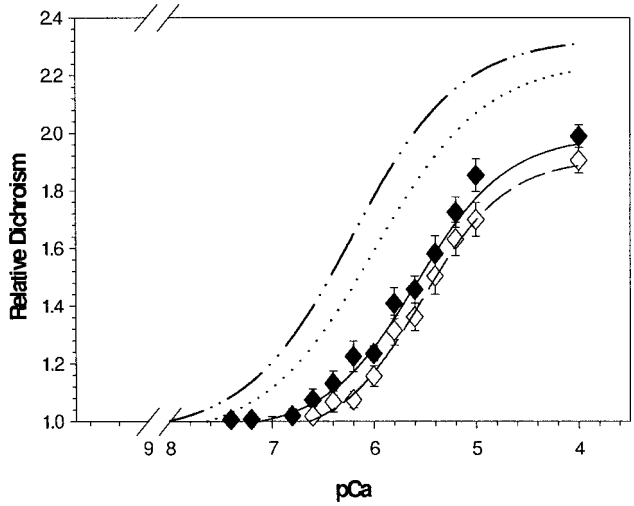

FIGURE 5 Dichroism-[Ca^{2+}] relations obtained at SL = 2.4 (♦; ——) and 2.0 (◇; — — —) μm initial SL are shown when force was inhibited with 1.0 mM Vi in all solutions. Data (means ± SEM) were obtained from five skinned trabeculae. The curves were generated from the mean values of pCa_{50} and n_H obtained from the individual preparations. Control dichroism-[Ca^{2+}] relations at 2.4 (— · · —) and 2.0 (· · ·) μm initial SL from Fig. 4 *B* are included for comparison.

The data suggest an important contribution of strong cross-bridge binding to thin filament activation in skinned fast skeletal fibers. For example, the slope of force-Ca^{2+} relations is much steeper than for the Ca^{2+} dependence of dichroism (Fig. 2; Table 1) (see also Martyn et al., 1999). This implies that whereas activation of isometric force is cooperative, Ca^{2+} binding to sTnC shows little cooperativity in skinned skeletal fibers. Activation of thin filaments by cycling cross-bridges is probably the source of the apparent cooperativity of force-Ca^{2+} relations because strong cross-bridge binding is necessary for efficient activation of skeletal muscle thin filaments (Geeves and Lehrer, 1994; Lehrer, 1994; Lehrer and Geeves, 1998). Also, the decrease in both the slope and pCa_{50} of force-Ca^{2+} relations following force and stiffness inhibition with BDM could be explained by a loss of cooperative thin filament activation by cycling cross-bridges, as observed by others (Horiuti et al., 1988; Higuchi and Takemori, 1989).

A role for cycling cross-bridge attachment in determining the SL dependence of skeletal thin filament activation is supported by the observation that the probability of cycling cross-bridge attachment decreases at short SL, probably because of the accompanying increase in LS (McDonald et al., 1997). This suggests that increasing LS at shorter SL decreases thin filament activation by cross-bridges and thereby the Ca^{2+} sensitivity of force. In support of this idea, phosphorylation of myosin regulatory light chains disorders cross-bridges on the surface of skeletal thick filaments, increases the kinetics of force development, and reduces the effects of SL on force (Sweeney et al., 1994; Levine et al., 1996, 1998; Yang et al., 1998). Thus, interventions that increase the probability of strong cross-bridge binding compensate for decreased cross-bridge binding at short SL and greater LS. However, the data in Fig. 2 *A* and work by others suggest that maximum Ca^{2+}-activated force changes little with changes in SL from 2.4 to 2.0 μm, whereas the Ca^{2+} sensitivity of force decreases over the entire range (Stephenson and Wendt, 1984; Martyn and Gordon, 1988). This could be explained if at saturating [Ca^{2+}] thin filament activation by the combination of Ca^{2+} binding to sTnC and

cycling cross-bridges were sufficient to fully expose the myosin binding surface on skeletal thin filaments. In this case, altered SL or LS could have less effect on the probability of strong cross-bridge attachment to the thin filaments than at lower [Ca^{2+}], at which thin filament activation is less than maximal.

In summary, for skinned skeletal fibers our data support and extend the idea that the SL dependence of force-Ca^{2+} relations does not result from alteration in either the amount of Ca^{2+} bound to sTnC or changes in the apparent Ca^{2+} affinity of sTnC. In stark contrast to skinned fast skeletal fibers, both the Ca^{2+} sensitvity of force and dichroism are SL dependent in cardiac muscle, and cycling cross-bridge binding both enhances Ca^{2+} binding to cTnC and contributes to the mechanism of SL regulation of thin filament activation.

SL dependence of force-[Ca^{2+}] relations and Ca^{2+} binding to cTnC in skinned myocardium

In skinned myocardium, decreasing SL causes an increase in the pCa_{50} values of both force and dichroism-[Ca^{2+}] relations, with the effect being slightly larger on force (Fig. 4, *A* and *B*; Table 2). This result is generally consistent with studies of the effects of SL on force-[Ca^{2+}] relations (Kentish et al., 1986) and Ca^{2+} binding to cardiac thin filaments (Hofmann and Fuchs, 1987b; Wang and Fuchs, 1994). We have previously shown that the 5′ATR label attached to Cys 84, near the hydrophobic binding pocket of cTnC, is sensitive to both Ca^{2+} binding to site II and strong, cycling cross-bridge attachment (Martyn et al., 2001). Thus, the decrease of pCa_{50} with dichroism at short SL (Fig. 4 *A*) could result from decreased force and strong cross-bridge binding and decreased Ca^{2+} binding to cTnC. As for skeletal muscle, decreased Ca^{2+} sensitivity of force at short SL appears to result from the accompanying increase in LS (McDonald and Moss, 1995; Wang and Fuchs, 1995; Fuchs and Wang, 1996).

Several studies indicate that the effects of SL and LS on force-[Ca^{2+}] relations in cardiac muscle are mediated through altered cross-bridge binding. For example, interventions that strengthen cross-bridge attachment (Fukuda et al., 2000) or activate cardiac thin filaments with exogenous strong binding myosin (Fitzsimons and Moss, 1998) decrease the SL dependence of force. Also, inhibition of active force eliminates the effects of SL on dichroism (Fig. 5; Table 2) and Ca^{2+} binding (Hofmann and Fuchs, 1987b; Wang and Fuchs, 1994). However, to suggest that the decreased pCa_{50} of dichroism at short SL (Fig. 4 *B*) results from decreased cross-bridge binding alone may be too simplistic. This is because although maximum Ca^{2+}-activated (pCa 4.0) force and stiffness decreased ~40% when SL was decreased from 2.4 to 2.0 μm (Fig. 4 *A*), there was only a small (6%) corresponding decrease in dichroism (Fig. 4 *B*). This result would seems to imply that at saturating [Ca^{2+}] (pCa 4.0) the structure of cTnC is maximally perturbed and relatively insensitive to strong cross-bridge binding. However, Ca^{2+} binding to isolated cTnC by itself does not fully perturb the N-terminal structure of cTnC (Sia et al., 1997; Spyracopoulos et al., 1997, 1998; Li et al., 1999), unlike sTnC (Herzberg and James, 1985, 1988). Furthermore, the structure of cTnC appears to be an equilibrium distribution between several states, even at saturating [Ca^{2+}] (Dong et al., 1996; Hazard et al., 1998). These observations indicate that at pCa 4.0 Ca^{2+} binding alone to site II on cTnC may not be enough for complete perturbation of cTnC structure.

The comparatively small decrease in dichroism at short SL and pCa 4.0 (Fig. 4 *B*; Table 2), compared with long SL, contrasts with the change in dichroism resulting from force inhibition with 1.0 mM Vi (Fig. 5; Table 2). At pCa 4.0, inhibition of force to 6% of maximum caused a 20% decrease in dichroism, strongly implying that a significant fraction of activation-induced changes in cTnC structure at saturating [Ca^{2+}] resulted from the attachment of cycling cross-bridges. If the effects of Ca^{2+} and cross-bridges from the overlap zone alone are considered, this value increases to 32% (see above). Therefore, in the simplest case, a 40% decrease in active force and cross-bridge attachment at 2.0 μm SL (Fig. 4 *A*) should cause a larger (13%) decrease in dichroism than was observed (Fig. 4 *B*; Table). The smaller than expected change in cTnC structure at saturating [Ca^{2+}] could indicate that at force levels above 50% of maximum the fraction of attached cross-bridges is high enough to contribute maximally to thin filament activation at pCa 4.0. Alternatively, to the extent that the structural changes in 5′ATR-labeled cTnC are determined by both Ca^{2+} binding to site II and strong cross-bridge attachment (Martyn et al., 2001), decreased maximal force with less corresponding change in cTnC structure could indicate an internal load at short SL. Internal loads would be expected to diminish force but not necessarily alter the structure of cTnC. However, the observation that decreasing SL caused both maximum force and stiffness to decrease to the same extent argues that a decrease in the degree of strong cross-bridge binding was the primary cause of force decline.

Our observations that inhibition of force by 1.0 mM Vi caused decreased pCa_{50} and maximum magnitude of Ca^{2+}-induced changes in 5′ATR-labeled cTnC structure (Fig. 5; Table 2) is further evidence that cycling cross-bridges contribute to thin filament activation in cardiac muscle. This is consistent with decreased Ca^{2+} binding to cardiac thin filaments when force is inhibited with Vi (Hofmann and Fuchs, 1987b; Wang and Fuchs, 1994). Likewise, the decreased effect of SL on dichroism-[Ca^{2+}] relations following force inhibition (Fig. 5; Table 2) is similar to observations of cardiac thin filament Ca^{2+} binding (Hofmann and Fuchs, 1987b; Wang and Fuchs, 1994). The diminished effect of SL on the Ca^{2+} sensitivity of structural changes in cTnC when force is inhibited strongly supports the idea that the

effect of SL or LS on force-$[Ca^{2+}]$ relations in cardiac muscle is the result of altered strong cross-bridge attachment.

CONCLUSION

In skinned fast skeletal fibers maximum force is relatively unaffected by changes in SL over the range investigated, although the Ca^{2+} sensitivity of force increased with increasing SL. This implies that at saturating $[Ca^{2+}]$ thin filaments may be fully activated so that changes in the probability of strong cross-bridge binding from either altered SL or LS has less effect on thin filament myosin binding site availability. Thus, in skinned skeletal fibers, force exhibits a strong SL dependence only if the thin filaments are sub-maximally activated and Tm is not fully in the open state (McKillop and Geeves, 1993; Geeves and Conibear, 1995). By contrast, maximum force in cardiac muscle is more SL dependent, and Ca^{2+} binding to cTnC is enhanced by cycling cross-bridge binding, even at saturating $[Ca^{2+}]$. We further observe that force inhibition results in decreased thin filament activation at saturating $[Ca^{2+}]$ (as evidenced by structural changes in cTnC) and diminished SL dependence of thin filament activation. Our results suggest the possibility that in cardiac muscle the steep SL dependence of force (even at saturating $[Ca^{2+}]$) results from an inability to achieve complete activation of cardiac thin filaments, particularly at shorter SL.

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