Ca^{2+}-dependence of structural changes in troponin-C in demembranated fibers of rabbit psoas muscle

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ABSTRACT The Ca^{2+}-dependence of structural changes in troponin-C (TnC) has been detected by monitoring the fluorescence from TnC labeled at Methionine-25, in the NH_2-terminal domain, with danzylaziridine (TnC-DANZ) and then exchanged for endogenous TnC in glycerinated single fibers. The fluorescence-pCa relation obtained from fibers stretched to a sarcomere length > 4.0 μm evidenced two transitions: a small one, attributable to the binding of Ca^{2+} to the high affinity, Ca^{2+}-Mg^{2+}-binding sites of TnC; and a large one, attributable to the binding of Ca^{2+} to the low affinity, Ca^{2+}-specific binding sites of TnC. In the fluorescence-pCa relation determined with fibers set to a sarcomere length of 2.4 μm, hence obtained in the presence of cycling cross-bridges, the large transition had the same Ca^{2+}-dependence as did the development of tension. These results indicate that the NH_2-terminal globular domain of TnC is modified by the binding of Ca^{2+} to sites located in both globular domains and that the structural changes in TnC resulting from the binding of Ca^{2+} to the low-affinity sites, but not to the high-affinity sites, are directly associated with the triggering of contraction.

INTRODUCTION

Tension development in a muscle cell results from the interaction of myosin, which is polymerized into thick filaments, with actin, which is polymerized into thin filaments (for review see Cooke, 1986). The regulation of this interaction occurs in a skeletal muscle cell through the concerted action of tropomyosin (Tm) and troponin (Tn), both of which are constituents of thin filaments (for review see Leavis and Gergely, 1984). Tension development is triggered by the binding of Ca^{2+} to troponin-C (TnC), one of the three subunits of Tn and a homologue of the cellular Ca^{2+}-binding protein calmodulin. This report presents an investigation of the Ca^{2+}-dependence of structural changes in TnC and of the relation of these structural changes to tension development in single fibers from rabbit psoas muscle.

X-ray diffraction patterns of crystalline TnC, obtained from skeletal muscle and grown at pH 5, reveal an elongated, dumbbell shape for TnC: the NH_2-terminal and the COOH-terminal globular domains, each comprising ~60 residues, are spatially separated by a nine-turn α-helix (Herzberg and James, 1985; Sundaralingam et al., 1985). Both globular domains contain two Ca^{2+}-binding sites. The two sites in the COOH-terminal domain evidence not only a high affinity for Ca^{2+}, but also an appreciable affinity for Mg^{2+} (Potter and Gergely, 1975; Leavis et al., 1978). These sites are called the Ca^{2+}-Mg^{2+}-binding, or high-affinity, sites. The association of TnC with the regulatory complex requires that either Ca^{2+} or Mg^{2+} occupy these two sites (Cox et al., 1981; Zot and Potter, 1982). Being essentially specific in their binding of Ca^{2+}, the two sites in the NH_2-terminal domain are called the Ca^{2+}-specific, or low-affinity sites. Biochemical evidence (Potter and Gergely, 1975; Potter et al., 1976) and kinetic modeling (Johnson et al., 1979; Robertson et al., 1981) point to a regulatory role for the low-affinity sites in triggering tension development.

The results of several recent experiments indicate that TnC in solution near pH 7 may exist in a compact shape and that the functional roles of the two classes of Ca^{2+}-binding site may be indistinct. For example, x-ray scattering (Heidorn and Trewhella, 1988) and fluorescence energy transfer (Wang and Cheung, 1986; Wang et al., 1987; Cheung et al., 1989) measurements of TnC

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Abbreviations used in this paper: ATP, adenosine 5'-triphosphate; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CP, phosphocreatine; CPK, creatine phosphokinase; DANZ, dansylaziridine, aziridine-1-((5-)dimethylamino)-1-naphthalenyl)sulfonyl); DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; 1,5-IAEDANS, 5-(2-((iodoacetyl) amino)ethyl)aminonaphthalene-1-sulfonic acid; IANBD, 4-(*N*-(iodoacetoxy)ethyl-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3- diazole; MLCK, myosin light chain kinase; MOPS, 3-[*N*-morpholino]propane-sulfonic acid; Tm, tropomyosin; Tn, troponin; TnC, Ca^{2+}-binding subunit of Tn; TnI, inhibitory subunit of Tn; TnT, Tm-binding subunit of Tn; TRIS, Tris[hydroxymethyl]amino-methane.

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in solution near pH 7 suggest the presence of a bend in the central α-helix that lessens the separation between the two globular domains. Such a bend would explain the observation that both globular domains of TnC interact with a short segment, spanning residues 96–116, of troponin-I (TnI), the inhibitory subunit of Tn (Leszyk et al., 1990). Fragments of TnI containing these residues bind to actin and inhibit the actomyosin ATPase (Syska et al., 1976; Nozaki et al., 1980; Talbot and Hodges, 1981; Grand et al., 1982). The interaction between residues 96–116 of TnI and the two globular domains of TnC raises the possibility that Ca^{2+}-induced structural changes in both globular domains play a role in triggering contraction.

We report a test of two hypotheses that address the emerging concept of an interaction between the two globular domains of TnC. The first hypothesis holds that the binding of Ca^{2+} to each class of Ca^{2+}-binding site affects the structure of both globular domains of TnC (cf Grabarek et al., 1986, and Wang and Gergely, 1986). The second hypothesis, which stems from the first, holds that the binding of Ca^{2+} to both classes of Ca^{2+}-binding site contributes to the triggering of contraction (cf Ebashi et al., 1980). We tested the two hypotheses by monitoring the fluorescence from TnC labeled at Met-25 with dansylaziridine (TnC-DANZ) and exchanged for endogenous TnC in demembranated single fibers. The quantum yield of the fluorophore is highly sensitive to its environment; hence, a structural change in TnC that alters the hydrophobicity of the fluorophore's environment will lead to a change in the fluorescence.

METHODS

TnC purification and labeling

TnC was purified from rabbit fast-twitch back and leg muscles according to the method of Greaser and Gergely (1971). Met-25 in TnC was labeled with dansylaziridine (aziridine-1-((5-(dimethylamino)-1-naphthalenyl)sulfonyl), D-151, Molecular Probes, Eugene, OR) by a procedure similar to that of Grabarek et al. (1983), who modified the method of Johnson et al. (1978). TnC (2 mg/ml) was incubated for 2 h in a solution that contained 0.1 M KCl, 20 mM Tris, pH 8.0, 1 mM EDTA, and ~10 mM DTNB. Following the 2 h incubation with DTNB to block the reactive SH-group of Cys-98, the sample was dialyzed six times during 48 h against a solution that contained 90 mM KCl, 10 mM MOPS, pH 7.0, and 0.1 mM $CaCl_2$. After dialysis, the sample was incubated at 20–24°C in a freshly prepared solution of DANZ in *N,N*-dimethylformamide either for 4 h in the presence of 1.2 mol DANZ per mol TnC or for 18 h in the presence of 1.6 mol DANZ per mole TnC. Covalently labeled TnC (TnC-DANZ) was separated from free DANZ and noncovalently bound DANZ by column chromatography on Sephadex G-25. Three columns were used: the first was equilibrated with 90 mM KCl, 10 mM MOPS, pH 7.0, and 0.1 mM $CaCl_2$; the second was equilibrated with 8 M Urea, 0.3 M KCl, 50 mM Tris, pH 7.2, and 3 mM EDTA; the third was equilibrated with 0.1 M KCl, 10 mM MOPS, pH 7.0, and 0.1 mM $MgCl_2$. Between the second and third chromatographic steps, the sample was incubated with 10 mM dithiothreitol for 30 min at 22–24°C to unblock Cys-98.

The extent of labeling was estimated by using the absorption coefficient for the probe at 350 nm (3,980 $M^{-1}cm^{-1}$, Johnson et al., 1978) and by measuring the protein concentration by the method of Bradford (1976), with bovine serum albumin as the protein standard. For the 4 h labeling, the extent of labeling was 0.25 mole Danz per mole TnC; for the 18 h labeling, the extent was 0.94. Fig. 1 displays the fluorescence-pCa relation obtained with samples of isolated TnC from the two preparations. Note that, in agreement with the observation of Johnson et al. (1978), the sole appreciable effect of the increase in the extent of labeling is a decrease in the relative magnitude of the Ca^{2+}-induced change in fluorescence. This decrease results from an increase in the basal fluorescence measured at pCa 8.5.

Experimental apparatus

The 200-μL chamber, constructed with plexiglass sandwiched between a glass slide and a glass cover slip, had three entry ports. Sandblasted, stainless steel rods were passed through two of the ports to allow suspension of the fiber between a post fixed in space and a silicon strain-gauge (model AE801, Aksjeselskapet Micro-Elektronikk, Norway). Solutions were injected via the third port, and a vacuum line for removal of solution was placed adjacent to the transducer's port. The chamber was mounted on the stage of a Zeiss standard microscope equipped with a thermoelectric device to control the chamber's temperature and with the components necessary for quantitative epi-fluorescence microscopy. The optical components included a 50 W/DC mercury vapor lamp (HBO 50W/3, OSRAM GmbH, FRG), a 12% neutral density filter, an epi-fluorescence filter set (model 487702, Zeiss, Germany; includes a 365-nm band-pass filter, a 395-nm dichromatic beam splitter, and a 420-nm long-pass filter), and a photomultiplier tube (R928HA, Hamamatsu Photonics KK, Japan). Three objectives were used during the course of the investigation: a Zeiss 10× (N.A. 0.30) Neufluar objective, a Zeiss 40× (N.A. 0.85) Achromat objective, and a Zeiss 40× (0.75) water-immersion objective. The photomultiplier tube was operated within the range of its linear response. The fluorescence from the dye, dissolved in ethanol, was linearly proportional to the concentration of dye from 1.0 to 100 μM, which encompasses the concentration of TnC-DANZ within the fibers.

Demembranated fiber preparation

Bundles of rabbit psoas muscle were glycerinated for 18–24 h at 4°C in a solution that contained 100 mM KCl, 9 mM $MgCl_2$, 4 mM Na_2ATP, 5 mM K_2EGTA, 10 mM MOPS, pH 7, and 50% *v/v* glycerol. Subsequently, the bundles were stored in a similar solution at −20°C for up to 6 mo. Single-fiber segments were isolated from a bundle and mounted between the two hooks in the chamber. To ensure the complete disruption of sarcoplasmic reticulum, we treated fibers for 30 s with relaxing solution containing 0.5 wt% CHAPS.

TnC exchange

Endogenous TnC was removed from a demembranated skeletal fiber by immersing the fiber in a divalent cation-free, low ionic strength solution [20 mM tris (pH 7.8 at 10 or 20°C), 5 mM K_2EDTA; Cox et al., 1981] for 5 min at either 10°C or 20°C. For a fiber set to a sarcomere length of 2.6–2.4 μm, extraction at 20°C typically led to the removal of ~60% of the TnC, as judged from the reduction of Ca^{2+}-activated tension relative to pre-extraction values and from the change in TnC content quantitated with gel electrophoresis (Yates, Coby, Luo, and Gordon, manuscript in preparation). Extraction at 10°C typically led to

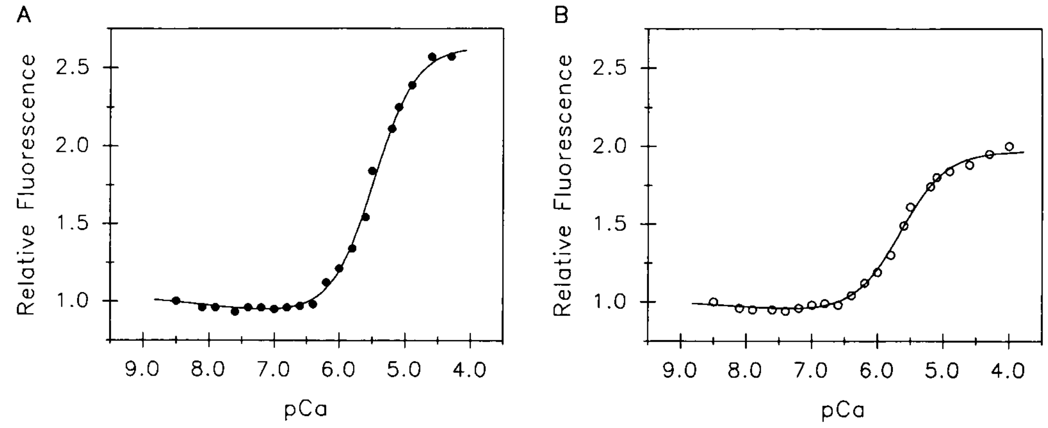

FIGURE 1 Fluorescence-pCa relations for isolated TnC-DANZ. (*A*) Minimally labeled TnC (89g2b). Fitted curve has $\mathrm{pCa}_{1/2}$ (2) = 5.47 and n_{Hill} (2) = 1.4. (*B*) Extensively labeled TnC (89f13*a*). Fitted curve has $\mathrm{pCa}_{1/2}$ (2) = 5.63 and n_{Hill} (2) = 1.3. The ordinate is the fluorescence relative to that measured at pCa 8.5 with the indicated species of TnC-DANZ. Both titrations were performed in the presence of 130 mM Kpropionate, 50 mM MOPS, and 1 mM K_2EGTA. Ionic strength, 0.135 M; pH 7.0; 20°C. Following each addition of $CaCl_2$, KOH was added to maintain pH 7.0. Less than 2% increase in the volume of the sample occurred because of these additions. The fluorescence measurements were made with a SLM-AMINCO Fluoro-Colorimeter II, which was equipped with a 300–400 nm band-pass filter (UG-1, Schott Optical Glass, Duryea, Pennsylvania) for the exciting light and a 418 nm long-pass filter (KV-418, Schott Optical Glass, Duryea, Pennsylvania) for the emitted light. The photomultiplier was oriented at a 90° angle with respect to the exciting light.

the removal of ~30% of the TnC, as judged from the reduction of Ca^{2+}-activated tension relative to pre-extraction values. An extracted fiber was soaked for 30 min in a relaxing solution that contained 10 μM TnC-DANZ and then was rinsed with three exchanges of relaxing solution.

The tension-pCa relation determined from fibers incorporated with TnC-DANZ, when compared to that determined from control, unextracted fibers, evidenced a decrement of 6% ($p < 0.025$) in the peak isometric tension, a decrement of 0.16 pCa unit ($p < 0.005$) in the $\mathrm{pCa}_{1/2}$, and a decrement of 0.23 ($p < 0.025$) in the Hill coefficient. No statistically significant difference, however, was observed when the tension-pCa relation from fibers incorporated with TnC-DANZ was compared to that from fibers incorporated with unlabeled TnC. We conclude, as did Zot et al. (1986), that extracted fibers regulated by TnC-DANZ do not appreciably differ in their tension-pCa relations from extracted fibers regulated by unlabeled TnC.

Solutions

For each solution, the equilibrium concentration of ligands and ions was found by solving the multiple binding equilibria of the constituents with published affinity constants (Martell and Smith, 1974, 1982; O'Sullivan and Smithers, 1979; Ellis and Morrison, 1982) corrected for the experimental temperature, pH, and ionic strength (Phillips et al., 1963, 1966; Martell and Smith, 1974, 1982; Khoo et al., 1977; O'Sullivan and Smithers, 1979; Ellis and Morrison, 1982). Solutions are described in the following list. Propionate was the major anion.

(*a*) 1 mM Mg^{2+} Rigor: $10^{-9.2}$ to $10^{-4.0}$ M free Ca^{2+}, 15 mM EGTA, 1 mM free Mg^{2+}, 0.13 M K^+, 0.04 M to 0.12 M total MOPS to bring the ionic strength to 0.2 M (pH 7.0 at 10°C).

(*b*) 0.05 mM Mg^{2+} Rigor: $10^{-9.2}$ to $10^{-4.0}$ M free Ca^{2+}, 15 mM EGTA, 3 mM EDTA, 0.05 mM free Mg^{2+}, 0.13 M K^+, 0.15 M to 0.23 M total MOPS to bring the ionic strength to 0.2 M (pH 7.0 at 10°C).

(*c*) Standard MgATP-containing solution: $10^{-9.2}$ to $10^{-4.0}$ M free Ca^{2+}, 10 mM EGTA, 1 mM free Mg^{2+}, 3 mM MgATP, 15 mM CP, 20 units/ml CPK, 0.13 M K^+ and Na^+, 0.14 M to 0.20 M total MOPS to bring the ionic strength to 0.2 M (pH 7.0 at 10°C).

(*d*) Standard MgATP-containing solution with 4 wt% Dextran T-500: same as in (*c*), with the addition of 4 wt% Dextran T-500. Vigorous shaking was required. The addition did not alter the pH.

Equilibrium measurements

Fig. 2 provides an example of tension and fluorescence measurements recorded simultaneously during titration of a fiber. The fluorescence measurements were corrected for background fluorescence, which was measured from each experimental solution in the absence of a fiber. The presence of a fiber during the determination of background fluorescences did not appreciably affect the determination. Corrected fluorescence measurements were normalized to the basal fiber fluorescence, measured in the absence of added Ca^{2+}. The basal fiber fluorescence, before each normalization calculation, was corrected for the extent of photobleaching that had occurred between the time of measurement of basal and Ca^{2+}-elicited fluorescences. The relation between basal fluorescence and duration of UV light exposure was determined experimentally.

Corrected, normalized fluorescence measurements from a full titration were fitted by nonlinear regression with two forms of the Hill equation (1910): one that allows for the presence of one transition in the determined relation, and one that allows for the presence of two transitions. In the following equation, the former form has *i* equal to 1, and the latter form has *i* equal to 2:

$$Y = A_0 + \sum_{j=1}^{i} \frac{A_j}{1 + 10^{n_j(\mathrm{pCa} - \mathrm{pCa}_{1/2j})}},$$

where Y is the fluorescence, pCa equals $-\log_{10}([\text{free } Ca^{2+}])$, A_0 is the fluorescence in the absence of Ca^{2+}, A_j is the change in fluorescence of the j^{th} transition, $\mathrm{pCa}_{1/2j}$ is the pCa at the midpoint of the j^{th} transition, and n_j is the Hill term of the j^{th} transition. Tension measurements from

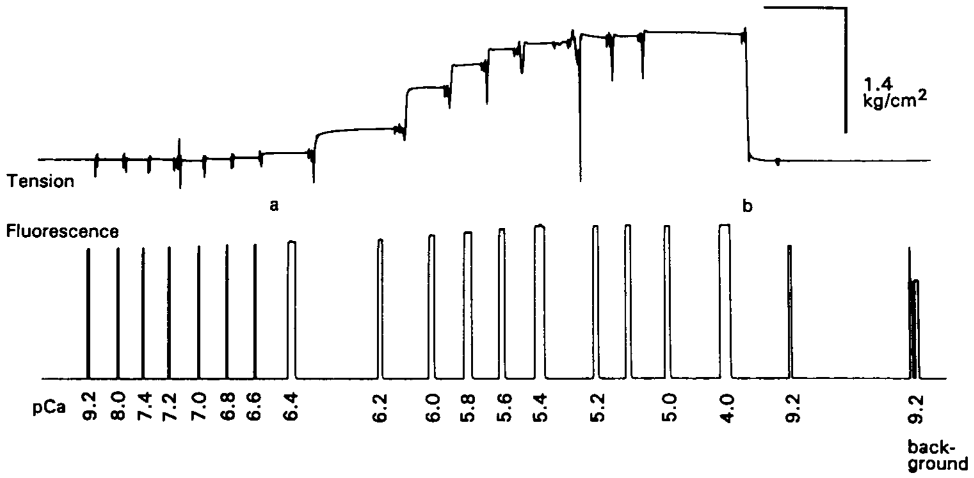

FIGURE 2 Simultaneously recorded tension and fluorescence. Fiber was titrated in the presence of 3 mM MgATP, 1 mM free Mg^{2+}, 15 mM CP, 20 units/ml CPK, and 4 wt% Dextran T-500. Ionic strength 0.2 M; pH 7.0; 10°C. 10× objective (N.A. 0.30) was used. Fiber 88g24. Horizontal scale bar represents 250 s before (*a*) and after (*b*) and represents 100 s between (*a*) and (*b*). See Fig. 4 *B*, for tension- and fluorescence pCa relations.

individual titrations were fitted with the Hill equation having one transition.

Statistical analysis and model identification

The X^2 value and the sum of squared residuals (Re^2) were calculated for each fit. For each form of the Hill equation, the fit producing the lowest X^2 value was considered the best. Two methods were used to determine whether the two-transition fit represented a significant improvement over the one-transition fit. The first method, the "F_X test for the addition of terms," is based on the "extra sum of squares" principle (Draper and Smith, 1966) and entails calculating the following F-statistic: $F = [(Re_1^2 - Re_2^2)/Re_2^2] \times [df_2/(df_1 - df_2)]$, where Re_i^2 is the sum of squared residuals for the i^{th} model, and where df_i is the number of degrees of freedom associated with the fit to the i^{th} model. The probability that the added terms are nonzero was found by comparing the calculated F-value with the values listed in a table of the F-distribution. The second method, the MAICE procedure, entails calculating Akaike's "an information criterion estimate," known as AIC, by the following equation (Akaike, 1974, 1976; Tanabe, 1976; Yamaoka, 1978): $AIC = N \times Ln(Re^2) + 2(p)$, where N is the number of data, Re^2 is the sum of residuals squared, and p is the number of parameters in the model being evaluated. The form of the Hill equation that gave the fit with the lowest AIC estimate was considered the preferred. We used the Student's t-distribution to determine the significance of apparent differences in means (Hoel, 1984).

Precautions

For the titrations performed on fibers set to a sarcomere length of 2.4 μm, an external pressure of 24 torr was applied to the fibers through the addition of 4 wt% Dextran T-500 to the bathing solution. The osmotic compression of the demembranated fiber afforded by this addition minimized changes in the lattice spacing during activation of the fiber (Millman, 1986). Changes in sarcomere length that develop during contraction distort the fluorescence signal by changing the density of fluorophores within the optically sampled volume. Use of a 10× objective (N.A. 0.30) permitted sampling from one third to one half of the fiber's length and thus minimized the distorting influence of changes in sarcomere length.

RESULTS

Presence and origin of the two transitions in the fluorescence-pCa relation

The Ca^{2+}-dependence of structural changes in TnC-DANZ incorporated into fibers can be determined most reliably in the absence of actomyosin interaction, hence from fibers stretched to a sarcomere length greater than 4.0 μm, beyond which thin filaments do not overlap thick filaments. These fibers herein are called highly stretched fibers. Fig. 3 presents results that are illustrative of those obtained in paired titrations, at 0.05 and 1.0 mM free Mg^{2+}, of single, highly stretched fibers incorporated with minimally labeled TnC and bathed in rigor solution. In the figure, the results obtained at 0.05 mM free Mg^{2+} clearly are fitted best with the form of the Hill equation allowing two transitions. Even for the results obtained at 1 mM free Mg^{2+}, inclusion of a second transition in the equation is justified ($p < 0.01$) when the residuals of the fits are analyzed with the F_X test for adding additional terms. The MAICE score for the one-transition model exceeds that for the two-transition model by 9.4; therefore, the MAICE procedure likewise selects the two-transition model.

The two-transition model remains favored when the

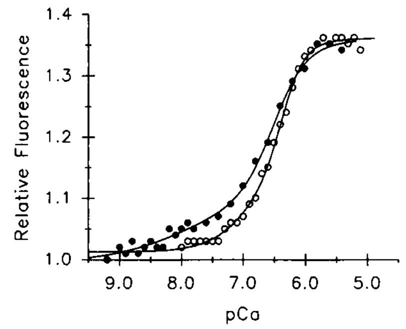

FIGURE 3 Fluorescence-pCa relations obtained from a highly stretched fiber incorporated with minimally labeled TnC. Titrations performed in the presence of 1 mM free Mg^{2+} (○) and 0.05 mM free Mg^{2+} (●). For 1 mM free Mg^{2+}, fitted curve has $pCa_{1/2}$ (1) = 6.96, n_{Hill} (1) = 1.25, $pCa_{1/2}$ (2) = 6.37, and n_{Hill} (2) = 2.36. For 0.05 mM free Mg^{2+}, fitted curve has $pCa_{1/2}$ (1) = 7.85, n_{Hill} (1) = 0.63, $pCa_{1/2}$ (2) = 6.51, and n_{Hill} (2) = 1.63. Fiber 89h5. Ionic strength, 0.2 M; pH 7.0; 10°C.

paired titrations from four fibers are considered. The average MAICE score difference for the results obtained at 1 mM free Mg^{2+} is 7.3 in favor of the two-transition model. This difference roughly means that the estimated likelihood ratio of the two- to the one-transition model is ~800. For the results at 0.05 mM free Mg^{2+}, the average MAICE score difference rises to 12.3, and the estimated likelihood ratio consequently rises to ~9,000 in favor of the two-transition model. The results from the MAICE procedure and from the F_X test for adding additional terms are given in Table 1.

Table 2 presents for several conditions the mean value for each analyzed term of the two-transition model fitted to the results of separate titrations. The fluorescence-pCa relation determined at 1 mM free Mg^{2+} is described by an initial transition that has a magnitude of 0.12, a $pCa_{1/2}$ of 6.87, and a Hill coefficient of 1.39, and by a second transition that has a magnitude of 0.20, a $pCa_{1/2}$ of 6.33, and a Hill coefficient of 2.74. The range in pCa over which the first transition occurs matches that over which Ca^{2+} binds to the high-affinity binding sites of TnC (Potter and Gergely, 1975). The lack of apparent cooperativity in this transition agrees with the reported lack of cooperativity in the binding of Ca^{2+} to the high-affinity sites in the presence of Mg^{2+} (Jahnke and Heilmeyer, 1980). The second transition has a $pCa_{1/2}$ compatible with the published binding constant for the low-affinity sites (Potter and Gergely, 1975) and evidences apparent cooperativity, in accord with the results that Grabarek et al. (1983) obtained with TnC-DANZ reconstituted into regulated actin filaments.

To test whether the first transition truly stems from the binding of Ca^{2+} to the high-affinity sites, we examined the sensitivity of this transition to a reduction in concentration of free Mg^{2+} from 1 mM to 0.05 mM. For four paired titrations at 1.0 and 0.05 mM free Mg^{2+}, the reduction in free Mg^{2+} concentration leads to an increase of 0.58 pCa unit in the $pCa_{1/2}$ of the first transition (Table 2). The observed increment, significant at the $p = 0.01$ level, agrees with the anticipated increment of 0.62 pCa unit in the apparent Ca^{2+}-affinity of the high-affinity sites, based on the reported Mg^{2+}-affinity of 4×10^3 M^{-1} of these sites (Potter and Gergely, 1975). The reduction in free Mg^{2+} concentration also leads to an increase of 0.12 pCa unit in the $pCa_{1/2}$ of the second transition. The increment, significant at the $p = 0.01$ level, suggests an apparent binding affinity for Mg^{2+} of 340 M^{-1}, similar to

TABLE 1 **Statistical identification of appropriate model for fluorescence-pCa relations**

Solution	Labeling	Points per titration	Number of titrations	Significance of added transition		Mean difference in MAICE[§]	Estimate of MLR[‖]
				$p \leq 1\%$	$1\% < p \leq 5\%$		
1 mM free Mg^{2+} rigor	Minimal	28–32	4	1	2	7.3	8×10^2
0.05 mM free Mg^{2+} rigor	Minimal	26–27	4[*]	2	1	12.3	9×10^3
			6[‡]	4	1	14.8	3×10^4
0.05 mM free Mg^{2+} rigor	Extensive	26–27	5	4		15.8	6×10^4
1 mM free Mg^{2+} and 3 mM MgATP	Extensive	16–17	6	2	1	10.2	3×10^3

The significance of the addition of a second transition was evaluated with "F_X test for the addition of terms" (see Methods). The ability of the one- and two-transition models to fit the fluorescence-pCa data was evaluated by MAICE (see Methods). The analyzed relations were determined with highly stretched fibers. [*] Paired with titrations performed in the presence of 1 mM free Mg^{2+}. [‡] Total number of titrations performed in the presence of 0.05 mM free Mg^{2+}. [§] Calculated as $(1/N) \times \Sigma_{i=1}$ [(MAICE for one-transition model fitted to the ith titration's results) − (MAICE for two-transition model fitted to the same results)], where N is the total number of titrations. [‖] Mean expected likelihood ratio of the two transition model to the one-transition model. Calculated as $\exp\{(1/2) \times [(\text{mean difference in MAICE}) + (2p - 2q)]\}$, where p and q are the numbers of terms in the two-transition model and the one-transition model.

Allen et al. Ca²⁺-dependent Structural Changes in TnC 403

TABLE 2 **Fluorescence-pCa relations determined with highly stretched fibers**

Solution	Labeling	Titrations; Fibers	First transition A	First transition $pCa_{1/2}$	First transition n_H	Second transition A	Second transition $pCa_{1/2}$	Second transition n_H
1 mM free Mg^{2+} Rigor	Minimal	4;4	0.12	6.87	1.39	0.20	6.33	2.74
			(0.03)	(0.07)	(0.45)	(0.05)	(0.07)	(0.56)
0.05 mM free Mg^{2+} Rigor	Minimal	4;4*	0.13	7.45	1.04	0.21	6.45	2.16
			(0.04)	(0.31)	(0.44)	(0.03)	(0.05)	(0.41)
		6;6‡	0.12	7.55	0.93	0.21	6.42	2.13
			(0.04)	(0.31)	(0.41)	(0.02)	(0.06)	(0.33)
0.05 mM free Mg^{2+} Rigor	Extensive	5;4	0.06	7.63	0.92	0.16	6.47	2.05
			(0.02)	(0.11)	(0.17)	(0.02)	(0.05)	(0.10)
1 mM free Mg^{2+} and 3 mM MgATP	Extensive	6;4	0.12	6.84	1.32	0.19	6.22	2.17
			(0.02)	(0.14)	(0.63)	(0.05)	(0.15)	(0.40)

In all cases, the sarcomere length of each fiber was greater than 4.0 μm; thus, there was no overlap of thick and thin filaments. The standard deviation for each value is given in parentheses.

*Paired with titrations performed in the pressure of 1 mM free Mg^{2+}. ‡Total number of titrations performed at 0.05 mM free Mg^{2+}.

the value of 200 M^{-1} reported by A. S. Zot and Potter (1987) for the apparent Mg^{2+}-affinity of the low-affinity sites.

A biphasic fluorescence-pCa relation could possibly result from the presence of fluorophores on both domains. Johnson et al. (1978), however, documented that, for TnC-DANZ labeled to the extent of 0.5 mol DANZ per mol protein, all fluorescence emanates from the tryptic fragment spanning residues 21 to 37. To test, nonetheless, whether the first transition might be caused by fluorophores covalently bound to the COOH-terminal domain of TnC, we employed a strategy similar to that used by Grabarek et al. (1986): we contrasted results obtained with minimally labeled TnC to those obtained with extensively labelled TnC. An increase in the extent of labeling should be accompanied by an increase in the likelihood of the labeling of sites other than Met-25. If the labeling of sites other than Met-25 accounts for the appearance of the first transition, then the magnitude of the first transition of the relation determined with extensively labeled TnC will exceed that of the relation determined with minimally labeled TnC.

The comparison of results has been made for titrations performed at 0.05 mM free Mg^{2+}, at which the two transitions are distinct. The obtained titration results, summarized in Table 2, are opposite to the prediction: accompanying the increase in extent of labeling are a 0.07 decrement in the magnitude of the first transition and a 0.05 decrement in that of the second. Both decrements are significant ($p < 0.025$). The increase in the extent of labeling alters neither the $pCa_{1/2}$ nor the Hill coefficient of either transition in a statistically significant way. Thus, an increase in the basal, Ca^{2+}-independent fluorescence, to which the Ca^{2+}-induced fluorescence increases are normalized, is the sole apparent consequence of an increase in the extent of labeling. This same conclusion was drawn from a similar comparison with TnC-DANZ free in solution (data given in Fig. 1 and described in Methods section).

Fluorescence-pCa relations obtained in the presence of MgATP

Two transitions also are found in the relation determined with highly stretched fibers incorporated with extensively labeled TnC and bathed in a solution that contains 1 mM free Mg^{2+} and 3 mM MgATP. Fig. 4*A* depicts the results obtained in a single titration; Table 2 offers the mean values for the fitted terms in the two-transition model. The $pCa_{1/2}$ values of the relation statistically are indistinguishable from those of the relation determined with fibers incorporated with minimally labeled TnC and bathed in 1 mM free Mg^{2+} rigor solution. Thus, the presence of MgATP does not affect the $pCa_{1/2}$ values of the relation.

The presence of MgATP, however, does affect the magnitude of the first transition. Because the extent of labeling of TnC also affects the magnitude, the effect of MgATP upon the first transition is salient only through a comparison of the relation determined in the presence of MgATP to the relation determined with fibers incorporated with extensively labeled TnC and bathed in 0.05 mM free Mg^{2+} rigor solution. The magnitude of the first transition is increased in the presence of 3 mM MgATP by 0.06, with a 95% confidence interval of ±0.03. That the increment stems from the increase in the concentration of free Mg^{2+} is untenable because of the insensitivity of the first transition's magnitude to changes in free Mg^{2+} concentration for the relation obtained from fibers incorporated with minimally labeled TnC and bathed in rigor solution. The effect of MgATP upon the transition

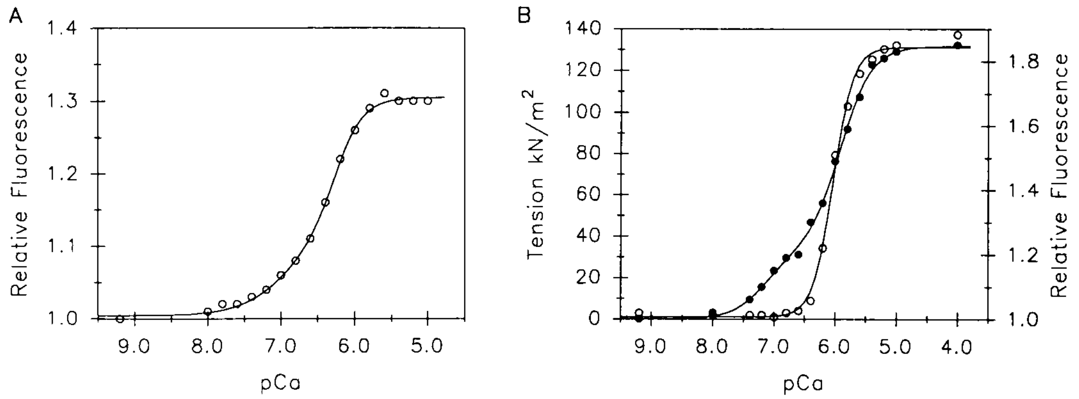

FIGURE 4 Fluorescence-pCa relations obtained from two fibers incorporated with extensively labeled TnC and set to different sarcomere lengths. (*A*) Sarcomere length >4.0 μm. Fitted curve has $pCa_{1/2}$ (1) = 6.90, n_{Hill} (1) = 1.28, $pCa_{1/2}$ (2) = 6.26, and n_{Hill} (2) = 2.40. Fiber 90c28b. (*B*) Sarcomere length set to 2.4 μm. Fitted curve for fluorescence (●) has $pCa_{1/2}$ (1) = 7.14, n_{Hill} (1) = 1.6, $pCa_{1/2}$ (2) = 5.92, and n_{Hill} (2) = 1.7. Fitted curve for tension (○) has $pCa_{1/2}$ = 6.03 and n_{Hill} = 2.7. Cross-sectional area of fiber, 5,385 μm^2; length of fiber, 0.9 mm. Fiber 88g24. Both titrations performed in the presence of 3 mM MgATP and 1 mM free Mg^{2+}. Ionic strength, 0.2 M; pH 7.0; 10°C. 4 wt% Dextran T-500 was added to solutions bathing the unstretched fiber.

associated with the high-affinity sites, thought to play a role in the structural stability of TnC within the thin filament (Cox et al., 1981; Zot and Potter, 1982), is reminiscent of the influence of MgATP upon the conformational state of F-actin in "ghost" fibers, prepared from rat soleus muscle (Szczepanowska et al., 1987).

If the structural changes in TnC reflected by the second transition intimately and directly are involved in the regulation of tension development in fibers with overlapping thick and thin filaments, then the $pCa_{1/2}$ value for tension development may be anticipated to match that of the second transition, not that of the first. Fig. 4 *B* depicts a single titration that exemplifies seven titrations of four fibers set to a sarcomere length of 2.4 μm before each titration. Table 3 lists the mean values for the fitted terms in the two-transition model. One striking feature of the results is that the second transition in fluorescence exhibits a Ca^{2+}-sensitivity that matches that of tension: the mean $pCa_{1/2}$ of tension is 6.04, and the mean $pCa_{1/2}$ of the second transition is 5.96. The 0.08 pCa unit difference in the $pCa_{1/2}$ values fails to be significant ($p > 0.05$) and has a 95% confidence interval bounded by ±0.11 unit. The Ca^{2+}-sensitivity of the first transition is ~10× that of tension.

Contrasting the fluorescence-pCa relation determined in the presence of cycling cross-bridges to that in the absence confirms another apparent feature of Fig. 4: the mean magnitude of the second transition is enhanced by 0.32 in the presence of cycling cross-bridges. The enhancement, significant at the $p = 0.005$ level, has a 95% confidence interval of ±0.15. The mean magnitude of the first transition is unaffected by the presence of cycling cross-bridges.

Like its magnitude, the first transition's Hill coeffi-

TABLE 3 Fluorescence- and tension-pCa relations determined in the presence of 1 mM free Mg^{2+} and 3 mM MgATP

Sarcomere length	Titrations; fibers		First transition *A*	First transition $pCa_{1/2}$	First transition n_H	Second transition *A*	Second transition $pCa_{1/2}$	Second transition n_H
>4.0 μm	6;4	Fluorescence	0.12 (0.02)	6.84 (0.14)	1.32 (0.63)	0.19 (0.05)	6.22 (0.15)	2.17 (0.40)
2.4 μm	7;4	Fluorescence	0.13 (0.06)	7.01 (0.23)	2.97 (2.51)	0.50 (0.16)	5.96 (0.14)	1.76 (0.28)
		Tension				88 kN/m^2 (25)	6.04 (0.04)	2.41 (0.20)

In all cases, extensively labeled TnC was used. The standard deviation for each value is given in parentheses. For fibers set to a sarcomere length of 2.4 μm, the fluorescence- and tension-pCa relations were determined simultaneously. The tension-pCa data were modeled with the one-transition model.

cient statistically is unaffected by the presence of cycling cross-bridges. The Hill coefficient of the second transition, though, decreases by 0.41 in the presence of cycling cross-bridges and is less by 0.65 than the Hill coefficient of tension development. This 0.65 difference is significant ($p < 0.005$); nonetheless, the second transition, regardless of the presence of cycling cross-bridges, exhibits apparent positive cooperativity.

Because the $pCa_{1/2}$ value, the Hill coefficient, and the magnitude of the first transition are unaffected by the presence of cycling cross-bridges, and because the transition's sensitivity to free Ca^{2+} exceeds that for tension development by an order of magnitude, the structural changes in TnC-DANZ reflected by the first transition truly appear to play no direct, immediate role in the regulation of tension development. An opposite conclusion for the structural changes in TnC-DANZ reflected in the second transition is argued by the results of this section. Indeed, the second transition evidences not only a Ca^{2+}-sensitivity that is comparable with that of tension development, but also a dramatic increase in magnitude in the presence of cycling cross-bridges.

DISCUSSION

Salient amongst the obervations on the fluorescence-pCa relations that were obtained from fibers regulated by TnC-DANZ is the statistically validated presence of two positive transitions. The first, because of its high $pCa_{1/2}$ value, is attributable to the exchange of Ca^{2+} for Mg^{2+} at the high-affinity binding sites of TnC. The second, because of its low $pCa_{1/2}$ value, is attributable to the binding of Ca^{2+} to the low-affinity sites. Confirmation of these attributions comes from two observations: the appreciable Mg^{2+}-sensitivity of the first transition matches that of the high-affinity sites, and the slight Mg^{2+}-sensitivity of the second transition matches that of the low-affinity sites. The presence of two transitions requires that the fluorophore's environment change upon the binding of Ca^{2+} to both classes of site and hence suggests that the structure of the NH_2-terminal domain of TnC is sensitive to the binding of Ca^{2+} to sites located in both domains.

The initial studies with TnC-DANZ (Johnson et al., 1978, 1979), which were performed with TnC-DANZ free in solution, revealed the biphasic quality of the fluorescence-pCa relation. Subsequent studies with TnC-DANZ incorporated into reconstituted thin filaments (Grabarek et al., 1983; H. G. Zot and Potter, 1987) and into fibers (Zot et al., 1986; Guth and Potter, 1987; A. S. Zot and Potter, 1987), however, did not discern the biphasic quality of the fluorescence-pCa relation. The biphasic quality of the relation escaped detection in these studies presumably because the binding isotherms for the high-affinity and low-affinity sites of TnC, when associated with the thin filament, merge in the presence of millimolar free Mg^{2+}. In our studies, the resolution of two transitions in the fluorescence-pCa relations emanated from the determination of the relations at 0.05 mM free Mg^{2+}, a concentration at which the two binding isotherms separate.

Our observation of an interaction between the two domains of TnC has parallels in the work of others with different fluorescent derivatives of TnC. For example, Grabarek et al. (1986) documented the sensitivity of the response from the fluorophore 1,5-IAEDANS, attached at Cys-98, in the COOH-terminal domain, to the binding of Ca^{2+} to both classes of site in isolated TnC. The observed sensitivity became more apparent when TnC-AEDANS was complexed with TnI. Similarly, Rosenfeld and Taylor (1985) observed in their kinetic study with TnC labeled with IANBD at Cys-98 a transient in the fluorophore's response arising from the binding of Ca^{2+} to the low-affinity sites, in the NH_2-terminal domain of TnC. Our results with TnC-DANZ complement the results from these two studies by showing that the structure of the NH_2-terminal globular domain is modified by the binding of Ca^{2+} to the high-affinity sites, in the COOH-terminal globular domain, and by demonstrating that the interaction between the domains occurs in the fiber.

The conclusion that the structure of the NH_2-terminal globular domain of TnC is modified by the binding of Ca^{2+} to sites in both globular domains needs to be weighted against the possibilities that multiple sites are labeled and that labeling affects the structure and function of TnC. Johnson et al. (1978) documented that, for TnC-DANZ labeled to the extent of 0.5 mol DANZ per mol protein, all fluorescence can be localized to the tryptic fragment spanning residues 21 to 37. Our conclusion that an interaction occurs between the two domains of TnC rests upon results from studies with TnC-DANZ labeled to the extent of 0.25 mol DANZ per mol protein. Moreover, like Grabarek et al. (1983), we took the precaution of blocking with DTNB the sold cysteine of TnC before incubating TnC with the fluorophore.

An unequivocal answer to whether TnC-DANZ may differ from TnC in either structure or function has not emerged. Wang et al. (1983) observed that the rate constant, determined by changes in intrinsic tyrosyl fluorescence, for the release of Ca^{2+} from the high-affinity sites is $2.9\ s^{-1}$ for unlabeled TnC and is $1.8\ s^{-1}$ for TnC-DANZ. Johnson et al. (1978), however, reported no difference between TnC and TnC-DANZ in circular dichroism studies of the Ca^{2+}-induced increase in α-helicity. We found that the tension-pCa relation of fibers extracted and then incorporated with TnC-DANZ did not appreciably differ from the relation of extracted fibers incorporated with unlabeled TnC. Thus, if present, alterations in either the structure or the function of labeled TnC are subtle.

The fluorescence-pCa relation for both fiber-incorporated and isolated TnC-DANZ is biphasic; thus, the interaction is not dependent upon the presence of the other constituents of the thin filament. That an interdomain interaction occurs in isolated TnC suggests two possibilities: (*a*) that the central helix, though spatially separating the two domains, mediates an indirect interaction between the domains; and (*b*) that the central helix is bent to an extent allowing direct interaction between the two domains. Heidorn and Trewhella (1988) found that x-ray solution scattering data from rabbit skeletal TnC, as well as from its homologue, calmodulin, were best described by a model in which the central helix is bent such that a line between the two domains subtends an angle of 114.6°. The possible presence of such a bend lends credibility to the second possibility. Nonetheless, the central helix, regardless of its actual shape, could mediate an indirect interaction between the two domains of TnC. Because a considerable electric dipole moment is associated with an alpha-helix (reviewed by Wada, 1976), and because the three-dimensional structure of a globular domain is determined, in part, by electrostatic interactions (e.g., Sali et al., 1988), the binding of Ca^{2+} to the high-affinity sites could modify the structure of the NH_2-terminal domain by changing the alignment of the central helix with respect to the NH_2-terminal domain, hence by modifying the electric field of the NH_2-terminal domain.

The mechanism for the interaction between the two domains of TnC is likely to be altered when TnC is associated with the regulatory complex: the sign of the first transition in the fluorescence-pCa relation changes from negative to positive when TnC-DANZ is ensconced into the thin filament. The contribution of TnI and TnT to the interdomain interaction within TnC was noted by Grabarek et al. (1986) and by Wang and Gergely (1986) in their reports of the Ca^{2+}-induced changes in spectroscopic signals from TnC labeled at Cys-98.

Interaction between the two domains of TnC may allow a modulation of the apparent Ca^{2+}-association constant of the low-affinity binding sites by the binding of Ca^{2+} to the high-affinity sites. The possibility of this modulation prompts an evaluation of the hypothesis that the high-affinity sites are nonregulatory (Potter and Gergely, 1975; Robertson et al., 1981). Comparing the fluorescence-pCa relation with the tension-pCa relation reveals several features. The $pCa_{1/2}$ of the second transition in fluorescence-pCa relation matches the $pCa_{1/2}$ of tension development. The $pCa_{1/2}$ of the first transition exceeds that of the tension development by 1 pCa unit. The magnitude of the second transition, but not the first, is greatly enhanced in the presence of cycling cross-bridges. The development of tension and the second transition, but not the first, exhibit apparent positive cooperativity. Thus, our results, agreeing with the conclusion of Guth and Potter (1987), indicate that the structural changes in TnC induced by the binding of Ca^{2+} to the low-affinity sites, but not to the high-affinity sites, relate directly to the development of tension.

The relevance of the structural changes in TnC induced by the binding of Ca^{2+} to the high-affinity sites seems remote for the triggering of contraction. Our results, however, do not exclude a role for these structural changes in modulating the binding of Ca^{2+} to the low-affinity sites. For example, through the interaction between the two domains of TnC, the Mg^{2+}-sensitivity of the second transition of the fluorescence-pCa relations determined with highly stretched fibers might stem from the Mg^{2+}-sensitivity of the first transition, hence from the Mg^{2+}-sensitivity of the high-affinity sites. This possibility gains strength from the results of NMR spectroscopy performed during the titration with Ca^{2+} of TnC and its proteolytic fragments (Drakenberg et al., 1987; for differing view, see Tsuda et al., 1990). A thermodynamic evaluation of this possibility awaits the determination of the free energy coupling for the binding of Ca^{2+} to the two domains of TnC when in the regulatory complex. The physiological consequences of a modulatory role for the high-affinity sites, if true, can not be discounted as trivial. For example, Irving et al. (1989) reported that, with prolonged voltage-clamp depolarization, frog twitch fibers evidence an increase in the concentration of free Mg^{2+} of 0.8–1.4 mM, an increase sufficient to reduce the apparent Ca^{2+}-affinity of the low-affinity sites and hence to affect the rate of relaxation.

SUMMARY

Our results reveal that the structure of the NH_2-terminal domain of fiber-incorporated TnC is modified by the binding of Ca^{2+} to sites in both domains. Furthermore, the results reveal that the structural changes in TnC induced by the binding of Ca^{2+} to the low-affinity sites, but not to the high-affinity sites, play a direct, immediate role in the regulation of tension development.

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