Troponin C Modulates the Activation of Thin Filaments by Rigor Cross-Bridges

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ABSTRACT Extraction of troponin C (TnC) from skinned muscle fibers reduces maximum Ca^{2+} and rigor cross-bridge (RXB)-activated tensions and reduces cooperativity between neighboring regulatory units (one troponin-tropomyosin complex and the seven associated actins) of thin filaments. This suggests that TnC has a determining role in RXB, as well as in Ca^{2+}-dependent activation processes. To investigate this possibility further, we replaced fast TnC (fTnC) of rabbit psoas fibers with either CaM[3,4TnC] or cardiac TnC (cTnC) and compared the effects of these substitutions on Ca^{2+} and RXB activation of tension. CaM[3,4TnC] substitution has the same effect on Ca^{2+}- and RXB-activated tensions; they are reduced 50%, and cooperativity between regulatory units is reduced 40%. cTnC substitution also reduces the maximum Ca^{2+}-activated tension and cooperativity. But with RXB activation the effects on tension and cooperativity are opposite; cTnC substitution potentiates tension but reduces cooperativity. We considered whether tension potentiation could be explained by increased activation by cycling cross-bridges (CXBs), but the concerted transition formalism predicts fibers will fail to relax in high substrate and high pCa when CXBs are activator ligands. It predicts resting tension, which is not observed in either control or cTnC-substituted fibers. Rather, it appears that cTnC facilitates RXB activation of fast fibers more effectively than fTnC. The order of RXB-activated tension facilitation is cTnC > fTnC > CaM[3,4TnC] > empty TnC-binding sites. Comparison of the structures of fTnC, CaM[3,4TnC], and cTnC indicates that the critical region for this property lies in the central helix or N-terminal domain, including EF hand motifs 1 and 2.

INTRODUCTION

Both Ca^{2+} and rigor cross-bridges (RXBs) bind to the thin filaments and activate striated muscle contraction. Therefore, both promote movement of the troponin-tropomyosin regulatory strand from the relaxed to the active position (Guth and Potter, 1987; Lehman et al., 1994). There are fundamental differences, however, in the ways in which Ca^{2+} and RXBs accomplish this. Ca^{2+} binding to troponin C (TnC) strengthens interactions between TnC and troponin I, which disrupts inhibitory interactions between TnI and actin (Grabarek et al., 1992). This permits movement of the regulatory strand to the "on" position (Lehman et al., 1994). In contrast, RXBs, by virtue of their high affinity for F-actin, bind to thin filaments independently of Ca^{2+} and force the regulatory strand from the relaxed to the active position (Bremel and Weber, 1972; Engelman, 1985; Kress et al., 1986; Wakabayashi et al., 1985). Both Ca^{2+} and RXBs must overcome the links between the troponin-tropomyosin regulatory strand and actin that stabilize the strand in the inhibitory position.

Selective extraction of TnC from skinned fibers is an important tool for investigating thin filament regulation (for review see Moss, 1992). Because TnC is the effector of Ca^{2+} activation (Potter and Gergely, 1975; Weber and Murray, 1973), its removal decreases both tension and cooperativity between regulatory units (one troponin-tropomyosin complex and the seven associated actins). Without TnC, regulatory units are refractory to Ca^{2+}. Reduction of maximum Ca^{2+}-induced tension is proportional to the TnC extracted (Brandt et al., 1984b, 1987). Cooperativity, however, is reduced disproportionately to the TnC extracted. Although extraction of an average of one TnC from each regulatory strand reduces tension by 6.6%, it reduces cooperativity by 26% (Brandt et al., 1987). Extraction studies also provide evidence that TnC has an important role in RXB activation of the thin filament (Brandt et al., 1990). Although RXB activation is independent of Ca^{2+}, extraction of 80% of the TnC reduces maximum RXB-activated tension by ~40% and cooperativity by 50%.

The parallel effects of TnC extraction on Ca^{2+} and RXB activation suggest that TnC is essential for transmission of cooperativity, and Ca^{2+} and RXB activation may have steps in common. If so, substitution of fast skeletal TnC (fTnC) with other molecules known to modulate Ca^{2+} activation processes should act similarly on RXB-activated tension and cooperativity. To test this, we replaced the endogenous fTnC with a calmodulin/troponin C chimera, CaM[3,4TnC] (Brandt et al., 1994; George et al., 1990, 1993), or cardiac TnC (cTnC) and determined the effects on RXB-activated tension and cooperativity of skinned psoas fibers.

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MATERIALS AND METHODS

Preparation of TnC and the CaM[3,4TnC] chimera

fTnC and cTnC were isolated from rabbit erector spinae and soleus, respectively (Brandt et al., 1984b), or were bacterially expressed. cTnC cDNA was from George et al. (1990, 1993). fTnC cDNA was generated by reverse transcriptase polymerase chain reaction from rabbit erector spinae RNA. The cDNAs were inserted into pET14b, and their sequences were confirmed by dideoxynucleotide cycle sequencing using Sequenase 2.0 (US Biochemicals). To improve yield, the cDNAs were excised from the heat-inducible pUC-P_L expression vector (George et al., 1990) by cleavage at unique *Nco*I and *Nde*I restriction sites and inserted into pET14b (Novagen). Protein concentrations were determined by Bradford assays (George et al., 1990) and by Coomassie Brilliant Blue staining on sodium dodecyl sulfate-polyacrylamide gels (Schachat et al., 1985), using 1 mg/ml solution of CaM (confirmed by amino acid analysis) as a standard. Consistent with previous observations (Gulati, 1992; Putkey et al., 1991; Sweeney et al., 1990), bacterially expressed fTnC and cTnC were indistinguishable from those isolated from skeletal or cardiac muscle in their capacities to restore contractile function.

Solutions and skinned fiber preparation

Solution preparations followed those described previously (Brandt et al., 1990), except that rigor solution was made without 3-(*N*-morpholino)propanesulfonic acid and was, therefore, unbuffered with respect to pH. This increased the rate of TnC extraction.

Extraction procedure

Fibers were first washed twice with wash solution, then once with rigor solution at room temperature, and finally washed continuously for ~5 min in the muscle chamber with extraction solution at 35°C. Approximately 80% of the TnC was extracted.

Apparatus

The pCa/tension and pS/tension data were collected on an apparatus that automates solution mixing and data recording. This has been described previously (Brandt et al., 1980, 1984a). All of the measurements were made at 20°C.

Data reduction

The pS/tension points are fit to a modified substrate inhibition equation (Brandt et al., 1990), $P/P_o = (K_1/S + (S/K_2)^n)^{-1}$, where $S = [MgATP^{2-}]$, n (n_S) is the slope, and pK_2 is the midpoint of the tension increase between pS 4 and 5. In this range, tension increases as the concentration of RXBs increases with the cooperativity of increase quantified by n_S. The data are well fit between pS 4 and 5, which is the range of interest here. The data above the tension optimum, pS 5, are only approximated by the equation and are not germane to this report.

RESULTS

To assess RXBs as activators of contraction, tension was measured as a function of the substrate, usually expressed as pS (pS = $-\log[MgATP^{2-}]$). As the pS is raised above 4, tension gradually increases (Fig. 1) until pS 5, where an optimal mixture of RXBs and force-generating cross-bridges produces a maximum tension. Thereafter, tension falls as the pS rises above 5. The data are fit to a modified substrate inhibition equation (see Materials and Methods), and the solid line in Fig. 1 is drawn according to this equation from the best fit parameters. The equation accurately describes the data on the right side of the maximum because the points are mostly on the line. The slope of the curve, n_S, describes the cooperativity of thin filament activation by RXBs, and the pK_2 describes the tension midpoint. Because the n_S is about 5, the tension increase between pS 5 and 4 is cooperative (Brandt et al., 1990; Metzger, 1995). To distinguish the maximum RXB tension from the maximum Ca^{2+}-activated tension, we define the RXB maximum as the "optimal" tension. This is ~60% of the maximum tension (Brandt et al., 1972; Moss and Haworth, 1984; Reuben et al., 1971).

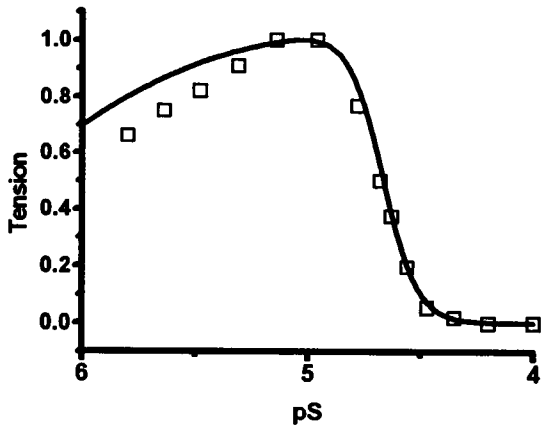

FIGURE 1 Plot of fiber tension against substrate concentration expressed as pS ($-\log[MgATP^{2-}]$). □, Data points. The solid line is drawn from the best-fitting parameters for the data fit to a modification of the classic substrate inhibition equation (Brandt et al., 1990). Tension peaks at the optimal ratio of RXBs and tension generating cross-bridges. Experiment D05a.12c: $pK_2 = 4.67$ and $n_S = 5.65$.

Three pS/tension curves are performed on each fiber: control, extracted, and reconstituted with a test protein. Before the start of each curve, the maximum response to pCa 4.75 is determined. The first columns of Tables 1 and 2, respectively, list the control maximum and optimal tensions. The middle columns list the tensions after extraction of ~80% of the TnC. This has a larger effect on maximum tension (83% lost) than on optimal tension (44% lost). The last columns give the absolute tensions after reconstitution with fTnC, CaM[3,4TnC], or cTnC.

The tensions recovered on reconstitution with CaM[3,4TnC] or cTnC are best compared to those recovered by reconstitution with purified fTnC, because exposure of fibers to three substrate curves and three pCa 4.75 tests

TABLE 1 Ca^{2+}-activated maximum tensions

	Control	Extracted	Reconstituted
fTnC	90.8 ± 8.8	10.2 ± 3.0	77.2 ± 5.7
CaM[3,4TnC]	86.6 ± 8.7	15.6 ± 5.0	45.6 ± 9.4
cTnC	95.6 ± 4.5	23.8 ± 5.0	54.2 ± 9.1

Mean ± SEM. $N = 5$ for each row. Tensions are in milligrams.

TABLE 2 Rigor cross-bridge-activated optimal tension

	Control	Extracted	Reconstituted
fTnC	51.8 ± 3.4	10.2 ± 3.0	45.8 ± 1.2
CaM[3,4TnC]	51.8 ± 7.6	30.4 ± 5.0	38.6 ± 5.4
cTnC	55.8 ± 3.8	32.0 ± 1.9	62.4 ± 4.7

Mean ± SEM. $N = 5$ for each row. Tensions are in milligrams.

reduces output. The absolute tensions of fibers reconstituted with fTnC are listed in Tables 1 and 2. They can also be expressed as percentage recovered/lost defined as the increment of tension gained on reconstitution divided by that lost on extraction, expressed as a percentage. On reconstitution with fTnC, 84% of the lost maximum tension and 76% of the lost optimal tension are restored (Fig. 2, *first bars of each group*). The percentage recovered/lost with fTnC reconstitution is 12% less for optimal than for maximum tension, although in absolute terms fibers recover to 85% and 88% of their preextraction maximum and optimal values, respectively (Tables 1 and 2, *top box, last column*). This 12% difference is because skinned fibers lose 15% of their capacity to generate tension (Metzger, 1995) with three complete substrate curves and three maximum Ca^{2+} tensions. The percentage of tension lost by extraction is less for optimal than for a maximum tension; therefore the 15% overall capacity loss has a bigger impact on the percentage recovered/lost optimal tension.

We reported previously that replacement of fTnC with CaM[3,4TnC] reduces the maximum tension and cooperativity or Hill coefficient (n_H) of the pCa/tension relationship (Brandt et al., 1994). We confirm maximum tension loss here and extend it to optimal tension; recovery of maximum and optimal tension with fTnC reconstitution is much larger than that for fibers reconstituted with CaM[3,4TnC] (Table 1). We calculate that after CaM[3,4TnC] reconstitution, 43% of the lost maximum tension and 42% of the lost optimal tension are recovered. These percentage recovered/lost are compared to those for fTnC in Fig. 2 (*first two bars of each group*). It is obvious that fibers reconstituted with CaM[3,4TnC] do not support as much maximum and optimal tensions as those reconstituted with fTnC. This is evident whether the percentage recovered/lost or the absolute recovered tensions are compared.

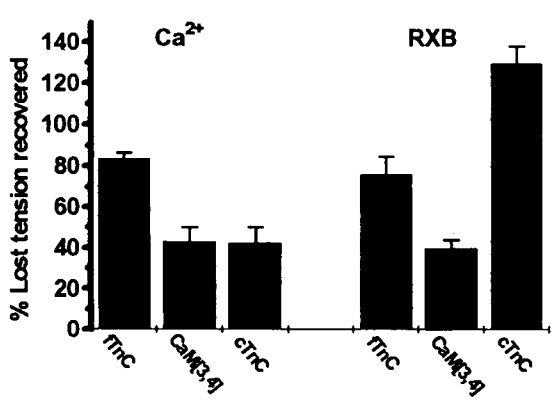

FIGURE 2 Histogram of the percentage recovered/lost tension (increment of tension recovered/increment of tension lost) for Ca^{2+} (*left three columns*) and for RXB-activated fibers (*right three columns*). The percentage recovered/lost (42%) is similar for maximum tensions of both CaM[3,4TnC] and cardiac TnC substituted. Recoveries are very different for optimal tension; the percentage recovered/lost tension with CaM[3,4TnC] replacement is 40%, and that with cTnC replacement is 130%. The percentage recovered/lost with fTnC reconstitution is 88%.

The EF hand motifs 1 and 2 of cTnC differ from those of fTnC. fTnC has two low-affinity Ca^{2+}-binding sites, whereas only one motif, 2, of cTnC binds Ca^{2+} and regulates contraction. cTnC and fTnC both have two structural or high-affinity Ca^{2+}-Mg^{2+}-binding EF hand motifs (3 and 4) that are required to bind the protein to the regulatory strand (Zot and Potter, 1982). Replacement of psoas fTnC with cTnC decreases maximum tension in the same manner as CaM[3,4TnC] substitution (Table 1); 42% of the lost tension is recovered (Fig. 2, *last bar of Ca^{2+} group*), whereas 56% of the absolute tension is recovered. The similarity between CaM[3,4TnC] and cTnC ends here, because the optimal percentage recovered/lost is 130% or 90% greater than that for CaM[3,4TnC] and 40% greater than that for fTnC (Fig. 2, *second group*). In spite of the 15% loss in tension-generating capacity with three pS/tension curves, the absolute optimal tension recovered in cTnC-substituted fibers is 112% of the initial control values (Table 2).

The effects of CaM[3,4TnC] substitution on the parameters, pK_2, n_S, and pS_{opt} (pS at optimal tension) are listed in Table 3. When 80% of the TnC is extracted, the n_S decreases from 5.5 to 2.5. This decrease is similar to the effect of extraction on the n_H (Hill coefficient) of the pCa/tension relationship (Brandt et al., 1990). Extraction of TnC shifts the pK_2 to a higher pS (Table 3), supporting the finding that extraction increases the affinity of myosin for ATP (Zhao et al., 1996). Replacement with fTnC or CaM[3,4TnC] moves the pKs back to values close to those of the controls. After reconstitution with CaM[3,4TnC] the n_S is 3.3, but after reconstitution with fTnC it is 5.3. This means that CaM[3,4TnC] supports less cooperativity with RXB activation than does fTnC, just as it supports less cooperativity with Ca^{2+} activation. Thus substitution with CaM[3,4TnC] has a parallel effect on tension and cooperativity for the two activating ligands, Ca^{2+} and RXBs, which supports the concept that TnC plays a common role in both modes of activation. In contrast, with cTnC substitution tension is enhanced, but this is accompanied with reduced, not enhanced, cooperativity; control n_S is 6, extracted n_S is 2.4, and reconstituted n_S is 2.6 (Table 3). The n_S of cTnC-reconstituted fibers is lower than that for CaM[3,4TnC]-reconstituted fibers; both are much lower than that for fibers reconstituted with fTnC.

DISCUSSION

In our first experiments, TnC is replaced with a chimera of CaM and TnC, CaM[3,4TnC], where the high-affinity

TABLE 3 Fitted pK_2s and n_ss of pS/tension curves

	pK_2 control	pK_2 extracted	pK_2 reconst.	n_s control	n_s extracted	n_s reconst.	pS_{opt} control	pS_{opt} extracted	pS_{opt} reconst.
fTnC	4.66 ± 0.01	4.94 ± 0.05	4.73 ± 0.01	5.79 ± 0.6	1.94 ± 0.1	5.34 ± 0.5	5.09 ± 0.11	5.45 ± 0.05	5.13 ± 0.03
CaM[3,4TnC]	4.71 ± 0.06	4.91 ± 0.11	4.81 ± 0.07	5.53 ± 1.34	2.46 ± 0.06	3.29 ± 0.26	5.08 ± 0.03	5.41 ± 0.09	5.23 ± 0.04
cTnC	4.63 ± 0.05	4.69 ± 0.04	4.09 ± 0.08	5.95 ± 1.9	2.35 ± 0.04	2.59 ± 0.22	5.11 ± 0.07	5.28 ± 0.06	4.76 ± 0.06

Mean parameters from the pS/tension curve-fitting procedure for the data of the fibers in Table 2. These are the pK_2, n_s, and pS_{opt}, each followed by SEM. The pS_{opt} is the pS at the calculated optimal tension.

Ca^{2+}/Mg^{2+} selective motifs 3 and 4 of cTnC are substituted into CaM. Consequently, the chimera retains the regulatory N-terminal of the CaM molecule but binds stably to the thin filament in relaxing solutions (pCa 8), whereas CaM binds only at about pCa 4 (Babu et al., 1988; Brandt et al., 1994). Even when extracted fibers are saturated with this chimera, or with CaM at pCa 4, maximum tension recovers to ~50% and cooperativity to ~60% of that of fibers reconstituted with fast TnC (Brandt et al., 1994). Here we show that CaM[3,4TnC] substitution has the same effect on RXB-activated fibers; ~40% of the lost optimal tension is recovered (Fig. 2).

Development of less than maximum and optimal tensions implies partial activation; however, this cannot be due to empty TnC- or Ca^{2+}-binding sites (Brandt et al., 1994). Similarly, at maximum RXB tension, the mix of RXBs and tension cross-bridges is optimal for tension development. With CaM[3,4TnC] substitution, the n_S is 60% of the original (Table 3); therefore it is likely that substitution of fTnC by CaM[3,4TnC] interferes with cooperativity such that the thin filament no longer activates as a unit, as it normally does (Brandt et al., 1984b, 1987, 1990; Fraser and Marston, 1995). Perhaps the substituted regulatory strands are functionally segmented, so sections turn on and off asynchronously in saturating Ca^{2+} or at optimal substrate. This could reduce both cooperativity and tension.

The strong parallel effect of the CaM[3,4TnC] chimera on maximum and optimal tensions and cooperativity suggests that Ca^{2+} and RXBs activate through common mechanisms. Perhaps the common component is that attached cycling cross-bridges (CXBs), in addition to Ca^{2+} and RXBs, are a third class of activator ligand (Guth and Potter, 1987; Lehrer, 1994; Moss, 1992; Williams et al., 1988). In this case activation in pCa 8 with RXBs is really a function of the sum of CXBs and RXBs. CXBs might be less efficient activators when CaM[3,4TnC] replaces fTnC and more efficient when cTnC replaces fTnC.

A problem with the hypothesis that CXBs are activator ligands is that once CXBs exist, how is relaxation achieved? We have introduced CXBs as activator ligands into the concerted transition formalism to illustrate this problem. In Fig. 3, *A* and *B*, the predicted force, fraction of thin filaments active, and fraction of myosin heads in rigor is plotted as a function of the pS. In Fig. 3 *A* the parameter that determines the contribution of CXBs to activation is assigned a value such that CXBs have little influence, whereas in Fig. 3 *B* this parameter is set so that CXBs have a discernible influence on activation. Under the latter conditions, relaxation is incomplete. Positive feedback from CXBs results in resting tension, even in high pCa and 10 mM substrate. Although there is substantial evidence that CXBs play a role in muscle activation, just what it is and how it is accomplished without the introduction of resting tension remain to be clarified. Perhaps a fraction of CXBs (for example, those near the end of the power stroke) can function as activator ligands and not block relaxation.

One can argue that the prediction of resting tension with CXBs as activator ligands is a unique result of the particular model tested here. The concerted transition formalism is widely employed by others, however, and the form used here can model regulation by Ca^{2+} and RXBs and the effects of TnC extraction (Brandt et al., 1987, 1990). It is also quite simple to do the calculations. We know of no other formalism of regulation that has been used to specifically examine the effects of CXBs as activator ligands.

A related issue is whether the RXBs formed in our experiments as the concentration of substrate is reduced are identical to rigor bridges formed in the complete absence of substrate. Certainly the two become theoretically indistinguishable as the substrate approaches zero, but the path to rigor is significant (Kawai and Brandt, 1976). We assume that RXBs are dissociated by substrate and replenished from the CXB pool.

Because we cannot readily explain our results using a hypothesis in which CXBs are activator ligands, other explanations must be examined. If CaM[3,4TnC] stabilizes the regulatory strand in the relaxed configuration more strongly than does fTnC, this might explain reduced tension, but it should result in increased cooperativity of activation. Another possibility may be that regulatory units incorporating CaM[3,4TnC] do not cooperate with neighboring units. This would be expected to reduce cooperativity, but the effect on tension is not clear.

Although these possibilities may help explain the effect of cTnC substitution on Ca^{2+} activation, they do not address the effect of this substitution on optimal tension. Optimal tension is enhanced above that of controls, and n_S is depressed. Interestingly, Metzger (1995) found that the n_S of skinned cardiac myocytes is 15, whereas that of slow skeletal fibers is 7 and that of fast skeletal fibers is 5. Thus cTnC in cardiac fibers is associated with an n_S that is much higher than that of fast fibers, whereas substitution into fast fibers depresses n_S below that of control fast fibers. An interesting question is, what do components of cardiac and fast fiber

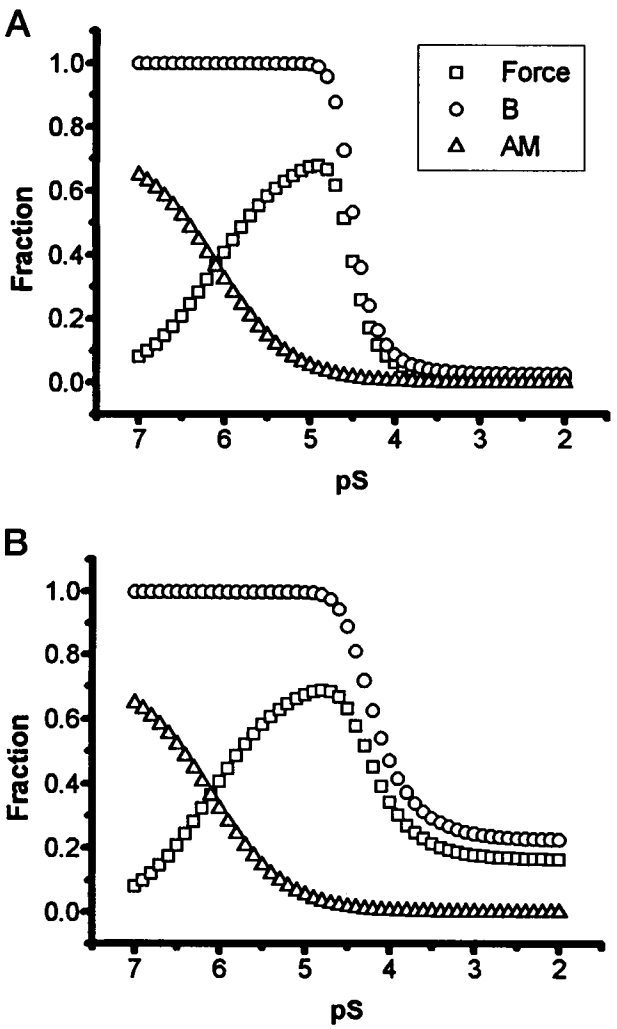

FIGURE 3 Plot of the predictions of the concerted transition formalism with cycling (CXBs) and rigor (RXB) cross-bridges as activator ligands. These are the fraction of cross-bridges in tension (□), the fraction in RXBs (△), and the fraction of thin filaments that are active (○). The effect of CXBs was studied by systematically varying the parameter $K_{CXB,B}/M_T$, that is, the normalized dissociation constant of CXBs for the active form of the regulatory strand. As this parameter decreases, CXBs become stronger activators. When the parameter is set to 60 (*A*), CXBs have no effect on the fraction of active thin filaments, and the equations generate a pS/tension relationship like that for a control fiber (see Fig. 1). When $K_{CXB,B}/M_T$ is set to 6 (*B*), there is a discernible effect of CXBs on the tension and the fraction of active thin filaments. However, under conditions where CXBs have discernible effects, the formalism predicts resting tension. This exists even in 10 mM substrate and at pCa 8 or higher. This analysis uses a system of equations published previously (Brandt et al., 1990); it is based on equilibrium kinetics, and sums together all attached cycling cross-bridge forms (CXBs), with the exception of those in rigor. About 4% of the myosin must be in rigor (RXB) to activate 50% of the maximum tension, and 10% must be in rigor to achieve optimal tension; these figures translate into about 8 and 20 RXBs per regulatory strand, respectively. The parameters used to calculate this figure using equations 3 and 4 from Brandt et al. (1990) are the same as those given in that report, except that the symbols AMS and AM were changed to CXB and RXB for this report, so that they would more obviously represent cycling and rigor cross-bridges. CXB = AMS and RXB = AM in the appendix of Brandt et al. (1990).

regulatory strands contribute to the environment that modulates cTnC's actions?

The effect of cTnC substitution on pK_2 may be a clue to the mechanism underlying cTnC's enhancement of RXB tension. A similar decrease in pK_2 and n_S with an increase in optimal tension occurs in control fibers when the background pCa is decreased from 8 to 6.5 (Brandt et al., 1972). It is doubtful, however, that a pure Ca^{2+}-like action is a model for the enhanced tension in cTnC-substituted fibers. If it were, maximum tension should be normal or enhanced. But cTnC-substituted fibers produce much less maximum tension when saturated with Ca^{2+} than do fTnC-reconstituted fibers. Potentiation of optimal tension in cTnC-substituted fibers is more likely to be due to a rearrangement of the Ca^{2+}-independent interactions of TnC than it is to the promotion of a Ca^{2+}-like effect. The cTnC substitution data also imply that the form of the regulatory complex on activation by RXBs differs from that on activation by Ca^{2+}. If so, there are three states for the regulatory strand, one relaxed and two active; one active form is favored by Ca^{2+} and one by RXB activation. This hypothesis, based on our physiological data, is in accord with three-dimensional reconstructions of relaxed, rigor, and Ca^{2+}-activated fibers (Lehman et al., 1994; Poole et al., 1996; Wakabayashi et al., 1985) and with fluorescence studies on skinned fibers labeled with DANZ-TnC (Guth and Potter, 1987). A three-state model for regulated thin filaments with one off and two active states has also been proposed (Geeves and Lehrer, 1994; McKillop and Geeves, 1993).

TnC extraction proportionately reduces maximum tension and reduces cooperativity more steeply (Brandt et al., 1984b, 1987). TnC extraction also reduces optimal tension and cooperativity by ~50% (Brandt et al., 1990; Metzger, 1995) (Tables 1–3). Replacing fTnC with CaM[3,4TnC] reduces maximum tension and cooperativity by about half (Brandt et al., 1994). Here we report this to be true also for optimal tension and cooperativity. Replacement of fTnC by cTnC reduces maximum tension and cooperativity by about 40% (Gulati et al., 1991; Moss et al., 1986). However, cTnC substitution enhances optimal tension above control levels while reducing cooperativity.

To summarize: TnC extraction minimizes optimal tension, replacement with CaM[3,4TnC] restores about half the lost optimal tension, replacement with fTnC restores most of the lost optimal tension, and replacement with cTnC results in above-control optimal tension. From these findings and those of others we conclude that TnC has three roles: 1) its historically well-defined action as the Ca^{2+} trigger for contraction (Potter and Gergely, 1975; Weber and Winicur, 1961), 2) its function as an essential link in the conduction of cooperativity along the regulatory strand (Brandt et al., 1984a,b, 1987, 1990), and 3) its ability to independently modulate RXB-activated optimal tension.

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