Isometric Force Redevelopment of Skinned Muscle Fibers from Rabbit Activated with and without Ca^{2+}

P. Bryant Chase,*‡ Donald A. Martyn,§ and James D. Hannon‡

*Department of Radiology, ‡Department of Physiology and Biophysics, and §Center for Bioengineering, University of Washington, Seattle, Washington 98195 USA

ABSTRACT Fiber isometric tension redevelopment rate (k_{TR}) was measured during submaximal and maximal activations in glycerinated fibers from rabbit psoas muscle. In fibers either containing endogenous skeletal troponin C (sTnC) or reconstituted with either purified cardiac troponin C (cTnC) or sTnC, graded activation was achieved by varying [Ca^{2+}]. Some fibers were first partially, then fully, reconstituted with a modified form of cTnC (aTnC) that enables active force generation and shortening in the absence of Ca^{2+}. k_{TR} was derived from the half-time of tension redevelopment. In control fibers with endogenous sTnC, k_{TR} increased nonlinearly with [Ca^{2+}], and maximal k_{TR} was 15.3 ± 3.6 s^{-1} (mean ± SD; n = 26 determinations on 25 fibers) at pCa 4.0. During submaximal activations by Ca^{2+}, k_{TR} in cTnC reconstituted fibers was approximately threefold faster than control, despite the lower (60%) maximum Ca^{2+}-activated force after reconstitution. To obtain submaximal force with aTnC, eight fibers were treated to fully extract endogenous sTnC, then reconstituted with a mixture of aTnC and cTnC (aTnC:cTnC molar ratio 1:8.5). A second extraction selectively removed cTnC. In such fibers containing aTnC only, neither force nor k_{TR} was affected by changes in [Ca^{2+}]. Force was 22 ± 7% of maximum control (mean ± SD; n = 15) at pCa 9.2 vs. 24 ± 8% (mean ± SD; n = 8) at pCa 4.0, whereas k_{TR} was 98 ± 14% of maximum control (mean ± SD; n = 15) at pCa 9.2 vs. 96 ± 15% (mean ± SD; n = 8) at pCa 4.0. Maximal reconstitution of fibers with aTnC alone increased force at pCa 9.2 to 69 ± 5% of maximum control (mean ± SD; n = 22 determinations on 13 fibers) and caused a small but significant reduction of k_{TR} to 78 ± 8% of maximum control (mean ± SD; n = 22 determinations on 13 fibers); neither force nor k_{TR} was significantly affected by Ca^{2+} (pCa 4.0). Taken together, we interpret our results to indicate that k_{TR} reflects the dynamics of activation of individual thin filament regulatory units and that modulation of k_{TR} by Ca^{2+} is effected primarily by Ca^{2+} binding to TnC.

INTRODUCTION

Measurements of stiffness, force, and the dynamics of thin filament regulatory proteins measured with low angle x-ray diffraction (Ford et al., 1986; Kress et al., 1986) indicate that activation of contraction by Ca^{2+} results from an increase in the availability of myosin-binding sites on actin. However, as suggested by others (Brenner, 1988; Chalovich et al., 1981), it is possible that Ca^{2+} regulates actomyosin interactions by a mechanism that involves more than simple modulation of the number of myosin binding sites on thin filaments. In the preceding manuscript (Martyn et al., 1994), we showed that cross-bridge kinetics as characterized by unloaded shortening velocity (V_{US}) were modulated by the level of thin filament activation whether force was achieved in a normal Ca^{2+}-dependent manner with sTnC or cTnC, or in a Ca^{2+}-independent manner with activating TnC (aTnC) (Hannon et al., 1993).

Both solution studies and experiments on skinned fibers indicate that Ca^{2+} regulates the kinetics of transitions within the actomyosin cross-bridge cycle (Brenner, 1988; Chalovich et al., 1981). For example, the rate of isometric tension redevelopment (k_{TR}) (Brenner and Eisenberg, 1986) has been reported to be strongly influenced by the level of Ca^{2+} activation in skinned skeletal fibers (Brenner, 1988; Metzger et al., 1989; Metzger and Moss, 1990, 1991; Millar and Homsher, 1990; Swartz and Moss, 1992; Sweeney and Stull, 1990; Walker et al., 1992). This result suggests that Ca^{2+} regulates a rate-limiting step or steps in the cross-bridge cycle during isometric force development. As with V_{US}, k_{TR} is faster in fast skeletal fibers than in slow fibers at all activation levels (Metzger and Moss, 1990); this difference persisted after exchange of fast and slow myosin light chain 2 (LC_2) isoforms in slow fibers and also persisted if contraction was activated either by Ca^{2+} or independently of Ca^{2+} by extracting whole troponin (Tn). These observations indicate that the isoform of myosin heavy chain is a primary determinant of k_{TR} (Metzger and Moss, 1990).

Although V_{US} appears to be regulated primarily through Ca^{2+} binding to TnC (Martyn et al., 1994), there is evidence that the regulation of k_{TR} may occur through Ca^{2+} binding to sites other than TnC. In fibers from which a fraction of the TnC had been removed, k_{TR} depended on [Ca^{2+}], but not the extent of thin filament activation or force (Metzger and Moss, 1991). To explain this result, it was hypothesized that a secondary Ca^{2+} binding site such as LC_2 may have a significant modulatory effect on k_{TR} (Metzger and Moss, 1991; Metzger and Moss, 1992). Observations that are consistent with a role for LC_2 in the regulation of k_{TR} include an increase in k_{TR} at submaximal [Ca^{2+}] when LC_2 is either partially extracted (Metzger and Moss, 1992) or when LC_2 is phosphorylated (Metzger et al., 1989; Sweeney and Stull, 1990).

To examine the relative contributions of thin filament regulatory units versus Ca^{2+} binding to sites other than TnC (such as LC_2) to modulation of k_{TR}, we have used aTnC to

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Address reprint requests to Donald A. Martyn, Center for Bioengineering, WD-12, University of Washington, Seattle, WA 98195.

Dr. Hannon's current address: Department of Anesthesiology, Mayo Clinic, Rochester, MN 55905.

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achieve force production in skinned rabbit psoas fibers in the absence of Ca^{2+} (Hannon et al., 1993). The results obtained at varying degrees of activation with aTnC (with aTnC activation, variations in $[Ca^{2+}]$ do not affect force) were compared with those obtained using Ca^{2+} binding to endogenous sTnC or cTnC reconstituted into fibers. Our results indicate that the variation of k_{TR} with activation, especially at submaximal levels, was influenced by the type of TnC present and presumably by the dynamics of activation of individual regulatory units. Furthermore, with sTnC or cTnC, the effects of $[Ca^{2+}]$ on k_{TR} appear to be mediated primarily through Ca^{2+} binding to TnC.

A preliminary report of these data has appeared in abstract form (Chase et al., 1993).

MATERIALS AND METHODS

Experiments were performed essentially as described in the preceding manuscript (Martyn et al., 1994). Glycerinated segments from individual, fast fibers from rabbit psoas muscle were prepared using published methods (Chase and Kushmerick, 1988). To dissect muscle fiber bundles for glycerination, rabbits were first euthanized with pentobarbital (120 mg·kg^{-1}) administered through the marginal ear vein. End compliance of the fiber segments was minimized by chemical fixation of the ends using focal microapplication of glutaraldehyde (5% in H_2O with 1 mg·ml^{-1} Na-fluorescein), with the fiber bathed in 50% glycerol/relaxing solution (v:v) and 10 mg·ml^{-1} soybean trypsin inhibitor added as a protein buffer to diffusion of fixative (Chase and Kushmerick, 1988). After fixation of the ends, fiber segments were treated for 10 min with the nonionic detergent Triton X-100 (1% v:v), and the ends were wrapped in aluminum foil T-clips for attachment to the mechanical apparatus. To obtain the unfixed fiber length (L_F), the length of the two fixed end regions was measured at the end of each experiment and was subtracted from the overall length as described (Chase and Kushmerick, 1988). At a relaxed sarcomere length (L_S) of 2.52 ± 0.03 μm (mean ± SD; n = 25), L_F was 1.55 ± 0.11 mm (mean ± SD; n = 25) and the diameter was 63 ± 10 μm (mean ± SD; n = 25). At pCa 4.0, L_S decreased to 2.34 ± 0.12 μm (mean ± SD; n = 25).

For mechanical measurements, the force transducer was a Model 400A with 2.2 kHz resonant frequency (Cambridge Technology, Watertown, MA). L_F was controlled using a model 300 servo motor (Cambridge Technology, Watertown, MA) tuned for a 300 μs step response. Helium-neon laser diffraction was used to monitor L_S continuously (Chase et al., 1993). Photomicrographs (400X) taken during steady state contractures were also used to determine L_S and diameter. Signals were recorded digitally with 12-bit resolution at a rate of 0.4–1.0 kHz per channel (1024 or 2048 points per channel) for k_{TR} measurements.

Fiber properties were maintained during continuous activation by using a protocol first described by Brenner (1983), as implemented by others (Chase and Kushmerick, 1988; Martyn et al., 1994; Sweeney et al., 1987). Measurements of isometric force and k_{TR} were made during the steady-state period before fiber shortening/restretch (Chase et al., 1993; Martyn et al., 1994; Sweeney et al., 1987). Force was normalized to cross sectional area using the measured diameter and assuming circular geometry. In maximum activating Ca^{2+} (pCa 4.0), the control force was 283 ± 83 mN·mm^{-2} (mean ± SD; n = 25); relaxed force (pCa 9.2) was 2.3 ± 0.9% (mean ± SD; n = 25) of the maximum Ca^{2+}-activated force.

k_{TR} experiments (Brenner and Eisenberg, 1986) were performed as illustrated in Figs. 1, 4, and 6. Steady-state isometric force was obtained from the initial portion of k_{TR} records (Figs. 1, 4, 6). The fiber was first shortened by ~20% L_F with a 4 L_F·s^{-1} ramp, which reduced the force to zero, followed by a rapid (600 μs), underdamped restretch to the initial isometric L_F. The subsequent tension redevelopment kinetics were characterized by: (i) the half-time of force recovery to the isometric level ($t_{1/2}$); (ii) the rate constant (k) from fitting the data to a monoexponential function, $y = A\,(e^{-kt}) + B$; and (iii) two rate constants (K, k) from fitting the data to a biexponential function, $y = C(1 - e^{-Kt}) + D(1 - e^{-kt}) + E$. An apparent rate constant was obtained by a linear transformation of the half-time estimate, extrapolating from 50 to 63.2% ($k_{TR} = (1.264 \cdot t_{1/2})^{-1}$). Exponential curve fits were performed using the Simplex method for nonlinear least-squares regression (Caceci and Cacheris, 1984). We chose the apparent rate constant estimated from $t_{1/2}$ as the best estimate of k_{TR} because: (i) it provides the least biased (i.e., model-independent) kinetic characterization; (ii) it was highly correlated with the rate constant from the monoexponential fit (R^2 = 0.947) and, thus, both estimates of k_{TR} resulted in qualitatively similar conclusions; and (iii) our data obtained without L_S control were, in general, better fit by the biexponential function than by the monoexponential function at higher forces, whereas the monoexponential fit was better at submaximal force.

Relaxing and activating solutions were calculated according to (Martyn et al., 1994; Martyn and Gordon, 1988) and contained (in mM): 5 Mg^{2+}-adenosine 5′-triphosphate (MgATP), 15 phosphocreatine (PCr), 1 orthophosphate (Pi), 15 [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), at least 40 3-[*N*-morpholino]propanesulfonic acid (MOPS), 135 $Na^+ + K^+$, 1 Mg^{2+}, pH 7.0, 250 units·ml^{-1} creatine phosphokinase (CK), and Dextran T-500 (4% w/v;

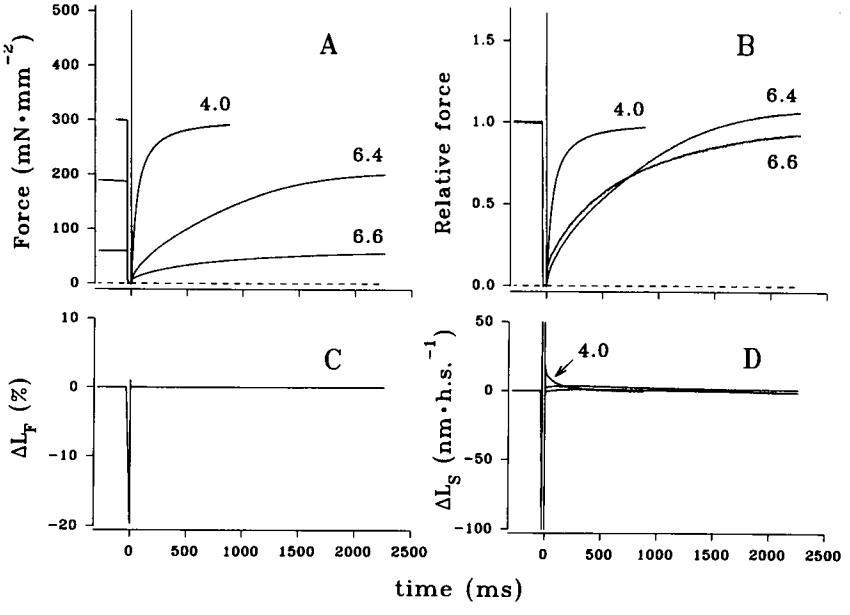

FIGURE 1 Determination of isometric tension redevelopment kinetics (k_{TR}) on a single glycerinated fiber from rabbit psoas muscle containing endogenous sTnC. Superimposed records were obtained using L_F feedback control at pCa 6.6, 6.4, and 4.0. Force was normalized to cross sectional area (A) or to the level of isometric tension immediately before the length release (B). The force baseline is indicated by a dashed line in A and B. k_{TR} derived from the half-time of force recovery was 1.6 s^{-1} at pCa 6.6, 1.7 s^{-1} at pCa 6.4, and was 16.4 s^{-1} at pCa 4.0. Corresponding records for ΔL_F and ΔL_S are shown in C and D, respectively. In terms of the relative change in L_S, the scale on the ordinate in D is expanded compared with that in C. In D the extreme, positive excursion off scale of the ΔL_S signal (>50 nm·h·s^{-1}) is an electronic artifact caused by temporary loss of the diffraction first order intensity on the detector during transient shortening. After the shortening artifact, the largest deviation in the L_S signal (<20 nm·h·s^{-1}) was recorded at pCa 4.0. L_F was 1.64 mm, and the diameter was 53.0 μm. Average isometric L_S was 2.51 μm at pCa 6.6, 2.44 μm at pCa 6.4, and 2.39 μm at pCa 4.0.

Pharmacia, Piscataway, NJ). Dextran T-500 was included in the bathing solutions to minimize covariation of myofilament lattice spacing (and fiber diameter) with force (Martyn and Gordon, 1988; Matsubara et al., 1985); fiber diameter was unaffected by activation at pCa 4.0, being 99 ± 4% (mean ± SD; n = 25) of that at pCa 9.2. The temperature was 12–13°C, and the ionic strength was 0.2 M.

TnC extraction from fibers was accomplished at 12°C by a 20 min incubation in a solution containing (in mM): 5 ethylenedinitrilotetraacetic acid (EDTA), 20 tris(hydroxymethyl)aminomethane (Tris), pH 7.2 (Cox et al., 1981) with 0.5 trifluoperazine (TFP; Aldrich Chemical Co., Milwaukee, WI) (Metzger et al., 1989). After this procedure, fibers exhibited no TnC by SDS-PAGE and no Ca^{2+}-activated force (Hannon et al., 1993); these were restored by reconstitution with purified sTnC. For sTnC or cTnC, reconstitution was allowed to proceed for 20 min (1–2 mg·ml^{-1} sTnC or cTnC) at pCa 9.2. Force increased during incubation at pCa 9.2 with 0.22 mg·ml^{-1} (~12 μM) aTnC (or a 1:8.5 molar ratio mixture of aTnC and cTnC) and was allowed to attain a steady level (10–20 min). Rabbit sTnC and cTnC were purified according to Potter (1982), and aTnC was prepared as described (Hannon et al., 1993).

Statistical comparisons were made using Excel version 4.0 (Microsoft Corporation, Redmond, WA).

RESULTS

Ca^{2+} activation of fibers with sTnC

As is evident both in individual traces from a single fiber (Fig. 1, *A* and *B*) and in the summary data (Fig. 2), k_{TR} increased nonlinearly with Ca^{2+} activation level in unextracted control fibers containing endogenous sTnC, in agreement with previous observations (Brenner, 1988; Metzger et al., 1989; Metzger and Moss, 1990, 1991; Millar and Homsher, 1990; Sweeney and Stull, 1990; Walker et al., 1992). Because of our concern about the effects of sarcomere shortening on k_{TR} measurements, as described by Brenner and Eisenberg (1986), we compared k_{TR} obtained using feedback control of either L_F or L_S (Fig. 3). L_S feedback control was implemented specifically for this comparison; during k_{TR} trials involving feedback, L_S was maintained at the pre-release value by a proportional control algorithm implemented in software. As noted previously (Brenner and Eisenberg, 1986), force recovered more rapidly (Fig. 3, *A* and *B*) and was better fit by a monoexponential function during trials with L_S control. In our study, k_{TR} measured with L_S control was 19.3% (3.6% SE) higher than that measured with L_F control (Fig. 3E), in contrast to the twofold difference observed by Brenner and Eisenberg (1986). There was a high correlation between paired measurements of k_{TR} obtained with L_S vs. L_F control (R^2 = 0.970; n = 12 pairs of observations on three fibers) over the entire range of activation examined in this study (Fig. 3E), qualitatively validating comparisons based on measurements obtained without L_S control. The absolute value for the maximum k_{TR} at pCa 4.0 obtained without L_S control (Table 1) is similar to comparable measurements made on rabbit psoas fibers (Brenner and Eisenberg, 1986; Metzger et al., 1989; Sweeney and Stull, 1990), when corrected for the effect of L_S control and differences in temperature.

The extraction/reconstitution protocol for TnC did not influence the results. Endogenous sTnC was completely extracted from two fibers, which were subsequently reconstituted with purified sTnC (Fig. 2). At all levels of Ca^{2+} activation tested, the values of k_{TR} obtained after reconstitution were indistinguishable from those obtained with unextracted control fibers (Fig. 2; ◇). Thus, the data in Fig. 2 demonstrate that k_{TR} decreased with reduced level of thin filament activation by Ca^{2+} in fibers containing sTnC, whether endogenous or reconstituted.

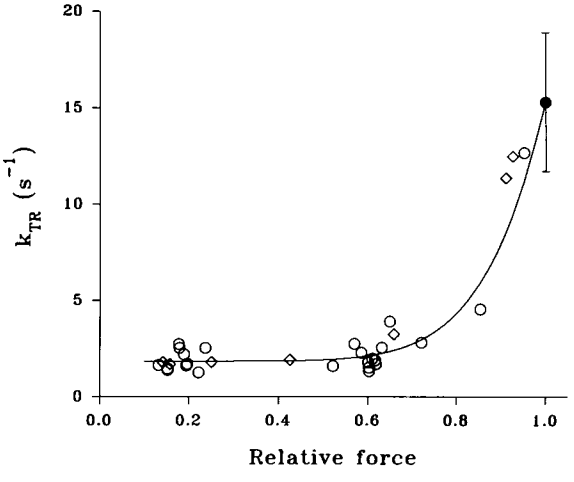

FIGURE 2 Relationship between k_{TR} and steady-state isometric force in fibers containing endogenous or reconstituted sTnC. Individual k_{TR} measurements were obtained as illustrated in Fig. 1 from 11 fibers containing endogenous sTnC at submaximal pCas (○), and the average k_{TR} at pCa 4.0 is also shown (●; mean ± SD; n = 26 determinations on 25 fibers). Force was normalized to the initial control force at pCa 4.0. Control force (100%) was 283 ± 83 mN·mm^{-2} (mean ± SD; n = 25). Data were also obtained from two fibers reconstituted with sTnC (◇) after full extraction of endogenous sTnC. The line was drawn by eye. Note the steep dependence of k_{TR} upon activation level for fibers containing endogenous sTnC.

Fibers reconstituted with aTnC

To test the hypothesis that Ca^{2+} modulates k_{TR} by binding to sites other than TnC (Brenner, 1988; Metzger and Moss, 1991), we also activated fibers to various degrees with aTnC in the presence and absence of Ca^{2+}. For comparison with results obtained from control fibers at submaximal [Ca^{2+}] (Fig. 2), partial activation was achieved by extracting endogenous sTnC and then reconstituting with a mixture of aTnC and cTnC (Martyn et al., 1994). Because such fibers contained both aTnC and cTnC, force consisted of both Ca^{2+}-sensitive and -insensitive components (Table 1). We have demonstrated that this protocol minimizes the potential for inhomogeneity in the distribution of aTnC throughout the fiber (Martyn et al., 1994). cTnC was selectively removed by a second extraction, resulting in only Ca^{2+}-insensitive force, presumably because of thin filament occupancy by aTnC only (Table 1; see Materials and Methods). At partial activation with aTnC, force was approximately 20–25% of initial control, whereas after full reconstitution with aTnC, force was approximately 70% of initial control (Table 1, Figs. 4 *A* and 5).

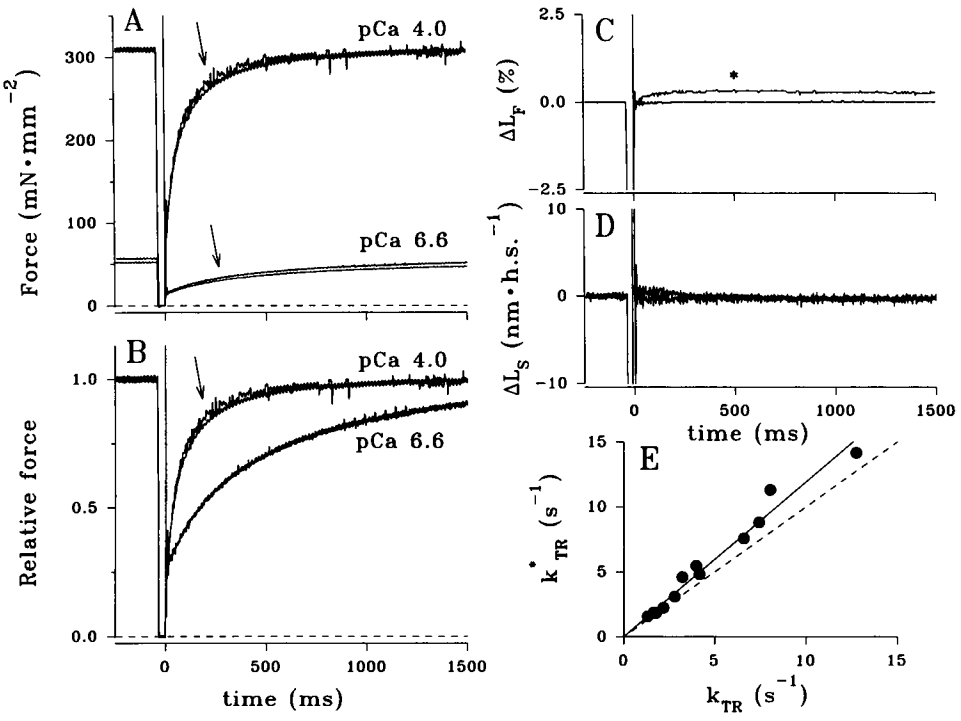

FIGURE 3 Comparison of k_{TR} measured with L_F vs. L_S control. Superimposed force records (*A*, *B*) are shown from trials on a single rabbit psoas fiber using L_F control or L_S control (*arrows*) at both 4.0 and 6.6; force was normalized to cross sectional area (*A*) or to the level of isometric tension immediately preceding the length release (*B*). In trials involving L_S control, the L_S clamp was initiated after the ramp release/restretch, and L_S during the clamp was maintained at the steady isometric value determined before the ramp release/restretch. The force baseline is indicated by a dashed line in *A* and *B*. At pCa 6.6, k_{TR} derived from the half-time of force recovery was 2.23 s^{-1} with L_S control (k^*_{TR}) and was 2.20 s^{-1} with L_F control (k_{TR}); corresponding values at pCa 4.0 were 14.2 s^{-1} (k^*_{TR}) vs. 12.8 s^{-1} (k_{TR}), respectively. Records of ΔL_F obtained during L_S control trials at pCa 4.0 (*) and pCa 6.6 are shown in *C*, and the corresponding ΔL_S traces are shown in *D* (records obtained during L_F control are omitted for clarity). Paired determinations of k_{TR} obtained from three fibers using L_S control (k^*_{TR}) vs. L_F control (k_{TR}) are shown in *E*; force was varied by altering $[Ca^{2+}]$. The linear least-squares regression, constrained to pass through the origin, is shown (——) with slope 1.193 (0.036 SE) and $R^2 = 0.970$; for comparison, the unity line $y = x$ is also shown (-----).

TABLE 1 Force and k_{TR} in fibers containing either a mixture of aTnC and cTnC or aTnC only

TnC	pCa	*n*	Normalized force	k_{TR} (s^{-1})	k'_{TR} (s^{-1})
s	4.0	26	1.00	15.26 ± 3.58	9.94 ± 2.03
a + c	9.2	14	0.16 ± 0.04	9.16 ± 0.99	7.23 ± 0.81
a + c	4.0	8	0.55 ± 0.05	6.41 ± 0.68	4.92 ± 0.33
P	9.2	15	0.22 ± 0.07	15.20 ± 2.22	11.32 ± 1.55
P	4.0	8	0.24 ± 0.08	14.81 ± 2.39	10.94 ± 1.83
F	9.2	22	0.69 ± 0.05	12.02 ± 1.27	8.70 ± 0.80
F	4.0	13	0.72 ± 0.04	11.91 ± 1.47	8.48 ± 0.94

k_{TR} was determined in control fibers containing endogenous sTnC (s). After complete extraction of endogenous sTnC (Materials and Methods), fibers were first reconstituted with a mixture of aTnC and cTnC (1:8.5 aTnC:cTnC; a + c). Subsequently, cTnC was extracted, which resulted in partial aTnC occupancy (P). Finally, the fibers were fully reconstituted with aTnC only (F). Values given are mean ± SD; *n* is the number of observations on a minimum of eight fibers. k_{TR} is derived from the half-time of force recovery; k'_{TR} is the rate constant derived from monoexponential fits to the data by nonlinear least-squares regression (Materials and Methods).

Fig. 4 illustrates the time course of tension redevelopment obtained at pCa 9.2 from a fiber that contained only aTnC. Endogenous sTnC was fully extracted, and the fiber was reconstituted with a mixture of aTnC and cTnC, followed by extraction of cTnC to obtain partial activation (P). Full activation (F) at pCa 9.2 was achieved by a subsequent incubation with aTnC. Unlike measurements obtained in control fibers, k_{TR} was similar at both activation levels (Fig. 4 *A*) and was actually faster at lower force (Fig. 4 *B*). Similar results were obtained from eight fibers (Fig. 5, Table 1). In fibers reconstituted partially with aTnC, the average value of k_{TR} was not significantly different from that obtained in control fibers at pCa 4.0 (Table 1). Full reconstitution of fibers with aTnC increased force (threefold) but resulted in a small but statistically significant decrease of k_{TR} (t-test; $p < 0.05$), presumably because of the prolonged duration of activation. During both partial and full activations with aTnC, addition of Ca^{2+} (pCa 4.0) caused no significant change in k_{TR} (Fig. 5, Table 1).

Fibers containing cTnC

To test whether the lack of activation dependence of k_{TR} in aTnC-reconstituted fibers resulted from inherent properties of cTnC, we examined the relation between k_{TR} and force in fibers containing cTnC. During the protocol for obtaining partial activation by aTnC alone, we measured k_{TR} when the fibers contained a mixture of aTnC and cTnC and, thus, Ca^{2+}-sensitive and -insensitive components. In fibers containing both aTnC and cTnC at pCa 9.2, k_{TR} was significantly lower than in control fibers at pCa 4.0 (t-test; $p < 0.05$) and was also

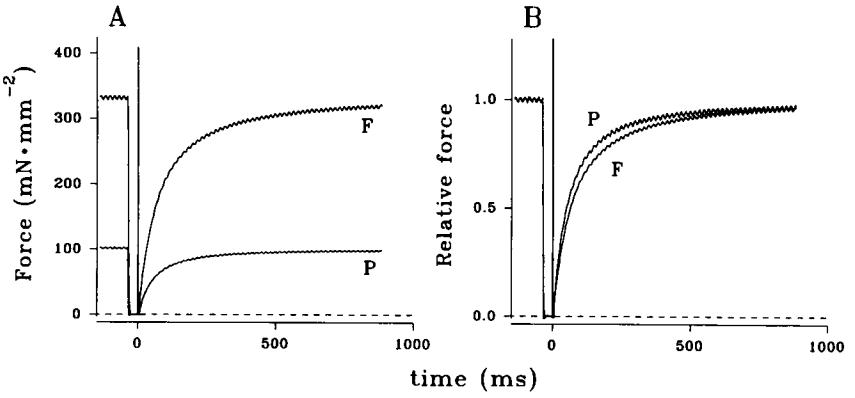

FIGURE 4 Superimposed force records during k_{TR} determinations using L_F feedback control in one fiber activated partially (trace marked *P*) or fully (trace marked *F*) with aTnC. The pCa was 9.2 in both records. Partial activation was achieved by: full extraction of endogenous sTnC, reconstitution with a mixture of aTnC:cTnC (1:8.5), and followed by extraction of cTnC. Full aTnC activation was achieved by subsequent reconstitution with aTnC only. Force was normalized to cross sectional area (*A*) or to the level of isometric tension immediately preceding the length release (*B*). The force baseline is indicated by a dashed line in both *A* and *B*. The restretch transient has been truncated in *B*. k_{TR} was 16.6 s^{-1} for partial aTnC reconstitution (*P*) and 12.8 s^{-1} for full aTnC reconstitution (*F*). L_F was 1.56 mm, and the diameter was 48.5 μm. Average isometric L_S was 2.34 μm during partial activation and was 2.21 μm at full aTnC activation.

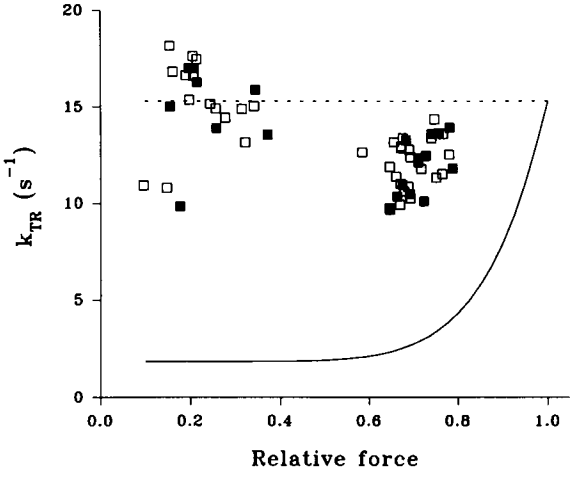

FIGURE 5 Relation between k_{TR} and the normalized steady-state isometric force obtained at pCa 9.2 (□) or pCa 4.0 (■) in fibers that contained aTnC only. Eight fibers were first partially, then fully, reconstituted with aTnC alone using the protocol involving reconstitution with a mixture of aTnC with cTnC (□, ■). Data are also included for an additional four fibers that were fully reconstituted with aTnC only. The solid line was redrawn from Fig. 2. For comparison at submaximal levels of activation, the dotted horizontal line shows the average control k_{TR} at pCa 4.0 for fibers containing endogenous sTnC. Note that k_{TR} in fibers reconstituted with aTnC was nearly the same as control (endogenous sTnC, pCa 4.0) at all levels of activation studied and was not significantly affected by altering the pCa from 9.2 to 4.0.

lower than in fibers partially reconstituted with aTnC only (t-test; $p < 0.05$) (Table 1). k_{TR} was further reduced by ~30% in fibers containing both aTnC and cTnC at pCa 4.0 (t-test; $p < 0.05$) (Table 1), suggesting that cTnC exerts a negative effect on k_{TR}.

Examination of three fibers reconstituted with cTnC showed that k_{TR} was relatively unaffected by [Ca^{2+}] and was elevated with respect to k_{TR} obtained at similar levels of Ca^{2+}-activated force before extraction of endogenous sTnC (Figs. 6, 7). Because k_{TR} in cTnC reconstituted fibers was much lower at submaximal forces than k_{TR} in fibers with partial aTnC alone, the lack of activation dependence with aTnC was not strictly caused by properties of cTnC. However, because we were unable to attain maximum control force with cTnC, we cannot be sure that the maximum possible value of k_{TR} with cTnC would not be higher. Taken together, the observed differences in the relationship between k_{TR} and force (Figs. 2, 5, 7) imply that the type of TnC influences the magnitude of k_{TR}, particularly at submaximal levels of activation.

DISCUSSION

Our experiments were designed to test the hypothesis that the kinetics of isometric force redevelopment vary with the level of force in a manner that is independent of the method of activation. We also tested the hypothesis that Ca^{2+} affects isometric tension redevelopment kinetics by binding to sites other than TnC. Our result that k_{TR} depended on the type of TnC in the fiber at similar force levels leads us to conclude that type-specific properties of TnC are important determinants of actomyosin kinetics during submaximal, isometric activation. Additionally, our result that k_{TR} was not significantly changed when the pCa was altered from 9.2 to 4.0 in fibers activated with aTnC leads us to conclude that, with sTnC or cTnC, Ca^{2+} modulates k_{TR} primarily by binding to TnC.

Role of Ca^{2+} in modulation of k_{TR}

In control fibers, k_{TR} was increased by approximately an order of magnitude over the range of pCas from 6.6 to 4.0 (Fig. 2). This result agrees with previous reports on fast fibers from rabbit (Brenner, 1988; Metzger et al., 1989; Metzger and Moss, 1992; Millar and Homsher, 1990; Swartz and

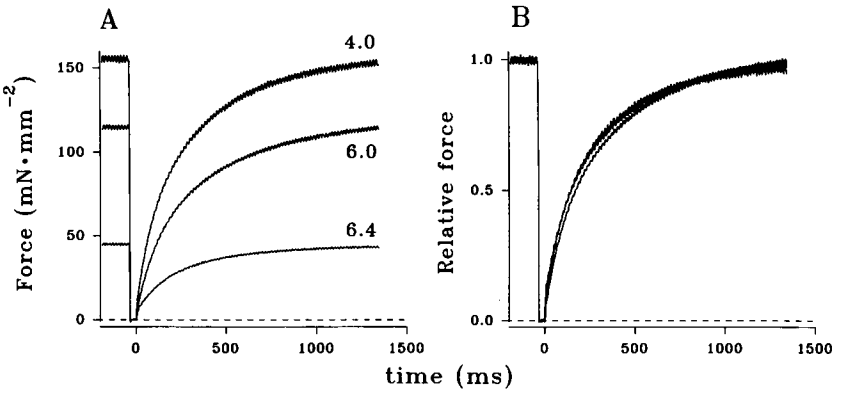

FIGURE 6 Superimposed force records obtained during k_{TR} determinations using L_F feedback control in one fiber that had been reconstituted with cTnC. pCas were 6.0, 6.4, or 4.0. Force was normalized to cross sectional area (A) or to the level of isometric tension immediately preceding the length release (B). The force baseline is indicated by a dashed line in both A and B. k_{TR} derived from the half-time of force recovery was 4.9 s^{-1} at pCa 6.6, 4.3 s^{-1} at pCa 6.4, and was 4.5 s^{-1} at pCa 4.0. The fiber is the same as in Fig. 1. L_F was 1.64 mm, and the diameter was 53.0 μm. Average isometric L_S was 2.47 μm at pCa 6.4, 2.41 μm at pCa 6.0, and 2.45 μm at pCa 4.0.

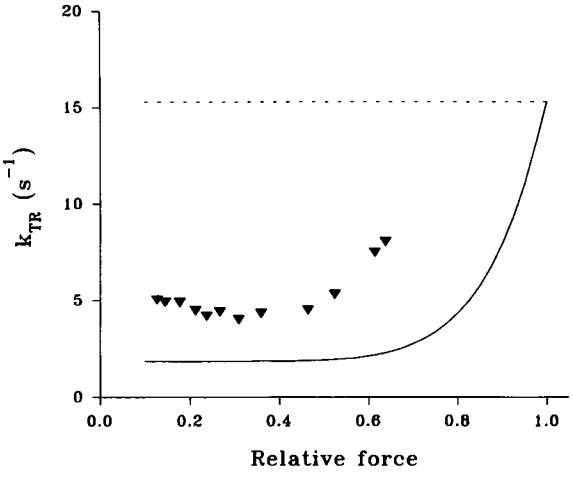

FIGURE 7 Relation between k_{TR} and steady-state isometric force in psoas fibers reconstituted with cTnC. Individual k_{TR} measurements were obtained as illustrated in Fig. 6 from three fibers at pCas from 6.6 to 4.0. Force was normalized to the initial control force at pCa 4.0 (endogenous sTnC). The solid line was redrawn from Fig. 2. For comparison at submaximal levels of activation, the dotted horizontal line shows the average control k_{TR} at pCa 4.0 for fibers containing endogenous sTnC. Note that k_{TR} in fibers reconstituted with cTnC lies above that for fibers containing endogenous sTnC.

Moss, 1992; Sweeney and Stull, 1990; Walker et al., 1992) and rat (Metzger et al., 1989; Metzger and Moss, 1990). Although we did not use L_S control during most k_{TR} measurements in our study, we demonstrated a close correlation between k_{TR} obtained with L_S vs. L_F feedback control at all levels of activation (Fig. 3 E) and found that with L_F control k_{TR} was underestimated by <20% (Fig. 3; Results). This correlation is most likely because of techniques that stabilize the striation pattern during prolonged activation (Materials and Methods) (Chase and Kushmerick, 1988). Thus, our conclusions are qualitatively unaffected by whether L_F or L_S was the feedback control variable during k_{TR} measurements.

To interpret k_{TR}, a simple two state model of steady-state force generation has been proposed in which the partitioning of cross-bridges between weakly attached, non-force-producing states versus strongly attached, force-producing states is composed of two terms (Brenner, 1988; Sweeney and Stull, 1990). The first term (nF) is affected by the number of cross-bridges turning over (Podolsky and Teichholz, 1970), whereas the second ($f/(f+g)$) is related to the kinetic equilibrium between attachment and detachment:

$$\text{force} = nF \cdot f/(f+g), \quad (1)$$

where n is the number of turning over cross-bridges, F is the average force per cross-bridge (and is assumed to be constant), f is the apparent rate constant for the transition into the force-generating state, and g is the apparent rate constant for the transition into the non-force-generating state. It is important to note that g is an isometric parameter and, therefore, is related to the $g(h)$ function for $h \geq 0$ in Huxley's (1957) two state model (i.e., g is related to $g_1(h)$ but not g_2). In this model, the rate of approach to the steady-state isometric force, k_{TR}, is:

$$k_{TR} = f + g. \quad (2)$$

Despite the known limitations of the two-state model, two recent analyses substantiating its utility concluded that f alone increased, whereas both g and nF remained constant during Ca^{2+} activation of skinned rabbit psoas fibers (Brenner, 1988; Sweeney and Stull, 1990) for forces >0.25 maximum. However, when activation is altered in a Ca^{2+}-independent manner, our results with aTnC illustrate that k_{TR} and, thus, the sum of f and g, was nearly independent of activation level (Fig. 5). If f did increase, as would be necessary to explain increased force (Eq. 1), then g must have decreased to an equal but opposite extent (Eq. 2). This is unlikely because it would imply that the ATPase rate (proportional to $n \cdot g \cdot f/(f+g)$) would depend nonlinearly on force and, over part of the range, ATPase would decrease as force increased; where ATPase has been measured in Ca^{2+}-activated skinned fibers containing sTnC, it varied linearly in proportion to isometric force (Brenner, 1988; Sweeney and Stull, 1990). Therefore, according to this model, it appears that aTnC altered force by affecting the number of cycling cross-bridges.

Such a dissociation of k_{TR} from force has also been reported under the following conditions: (i) at maximum Ca^{2+}-activation during cross-bridge inhibition by actin-binding fragments of caldesmon (Brenner et al., 1991); and (ii) at constant [Ca^{2+}] (either maximal or submaximal) after partial extraction of TnC (Metzger and Moss, 1991). These methods

of altering force, as with aTnC, most likely operate via modulation of n, rather than f or g. The observation that k_{TR} can remain constant as the level of force varies, as described above and in this study (Fig. 5), implies that cooperative interactions between adjacent regulatory units on the thin filament do not appear to play a dominant role in modulating k_{TR}, as concluded by Metzger and Moss (1991), at least for thin filaments that are partially occupied by TnC. However, we found that k_{TR} was reduced in fibers at pCa 9.2 containing both cTnC and aTnC, compared with aTnC alone (Table 1), which may imply some interactions between adjacent regulatory units. The significance of these observations is that there does not appear to be a unique relationship between k_{TR} and force; this relation is clearly influenced by the protein complement of the thin filament regulatory units and the method by which the thin filaments are activated.

Because partial extraction of TnC resulted in decreased force at constant [Ca^{2+}] without affecting k_{TR}, it was suggested that thin filament regulatory proteins play little or no role in determining k_{TR} and that Ca^{2+} modulates k_{TR} via binding to sites other than TnC (Metzger and Moss, 1991), perhaps LC_2 (Metzger and Moss, 1992). However, we found that in aTnC-activated fibers, k_{TR} was unaffected by altering [Ca^{2+}] between pCa 9.2 and 4.0 and that the absolute value of k_{TR} was similar to that obtained with control fibers at pCa 4.0 (Fig. 5, Table 1). Therefore, Ca^{2+} binding to sites other than TnC does not appear to be required for maximal activation of k_{TR} in skinned fibers.

The difference between our experiments involving thin filaments partially occupied by TnC and those of Metzger and Moss (1991) may lie in the nature of the TnC itself. aTnC is covalently stabilized in a configuration that appears functionally as if Ca^{2+} is always bound, irrespective of the solution [Ca^{2+}] (Hannon et al., 1993; Putkey et al., 1993), whereas the structure of sTnC (and cTnC) is presumably in a dynamic equilibrium between Ca^{2+}-bound and Ca^{2+}-free forms (Grabarek et al., 1992; Johnson et al., 1981). Thus, lowering [Ca^{2+}] would alter the dynamics of activation of individual thin filament regulatory units containing sTnC or cTnC, but not those containing aTnC. Therefore, it is possible that modulation of k_{TR} by Ca^{2+} in unextracted fibers is effected via a shift in the equilibrium between regulatory unit states associated with the Ca^{2+}-bound and Ca^{2+}-free forms of TnC. This interpretation could explain the Ca^{2+} dependence of k_{TR} after partial extraction of TnC (Metzger and Moss, 1991) without requiring a second regulatory site for Ca^{2+}.

Role of thin filament regulatory proteins in modulation of k_{TR}

Our data in fast fibers provide additional evidence for a central role of the thin filament regulatory proteins in modulating k_{TR}. At submaximal levels of activation, k_{TR} was elevated after reconstitution with cTnC (Figs. 6 and 7). At submaximum [Ca^{2+}], k_{TR} in fibers reconstituted with cTnC was 28–33% of the maximum obtained in control fibers, whereas k_{TR} was 9–18% of maximum in fibers containing sTnC. For comparison, we note that slow fibers, which contain the cardiac isoform of TnC (Parmacek and Leiden, 1989; Schreier et al., 1990; Wilkinson, 1980) as well as other cardiac ventricular isoforms of proteins such as β myosin heavy chain (Lompré et al., 1984) and LC_1 (Barton et al., 1985), exhibited a smaller relative change in k_{TR} than did fast fibers over the same range of pCas (Metzger and Moss, 1990). In accord with our hypothesis that the isoform of TnC affects k_{TR}, k_{TR} increased only threefold over the full range of activation in soleus fibers, compared with an 11-fold increase in fast fibers (Metzger and Moss, 1990). Although the maximum k_{TR} achievable is probably a property of the myosin isoform (Metzger and Moss, 1990), the dependence of k_{TR} upon the isoform of TnC (compare Figs. 2, 5, and 7) suggests to us that modulation of actomyosin kinetics at submaximal activation likely reflects the dynamic properties of individual thin filament regulatory units, which are influenced by the type of TnC.

Evidence suggesting that the effect of TnC isoform on k_{TR} could result from differences in Ca^{2+} binding kinetics comes from measurements of the Ca^{2+} dissociation rate constant from whole Tn complexes in solution: 23 s^{-1} for the skeletal isoform (Johnson et al., 1981) vs. 14.5 s^{-1} for the cardiac isoform in the absence of troponin I (TnI) phosphorylation (Robertson et al., 1982). Of course, validation of our hypothesis would require equivalent measurements in the intact, force-generating myofilament lattice. The importance of the dynamics of individual regulatory units in determining actomyosin kinetics is further supported by experiments using *N*-ethylmaleimide-modified myosin subfragment 1 (NEM-S1), which is thought to continuously activate individual regulatory units by tight binding to actin (Greene et al., 1987). At submaximal [Ca^{2+}] and submaximal force, NEM-S1 increased k_{TR} to the level found at full Ca^{2+} activation (Kraft et al., 1993; Swartz and Moss, 1992). Physiologically, this suggests that modulation of the kinetics of Ca^{2+} binding to TnC by cycling cross-bridges may, in turn, feed back to affect the kinetics of the actomyosin interaction itself (Gordon and Ridgway, 1987, 1990; Hannon et al., 1992).

CONCLUSION

Experiments with aTnC described here and in the preceding report (Martyn et al., 1994) have allowed us to demonstrate that Ca^{2+} regulates actomyosin kinetics primarily through binding to TnC, both during isometric force redevelopment and during unloaded shortening in skeletal fibers. Furthermore, we found that, unlike unloaded shortening (Martyn et al., 1994), isometric force redevelopment rate (and also f, in-so-far as the analysis of normal muscle activation by others (Brenner, 1988; Sweeney and Stull, 1990) is correct) was strongly influenced by the properties of TnC present and, thus, the dynamics of activation of individual regulatory units.

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