Kinetics of a Ca^{2+}-sensitive Cross-Bridge State Transition in Skeletal Muscle Fibers

Effects Due to Variations in Thin Filament Activation by Extraction of Troponin C

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ABSTRACT The rate constant of tension redevelopment (k_{tr}; 1986. *Proc. Natl. Acad. Sci. USA.* 83:3542–3546) was determined at various levels of thin filament activation in skinned single fibers from mammalian fast twitch muscles. Activation was altered by (*a*) varying the concentration of free Ca^{2+} in the activating solution, or (*b*) extracting various amounts of troponin C (TnC) from whole troponin complexes while keeping the concentration of Ca^{2+} constant. TnC was extracted by bathing the fiber in a solution containing 5 mM EDTA, 10 mM HEPES, and 0.5 mM trifluoperazine dihydrochloride. Partial extraction of TnC resulted in a decrease in the Ca^{2+} sensitivity of isometric tension, presumably due to disruption of near-neighbor molecular cooperativity between functional groups (i.e., seven actin monomers plus associated troponin and tropomyosin) within the thin filament. Altering the level of thin filament activation by partial extraction of TnC while keeping Ca^{2+} concentration constant tested whether the Ca^{2+} sensitivity of k_{tr} results from a direct effect of Ca^{2+} on cross-bridge state transitions or, alternatively, an indirect effect of Ca^{2+} on these transitions due to varying extents of thin filament activation. Results showed that the k_{tr}–pCa relation was unaffected by partial extraction of TnC, while steady-state isometric tension exhibited the expected reduction in Ca^{2+} sensitivity. This finding provides evidence for a direct effect of Ca^{2+} on an apparent rate constant that limits the formation of force-bearing cross-bridge states in muscle fibers. Further, the kinetics of this transition are unaffected by disruption of near-neighbor thin filament cooperativity subsequent to extraction of TnC. Finally, the results support the idea that the steepness of the steady-state isometric tension–calcium relationship is at least in part due to mechanisms involving molecular cooperativity among thin filament regulatory proteins.

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INTRODUCTION

During muscle contraction cross-bridges from the myosin-containing thick filament interact cyclically with binding sites on actin in the thin filament. This interaction, which requires chemical energy from the hydrolysis of adenosine triphosphate, underlies force production and shortening in muscle. Some information is available concerning the process by which chemical energy is coupled to mechanical work in striated muscle fibers (Hibberd and Trentham, 1986; Goldman and Brenner, 1987). The scheme in Fig. 1 shows biochemically identified intermediates of the actomyosin ATP hydrolysis reaction in solution. Recent findings indicate that the transition from the weakly bound state to the strongly bound state is coupled to the release of phosphate from the actomyosin complex (Hibberd, Dantzig, Trentham, and Goldman, 1985; but see Homsher and Millar, 1989). This step may in part limit the rate of formation of the force-bearing cross-bridge state in muscle fibers. On the other hand, ADP release appears to limit the rate of cross-bridge detachment, and thus limits maximum shortening velocity (Siemankowski, Wiseman, and White, 1985).

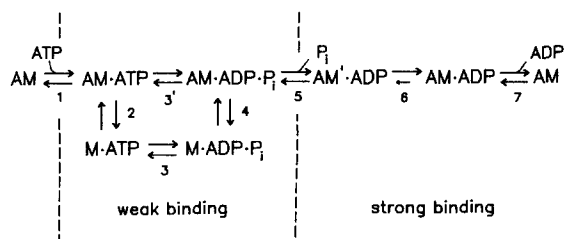An unresolved question is whether rates of transition among cross-bridge states are

FIGURE 1. Kinetic scheme of the actomyosin ATP hydrolysis reaction in skeletal muscle. A, actin; M, heavy meromyosin or myosin S1. The scheme is adapted from current models of ATP hydrolysis by actomyosin (Taylor, 1979; Sleep and Smith, 1981; Eisenberg and Hill, 1985; Hibberd and Trentham, 1986; Goldman and Brenner, 1987).

sensitive to the concentration of Ca^{2+}. A method has been developed to probe cross-bridge state transitions in muscle fibers (Brenner and Eisenberg, 1986). Once steady-state isometric force has developed, muscle length is rapidly shortened and re-extended, thereby reducing the number of attached cross-bridges to zero or nearly zero. Cross-bridges subsequently reattach and a steady-state distribution of cross-bridge states is attained. Thus, the rate of tension redevelopment is a measure of the transition(s) in the actomyosin ATP hydrolysis reaction that limits the rate of formation of strongly bound intermediates. In this study we investigated the basis of the Ca^{2+} sensitivity of the rate of tension redevelopment after the perturbations of muscle length. Earlier results showed that the rate constant of tension redevelopment (k_{tr}) increases with increased concentrations of Ca^{2+}, and is thought to be due to a direct effect of Ca^{2+} on an apparent rate constant that limits the formation of the force-bearing cross-bridge intermediate (Brenner, 1988; Metzger and Moss, 1989). However, the effect of Ca^{2+} to increase k_{tr} may instead be a manifestation of increased activation of the thin filament as the concentration of Ca^{2+} is increased. For example, at low concentrations of Ca^{2+} the transition to the strongly bound state may be slowed

due to steric constraints imposed by the partially activated thin filament. In an attempt to distinguish between these possibilities, k_{tr} was measured in skinned fibers in which thin filament activation was varied over a wide range independent of Ca^{2+} concentration. Thin filament activation was manipulated by reversibly extracting varying amounts of troponin C (TnC) from whole troponin complexes. After partial extraction of TnC, the k_{tr}–pCa relationship was unaltered while the Ca^{2+} sensitivity of steady-state isometric tension was markedly reduced as described previously (Brandt, Diamond, and Schachat, 1984; Moss, Giulian, and Greaser, 1985). Our findings are evidence that Ca^{2+} modulates k_{tr} via effects on an apparent rate constant that limits the rate of formation of force-bearing cross-bridges.

METHODS

Skinned Fiber Preparations and Experimental Apparatus

Fast-twitch skeletal muscle fibers were obtained from the superficial portion of the vastus lateralis (svl) muscles of adult female Sprague-Dawley rats and from psoas muscles of adult male New Zealand rabbits (only svl data are presented in the figures). Bundles of ∼50 fibers were dissected from each muscle while in relaxing solution (below), and were then tied with surgical silk to glass capillary tubes. Bundles were stored for up to 3 wk at −23°C in relaxing solution containing 50% (vol/vol) glycerol. Before each experiment, bundles were placed in relaxing solution containing 0.5% (wt/vol) Brij-58 for 30 min to disrupt the sarcoplasmic reticulum (Moss, 1979). Individual fibers were carefully pulled free from one end of the fiber bundle and mounted between a force transducer (model 407; Cambridge Technology, Inc., Cambridge, MA; sensitivity, 0.2 mV/μN; 1–99% response time, 100 μs; resonant frequency, ∼5 kHz; noise level at the output equivalent to 1 mg peak-to-peak) and DC torque motor (model 300s; Cambridge Technology, Inc.). The fiber was viewed through an inverted microscope (model IM; Carl Zeiss, Inc., Thornwood, NY) and its overall length was adjusted with a mechanical translator to set resting sarcomere length. Complete details of the mounting procedure and experimental set-up have been reported elsewhere (Moss, 1979; Metzger, Greaser, and Moss, 1989).

Solutions

Relaxing and activating solutions contained (in mmol liter^{-1}): 7 EGTA, 1 free Mg^{2+}, 4.4 total ATP, 14.5 creatine phosphate, 20 imidazole, 10 caffeine, and sufficient KCl to yield a total ionic strength of 180 mmol liter^{-1}. Experiments in which caffeine was not added gave qualitatively similar results except that there was a small (<0.1 pCa unit) rightward shift in the tension–pCa relationship. Solution pH was adjusted to 7.00 with KOH. Relaxing solution had a pCa (i.e., −log [Ca^{2+}]) of 9.0, while the pCa of the solution for maximal activation was 4.5. The computer program of Fabiato and Fabiato (1979) was used to calculate the final concentrations of each metal, ligand, and metal–ligand complex, using the stability constants listed by Godt and Lindley (1982). The apparent stability constant for Ca^{2+}-EGTA was corrected for ionic strength, pH, and an experimental temperature of 15°C (Fabiato and Fabiato, 1979).

Rate Constant of Tension Redevelopment

The experimental protocol for measuring the rate constant of tension redevelopment (k_{tr}) was a modification of the multistep protocol developed by Brenner and Eisenberg (1986). Measurement of k_{tr} involved a mechanical maneuver to completely dissociate myosin cross-bridges from actin in a steadily activated fiber, so that the subsequent rate of tension redevelopment reflects

the forward and reverse rate constants for the rate-limiting transition(s) in the cross-bridge cycle leading to formation of strongly bound, force-generating states. The fiber was first transferred from relaxing solution to an activating solution with pCa controlled in the range 6.4–4.5, and steady isometric tension was allowed to develop. The fiber was then rapidly (within 0.5 ms) shortened by ~200–300 nm/half-sarcomere, resulting in an abrupt reduction of force to zero, and the fiber shortened for 5–40 ms under unloaded conditions (i.e., at V_{max}). While shortening at V_{max}, the number of cross-bridges attached at any moment is ~20% of the total (Huxley, 1957; Julian and Sollins, 1975). To detach these remaining cross-bridges from actin, the fiber was rapidly (within 0.5 ms) re-extended to its initial length. Coincident with the restretch, force transiently increased due to positive straining of attached cross-bridges; however, since the amount of restretch (200–300 nm/half-sarcomere) was much greater than estimates of the working distance of the cross-bridge (~10 nm/half-sarcomere; Huxley and Simmons, 1971), attached cross-bridges dissociated and force rapidly declined to zero or very nearly zero. The redevelopment of force after this maneuver reflects the rate of reattachment of cross-bridges and the transition to strongly bound, force-producing states. During redevelopment of force, sarcomere length was held constant since in the absence of sarcomere length control k_{tr} would be underestimated due to end compliance (Brenner and Eisenberg, 1986). Sarcomere length was clamped to within 0.5 nm/half-sarcomere by servo-control of the position of the first order line of the laser diffraction pattern (Fig. 5; see also Metzger et al., 1989). Records of tension redevelopment were best fit by a first order exponential equation: $F_t = F_o$ $(1 - e^{-kt})$, where F_t is force at time t, F_o is maximum force, and k is k_{tr}. Complete details of the experimental protocol, curve fitting procedure, mechanical set-up, and sarcomere length control system are described elsewhere (Metzger et al., 1989).

Tension–pCa Relationship

At each pCa, steady isometric tension was allowed to develop, after which the fiber was rapidly (<0.5 ms) slackened to obtain the tension baseline. The fiber was then relaxed. The difference between steady tension and the tension baseline following the slack step was measured as total tension. To obtain active tension, resting tension measured at pCa 9.0 (~1% of total tension) was subtracted from total tension. The fiber was transferred to relaxing solution after each activation at a given pCa. Tension–pCa relations were determined in each fiber by expressing tensions (P) at various submaximal Ca^{2+} concentrations as fractions of the maximum value, P_o (i.e., isometric tension at pCa 4.5), obtained in each fiber at pCa 4.5. Every fourth contraction was at pCa 4.5 to monitor any decline in fiber performance (Moss, 1979).

Protocols for Extraction and Readdition of TnC

TnC was specifically extracted from the troponin complex by exposing the fiber to a solution containing 5 mM EDTA, 10 mM HEPES, and 500 μM trifluoperazine dihydrochloride (TFP; Smith, Kline and French Laboratories, Philadelphia, PA). The extraction solution was based on Cox, Comte, and Stein (1981), which we used in modified form (Zot and Potter, 1982) and with the addition of TFP (Metzger et al., 1989). The temperature during extraction was varied from 15 to 30°C. In initial experiments extraction was done at the higher temperatures since this was thought to provide a more effective means to dissociate TnC. It was subsequently found that extraction at 15°C gave similar results and was preferable since data were collected at this temperature. The time in extracting solution was also varied and greater amounts of TnC were extracted as duration was increased from 2 to 30 min. After extraction of TnC, steady isometric tension at pCa 4.5 varied between 0.04 and 0.75 P_o. Measurements of tension and k_{tr} were obtained as functions of pCa in the extracted state. Subsequently, the fiber was transferred to relaxing solution containing 0.3–0.5 mg/ml purified TnC in an attempt to reconstitute the
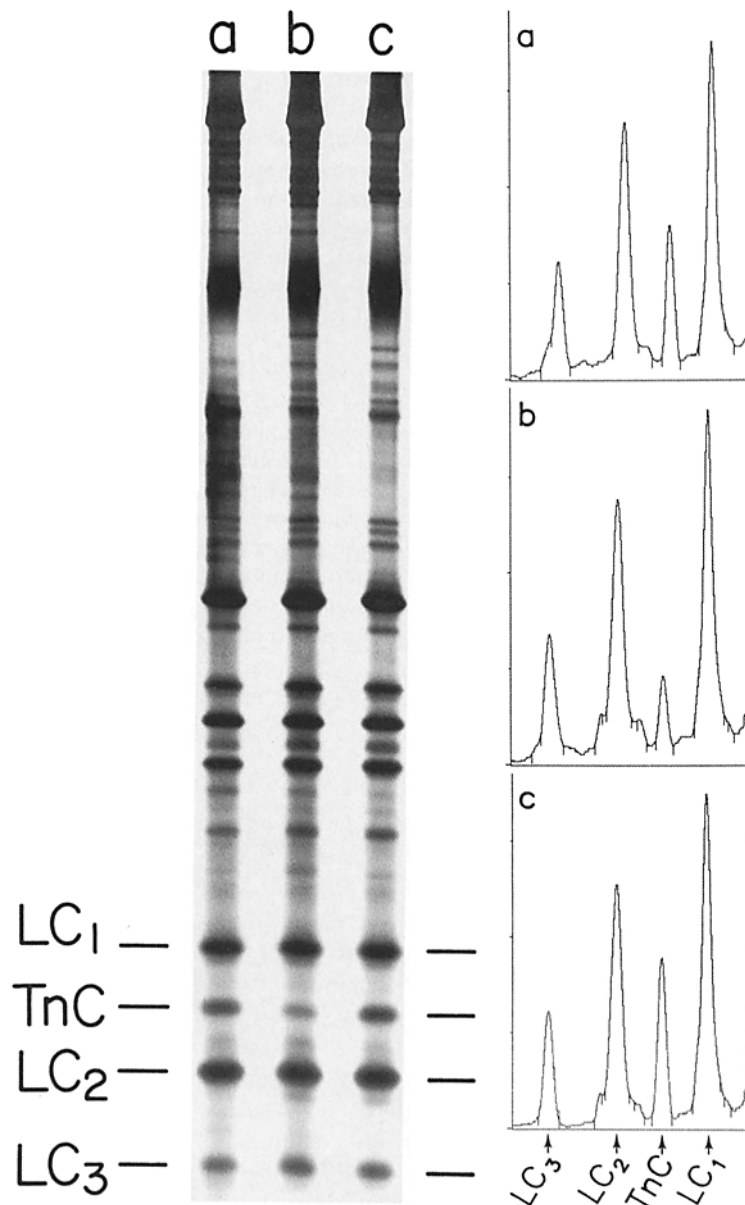
FIGURE 2. Sodium dodecyl sulfate polyacrylamide gels and densitometric scans of gels obtained from segments of a single svl fiber before (a) and after (b) the procedure to extract TnC and following readdition of TnC (c) to the extracted fiber. To quantitate the amount to TnC extracted, the ratio of $TnC/(LC_1 + LC_3)$ was determined for control, extracted, and reconstituted fiber segments by measuring the areas under the peaks corresponding to these proteins. The ratios from extracted or reconstituted fibers were then divided by the control ratio to determine the TnC content of the treated fiber. Calculated this way, 50% of endogenous TnC was extracted (b), while readdition of exogenous TnC was stoichiometric (c). Importantly, comparisons of the $LC_2/(LC_1 + LC_3)$ ratios indicated no alteration in LC_2 content by the procedure.

troponin complexes. TnC recombination was approximately stoichiometric since isometric tension increased to values greater than 0.94 P_o.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Before attachment of the fiber to the experimental apparatus, a 0.5–1-mm segment of the fiber was removed for analysis of the control protein composition by SDS-PAGE. The remaining portion of the fiber, ~5 mm in length, was mounted into connectors (Moss, 1979) to the motor and force transducer so that 2–3 mm were exposed to the bathing solution between the connectors and ~1 mm extended beyond the points of attachment. The free ends of the fiber were secured to the connecting wires with 10-0 suture and thus were exposed to the same solutions as the segment from which mechanical data were obtained. Contractile properties were determined in the control fiber and after extraction of TnC. After extraction, a 0.5–1-mm segment obtained from a free end of fiber was removed for protein analysis. Fibers were subsequently bathed in relaxing solution containing purified TnC in an attempt to reconstitute the thin filament. Mechanical measurements were obtained after readdition of TnC and a 0.5–1-mm segment of the fiber was then removed for protein analysis. Each fiber segment was placed in a 0.5-ml microfuge tube containing SDS sample buffer (10 μl/mm of segment length) and stored at −80°C for subsequent analysis of contractile and regulatory protein content by SDS-PAGE and scanning densitometry, as described previously (Moss et al., 1985). Thus, gels obtained from segments of the same fiber (control, TnC extracted, TnC add-back) were analyzed to quantitate protein composition at the different stages of an experiment (Fig. 2). Gels of fiber segments where TFP was added to the extraction solution were similar to those in the absence of TFP (Moss et al., 1985).

RESULTS

k_{tr} in Control Fibers

Original records of tension from a rat svl fiber during the protocol to determine k_{tr} are shown in Fig. 3 for various concentrations of Ca^{2+}. k_{tr} decreased from a mean maximum value of 22.9 ± 0.5 s^{-1} (20) to ~2 s^{-1} as free Ca^{2+} concentration was reduced from $10^{-4.5}$ to $10^{-6.3}$ M (see Metzger and Moss, 1990, for details). Fig. 4 presents a summary of k_{tr} as a function of steady isometric tension just before the release of muscle length, which was varied by altering the pCa of the activating solution. Of interest, the relationship between k_{tr} and relative tension shows that k_{tr} was low and essentially invariant up to ~0.50 P_o but increased dramatically at higher tensions. Thus, the relative k_{tr}–pCa relationship was shifted to the right of the relative tension–pCa relationship, indicating that the concentration of Ca^{2+} necessary for half-maximal activation (i.e., pCa_{50}) of k_{tr} was markedly greater than that for steady-state isometric tension (Brenner, 1988; Metzger et al., 1989; Metzger and Moss, 1990).

Results obtained from psoas fibers were generally similar to those from svl (e.g., pCa_{50} values obtained from tension–pCa and k_{tr}–pCa relations were 6.10 and 5.71, respectively), except that k_{tr} during activation at pCa 4.5 was significantly lower in psoas fibers (16.7 ± 0.7, n = 10).

Effect of Partial Removal of TnC on the k_{tr}–pCa Relationship

Fig. 2 shows SDS-PAGE gels of fiber segments obtained from a single svl fiber before and after the procedure to extract TnC and after readdition of TnC to the extracted
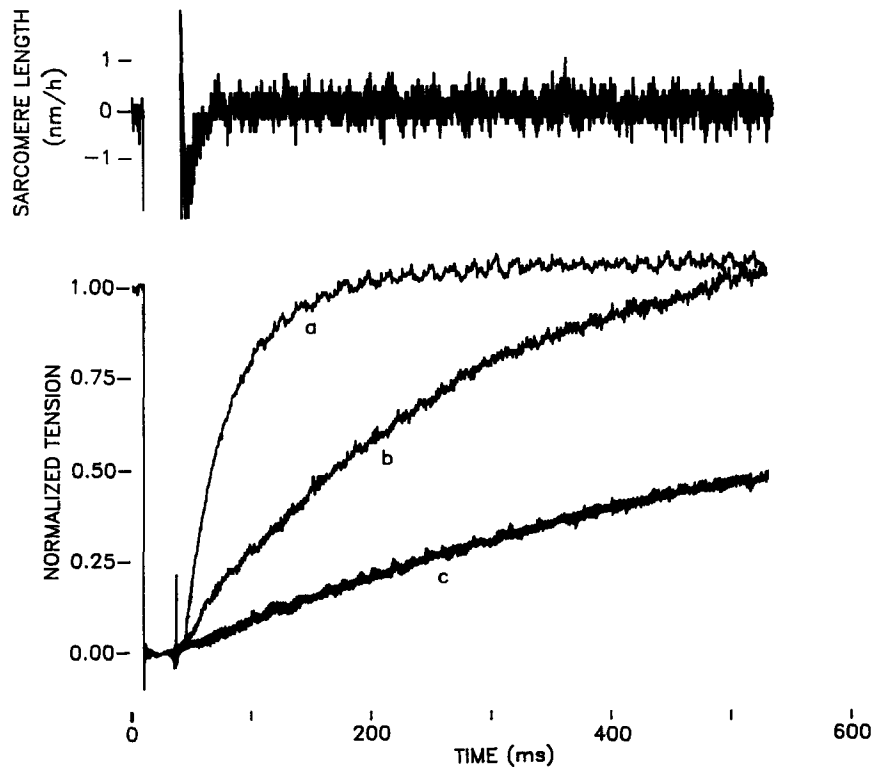
FIGURE 3. Original records of tension and sarcomere length obtained in the determination of k_{tr} in a single svl fiber at maximal and submaximal levels of Ca^{2+} activation. Record a was obtained at pCa 4.5. k_{tr} was 21 s^{-1} and steady-state isometric tension was 97 kN/m^2. Record b was obtained at pCa 6.0. k_{tr} was 5.1 s^{-1} and steady-state isometric tension was 0.54 P_o. Record c was obtained at pCa 6.2. k_{tr} was 1.8 s^{-1} and steady-state isometric tension was 0.05 P_o. In each case, sarcomere length was clamped at 2.54 μm during tension redevelopment. Fiber length was 2.48 mm.
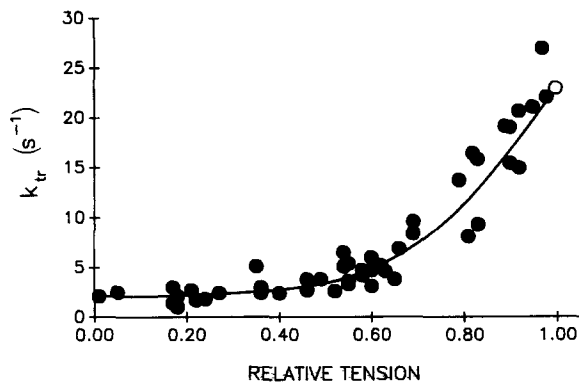

FIGURE 4. Summary of k_{tr} in svl fibers as a function of relative steady-state isometric tension before the length perturbation. Individual data points are shown, except that the open circle is the mean value ($n = 20$) obtained at pCa 4.5.

fiber. Densitometric scans of these gels indicate that the extraction procedure and add-back were specific for TnC since relative amounts of other regulatory and contractile proteins were unchanged following these procedures. These findings are similar to those reported earlier for TnC extraction and readdition in psoas fibers (Moss et al., 1985).
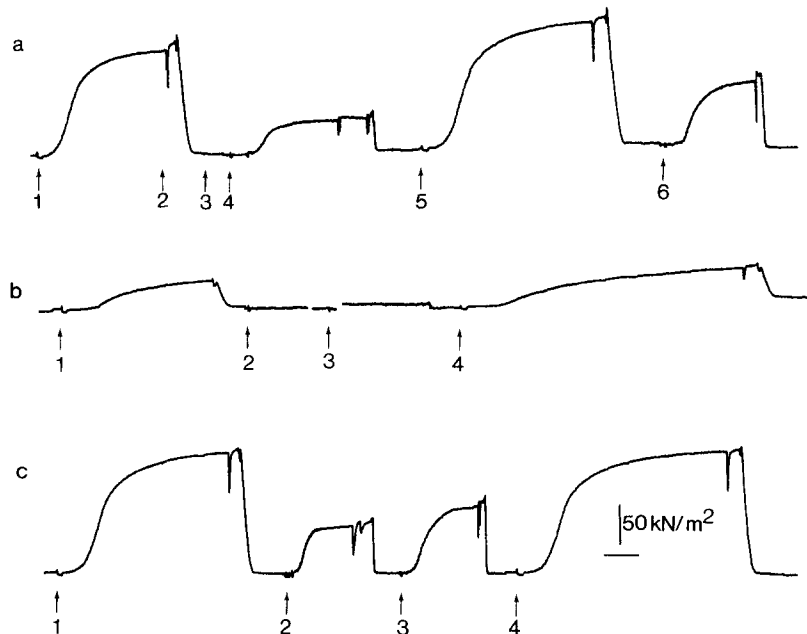

FIGURE 5. Effect of partial TnC extraction on the Ca^{2+} sensitivity of steady-state isometric tension in a single svl fiber. (a) Slow time base records of isometric tension. The fiber was activated at pCa 4.5 (1), and upon reaching steady-state tension slack was introduced to reduce tension to zero (2; not seen on slow time base) and the fiber was transferred back to relaxing solution and original length was restored (3). Subsequent activations were at pCa 6.0 (4), pCa 4.5 (5), and pCa 5.9 (6). (b) Tension records after partial extraction of TnC. Activations were at pCa 4.5 (1), pCa 6.0 (2; break in record represents 2 min), pCa 5.9 (3), and pCa 4.5 (4). (c) Tension records after reconstitution with purified TnC. Activations were pCa 4.5 (1), pCa 6.0 (2), pCa 5.9 (3), and pCa 4.5 (4). P_o was 128 kN/m^2. Horizontal calibration bar represents 10 s for pCa 4.5 records and 50 s for pCa 6.0 and 5.9 records.

Summary of Relative Tensions

	pCa 4.5	pCa 5.9	pCa 6.0
a	1.00	0.53	0.31
b	0.35	0.20	0.05
c	1.00	0.54	0.37

All values at pCa 4.5 were scaled to the control value at pCa 4.5, while values at pCa 5.9 and 6.0 were in each case normalized to tension at pCa 4.5 under the same conditions (i.e., control [a], TnC extracted [b], or TnC readded [c]).

Results in Fig. 4 indicate that at low relative tensions (that is, low levels of thin filament activation by Ca^{2+}) k_{tr} is reduced, perhaps due to an effect of the partially activated thin filament to modulate the kinetics of the cross-bridge transition from weakly to strongly bound states. The available data do not distinguish whether k_{tr} is affected directly by Ca^{2+} or modulated by a Ca^{2+}-sensitive property such as thin filament activation.

To discriminate between these mechanisms it was necessary to vary thin filament activation independently of the concentration of Ca^{2+}. This was accomplished by specifically extracting varying amounts of TnC from functional groups (defined structurally as seven actin monomers, one troponin complex, and one tropomyosin) on the thin filament. As increasing amounts of TnC were extracted there were progressive reductions in maximum Ca^{2+}-activated tension and the Ca^{2+} sensitivity of tension (Fig. 5).
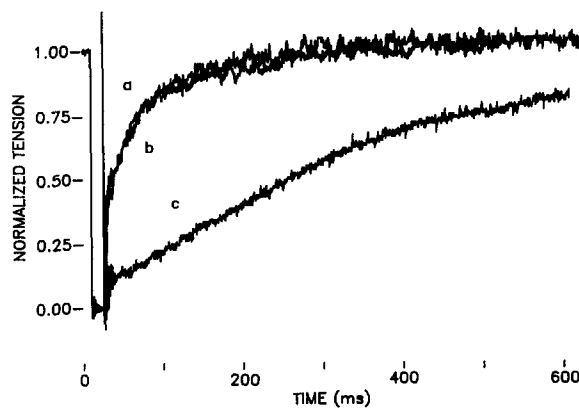

FIGURE 6. Effect of variations in thin filament activation on k_{tr} in a single svl fiber. The fiber was first activated at pCa 4.5 and k_{tr} was 21 s⁻¹ (trace *a*). Thin filament activation was reduced by increasing the pCa to 6.0. k_{tr} was 3.8 s⁻¹ and steady-state isometric tension was 0.46 P_o (trace *c*). TnC was then partially extracted so that steady-state tension at pCa 4.5 was 0.38 P_o (i.e., comparable to that in trace *c*). Under these conditions k_{tr} was 20 s⁻¹ (trace *b*) and similar to the control value at pCa 4.5 (trace *a*). End-to-end fiber length was 2.19 mm and sarcomere length during tension redevelopment was 2.52 μm (records not shown).

Fig. 6 shows records of tension redevelopment from a fiber activated with maximal (trace *a*) and submaximal concentrations of Ca^{2+} (trace *c*) before partial extraction of TnC, and at pCa 4.5 after extraction of TnC (trace *b*). Sufficient TnC was extracted so that steady isometric tension at pCa 4.5 approximated the tension at pCa 6.0 in the control. Under these conditions the rate of tension redevelopment at pCa 4.5 in the extracted fiber remained high (trace *b*) and was virtually identical to the control value at pCa 4.5 (trace *a*). Results obtained from psoas fibers were qualitatively similar (data not shown).

The relationship between k_{tr} and submaximal Ca^{2+} concentration was further explored in fibers in which thin filament activation at a particular Ca^{2+} concentration was varied by extraction and progressive add-back of purified TnC (Fig. 7). In this case, k_{tr} values at pCa 6.0 and 5.9 (Table I) were unaffected by partial extraction of TnC (Fig. 7 *b*) or following add-back of purified TnC (Fig. 7 *c*), while the Ca^{2+} sensitivity of steady tension varied markedly (tension records from this fiber are shown in Fig. 5). A composite of normalized tension redevelopment records from
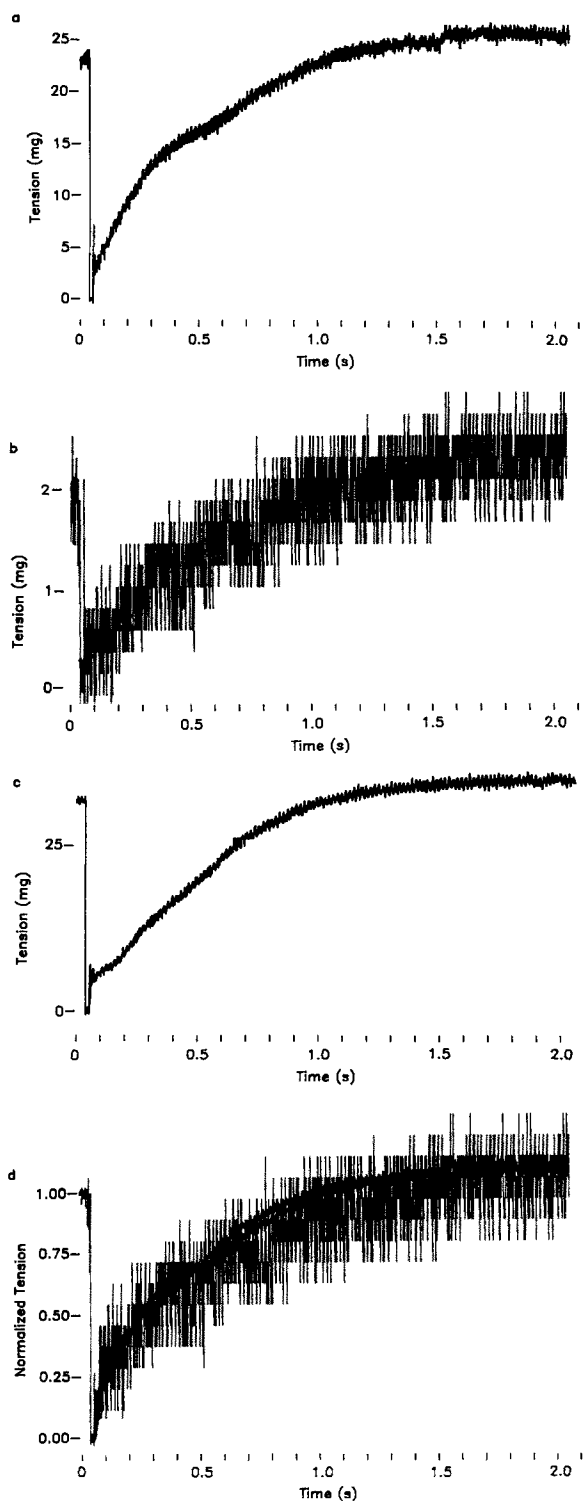
FIGURE 7. Effect of TnC extraction on k_{tr} in a single svl fiber at a submaximal concentration of Ca^{2+}. Before extraction, k_{tr} and relative tension at pCa 6.0 were 2.2 s^{-1} and 0.31 P/P_o, respectively (a; isometric tension records shown in Fig. 5). After partial extraction of TnC, tension at pCa 4.5 was 0.35 of the control tension at pCa 4.5. At pCa 6.0, normalized tension (i.e., pCa 6.0 tension/pCa 4.5 tension) was 0.05, indicating a marked reduction in the Ca^{2+} sensitivity of isometric tension, as shown previously (Brandt et al., 1984; Moss et al., 1985). However, k_{tr} after extraction of TnC was 2.1 s^{-1} at pCa 6.0 (b), a value comparable to that obtained at pCa 6.0 before extraction. In c, purified TnC was added to the fiber, resulting in complete recovery of maximum tension (1.00 P/P_o) and the Ca^{2+} sensitivity of isometric tension (i.e., pCa 6.0/pCa 4.5 tension ratio was 0.37). After reconstitution with TnC, k_{tr} was 2.2 s^{-1}. d is a composite of tension redevelopment records from a, b, and c. Fiber length was 2.18 mm and sarcomere length during tension redevelopment for records shown in panels a–c was 2.47 μm (sarcomere length records not shown).
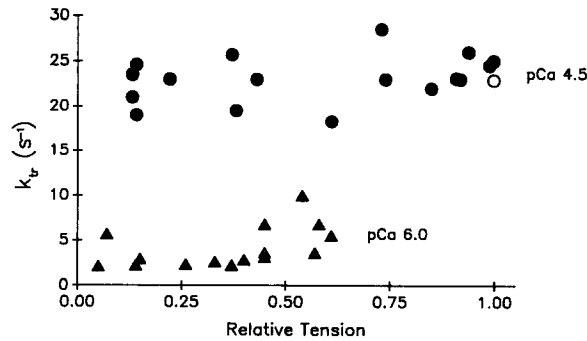
FIGURE 8. Summary of effects on k_{tr} in svl fibers due to variations in thin filament activation by partial extraction of TnC at maximal (pCa 4.5) and submaximal (pCa 6.0) concentrations of Ca^{2+}. Filled circles are k_{tr} values after the extraction of varying amounts of TnC. Open circle is mean control value at pCa 4.5. Filled triangles are k_{tr} values after the extraction of TnC at pCa 6.0. Open triangle is mean control value at pCa 6.0. Qualitatively similar results were obtained from psoas and soleus fibers (data not shown).

Fig. 7, *a*, *b*, and *c*, is shown in Fig. 7 *d*. The records of tension redevelopment superimpose, indicating that (*a*) k_{tr} is independent of thin filament activation varied by TnC extraction, and (*b*) the tension–pCa relationship bears no fixed relation to the k_{tr}–pCa relationship. Results obtained from psoas fibers also showed that k_{tr} at submaximal $[Ca^{2+}]$ was unaffected by TnC extraction despite a marked reduction in the Ca^{2+} sensitivity of steady-state tension (data not shown).

A summary of the effects of TnC extraction on k_{tr} in svl fibers at maximal and submaximal concentrations of Ca^{2+} is presented in Fig. 8 and Table I. In these experiments, various amounts of TnC were extracted so that relative isometric tension at pCa 4.5 varied between 0.13 and 0.81 P_0. In many of these fibers exogenous TnC was added to reconstitute Ca^{2+}-sensitive activation of the thin filament. In one experiment, TnC was extracted so that upon activation at pCa 4.5

TABLE I

Summaries of k_{tr} and Isometric Tension at Maximal and Submaximal Concentrations of Ca^{2+} before and after the Partial Extraction of TnC in svl Fibers

	Control	TnC extracted
pCa 4.5		
k_{tr}, s^{-1}	22.1 ± 1.0 (6)	23.1 ± 0.7 (16)
P/P_0	1.00 ± 0.0 (6)	0.30 ± 0.09 (6)
pCa 5.9		
k_{tr}, s^{-1}	3.0 (1)	3.03 ± 0.03 (3)
P/P_0	0.53 (1)	0.20 (1)
pCa 6.0		
k_{tr}, s^{-1}	3.6 ± 0.7 (5)	4.2 ± 0.6 (14)
P/P_0	0.45 ± 0.06 (5)	0.19 ± 0.09 (5)

Values are mean ± SEM (number of observations). P/P_0 value for TnC extracted data is the mean value obtained after TnC extraction (i.e., before readdition of TnC). Relative tension values obtained at pCa 5.9 and 6.0 following extraction are scaled to tension in each fiber at pCa 4.5 under the same conditions. In several experiments extracted fibers were reconstituted with exogenous TnC in a step-wise manner (see Methods) with k_{tr} data obtained at each step. This accounts for the greater *n* for k_{tr} measured in TnC extracted fibers. Following step-wise readdition of TnC, maximum tension was 0.95 ± 0.04 P_0.

isometric tension was 0.14 P_o and yet k_{tr} was 25 s^{-1}, similar to the control value of 26 s^{-1} at pCa 4.5. TnC was then added to this fiber so that upon activation at pCa 4.5 isometric tension increased to 0.94 P_o and k_{tr} was 26 s^{-1}. Qualitatively similar findings were obtained from rabbit psoas and rat slow soleus fibers (data not shown). These results show that k_{tr} varies as a function of Ca^{2+} concentration rather than the extent of thin filament activation as varied by partial extraction of TnC.

DISCUSSION

Evidence for a Ca^{2+}-sensitive Cross-Bridge State Transition in Skeletal Muscle

Previous results showed that the rate constant of tension redevelopment (k_{tr}) in skinned fibers is sensitive to the concentration of Ca^{2+} (Brenner, 1988; Metzger et al., 1989; Metzger and Moss, 1990). Here we tested whether k_{tr} depends on the concentration of Ca^{2+} per se or on a Ca^{2+}-sensitive property of muscle such as the level of thin filament activation. The level of thin filament activation was manipulated by reversible extraction of TnC, which disrupts near-neighbor molecular cooperativity among thin filament functional groups as inferred from the reduction in the Ca^{2+} sensitivity of steady-state tension (Brandt et al., 1984; Moss et al., 1985).

In untreated fibers, plots of k_{tr} versus steady-state isometric tensions showed that k_{tr} was low and virtually unchanged with tensions up to $\sim 0.50 \ P_o$ (Fig. 4). A possible interpretation of this finding is that k_{tr} is repressed at low concentrations of Ca^{2+} due to an effect of regulatory proteins in the partially activated thin filament. Our experiments were designed to vary thin filament activation over a wide range independent of Ca^{2+} concentration by extraction of TnC. Our main finding, that k_{tr} varies with [Ca^{2+}] rather than thin filament activation as modified by extracting TnC, provides evidence for a direct effect of Ca^{2+} on an apparent rate constant that limits formation of strongly bound, force-bearing cross-bridge states. Our results indicate that this cross-bridge state transition is unaffected by disrupting thin filament cooperativity by partial extraction of TnC. The results do not exclude the possibility that k_{tr} is reduced at low [Ca^{2+}] due to effects within individual functional groups, which may not be markedly affected by TnC extraction.

The cross-bridge state transition(s) that rate limits formation of the force-bearing state is unknown. Recent evidence suggests that AM'·ADP is the dominant force-bearing intermediate during contraction. For example, the rate of decline of rigor tension following the photolytic release of ATP from caged ATP has been shown to be dependent on the concentration of P$_i$, with rates increasing when [P$_i$] is elevated (Hibberd et al., 1985). Elevated [P$_i$] has also been shown to reduce steady-state isometric tension (Rüegg, Schädler, Steiger, and Müller, 1971; Hibberd et al., 1985). These findings suggest that P$_i$ release (step 5 in Fig. 1) is associated with force production in muscle. Further, k_{tr} is accelerated by increased concentrations of phosphate at maximal and submaximal concentrations of Ca^{2+} (Metzger, J. M., and R. L. Moss, unpublished observations). Thus, it seems likely that the Ca^{2+} sensitivity of k_{tr} involves, at least in part, an effect of Ca^{2+} on the phosphate release step or alternatively a state transition or isomerization that is in rapid equilibrium with the P$_i$ release step (Homsher and Millar, 1989), although there is at present disagreement as to the Ca^{2+} sensitivity of the rate of P$_i$ release (Millar and Homsher, 1990; Walker,

Lu, Swartz, and Moss, 1991). The step(s) that limits k_{tr} is unlikely to precede the P_i release step since these state transitions are thought to be in rapid equilibrium and come under the heading of weak binding states in Fig. 1 (but see Millar and Homsher, 1990). In keeping with this idea, in skinned fibers activated by photolytic release of Ca^{2+} from DM nitrophen, force development was markedly slower than the movement of cross-bridges from the thick filament toward the thin filament as determined by analysis of the 1,0 and 1,1 equatorial x-ray reflections during contraction (Brenner, Ferenczi, Irving, Kaplan, Simmons, and Towns-Andrews, 1989). This result may mean that a step (or steps) subsequent to cross-bridge attachment rate-limits the transition to the force-bearing state in skeletal muscle. Thus, at present, k_{tr} appears to be complex and is probably a manifestation of one or more state transitions in the hydrolysis scheme (Fig. 1).

Basis of Ca²⁺ Sensitivity of k_{tr}

The protein(s) involved in conferring Ca^{2+} sensitivity to an apparent rate constant in the actin–myosin ATP hydrolysis scheme is not known. It is generally thought that in mammalian skeletal muscle the contractile event is regulated solely at the level of the thin filament. Binding of Ca^{2+} to TnC initiates a series of events culminating in activation of the thin filament. Our findings show that partial removal of this protein does not alter the Ca^{2+} sensitivity of k_{tr}. However, it is plausible that TnC is involved in conferring Ca^{2+} sensitivity to k_{tr}, but via a mechanism that does not involve near-neighbor cooperativity apparent in the tension–pCa relation. That is, effects on k_{tr} due to Ca^{2+} binding to TnC may be the result of effects within a functional group rather than between functional groups.

Another possibility is that other proteins are involved in conferring Ca^{2+} sensitivity to k_{tr}. For example, recent findings suggest that myosin light chain 2 (LC_2) modulates the k_{tr}–pCa relation (Metzger and Moss, 1989). This result is intriguing since in various invertebrate muscles Ca^{2+} activates myosin–actin interaction by binding to myosin directly. In molluscan striated muscle the Ca^{2+} sensitivity of actin-activated myosin ATPase and tension is abolished by removal of LC_2 from myosin (Szent-Györgyi, Szentkiralyi and Kendrick-Jones, 1973; Simmons and Szent-Györgyi, 1978). Skeletal muscle LC_2 contains a Ca^{2+}–Mg^{2+} binding site which under steady-state conditions preferentially binds Ca^{2+} over Mg^{2+}. The role of this site in contraction under physiological conditions is unclear since Ca^{2+} would not displace Mg^{2+} during the time course of a twitch due to the slow rate of dissociation of Mg^{2+} (Bagshaw and Reed, 1977). However, during repetitive contractions Ca^{2+} would partially displace Mg^{2+} (Robertson, Johnson, and Potter, 1981), raising the possibility that this site may be important in modulating tension in vivo.

Comments on the Ca²⁺ Sensitivities k_{tr} and V_{max}

The present results provide evidence of a direct effect of Ca^{2+} on an apparent rate constant that limits formation of the force-bearing cross-bridge state. By contrast, there is disagreement as to whether Ca^{2+} affects maximum shortening velocity (V_{max}) in skeletal muscle. We have recently provided evidence that Ca^{2+} per se has no direct effect on V_{max} (except at very low levels of Ca^{2+}), but rather Ca^{2+} sensitivity of V_{max} is related to the level of thin filament activation which varies with $[Ca^{2+}]$ (Moss, 1986;

Metzger and Moss, 1988). Thus, the mechanisms underlying the Ca^{2+} sensitivity of k_{tr} and V_{max} differ qualitatively. This is consistent with the idea that the rate of tension development and maximum shortening velocity are thought to be rate limited at different points in the cross-bridge cycle (Huxley, 1957). Huxley (1957) proposed that V_{max} is determined by the detachment rate constant for cross-bridges that bear a compressive load which impedes filament sliding. V_{max} has been correlated with the rate of release of ADP from the actomyosin complex, as shown in the scheme in Fig. 1 (Siemankowski et al., 1985), which is subsequent to the transition(s) hypothesized to limit k_{tr} (i.e., transitions to strongly bound states).

That TnC extraction affects V_{max} but not k_{tr} suggests that disruption of thin filament near-neighbor interactions alters the rate of relative sliding of actin and myosin filaments but not the kinetics of the formation of the strongly bound force-bearing cross-bridge state.

Steady-State Isometric Tension: Implications of an Effect of Ca^{2+} on k_{tr}

The shape and position of the isometric tension–pCa relationship have been well documented in skinned skeletal muscle fibers. Hill plots of tension–pCa data characteristically show that the slope of the relation for tensions less than about half-maximal is quite steep in vertebrate fast-twitch muscle fibers (e.g., Brandt, Cox, and Kawai, 1980). It has been argued that the large Hill coefficients obtained from tension–pCa curves indicate the presence of molecular cooperativity within the vertebrate thin filament (Brandt et al., 1984; Moss et al., 1985). Recently, Brenner (1988) proposed that the position and steepness of the tension–pCa relation can be attributed to effects of Ca^{2+} on an apparent rate constant (f_{app}; adopted from A. F. Huxley's 1957 model) that limits formation of the force-bearing cross-bridge. In this scheme, as f_{app} (where $f_{app} = k_{tr} - g_{app}$, and g_{app} is the apparent rate constant for dissociation of strongly bound cross-bridges; see Brenner, 1988) increases with $[Ca^{2+}]$, the proportion of cross-bridges that are strongly bound and force-bearing also increases. Thus, by making f_{app} Ca^{2+} sensitive the position and steepness of the tension–pCa relationship could be markedly altered.

We sought to test the idea that cross-bridge kinetics directly determine the slope and position of the steady-state isometric tension–pCa relation. Thus, we examined the Ca^{2+} sensitivities of isometric tension and k_{tr} before and after partial extraction of TnC from the thin filament, since this procedure has been shown to (*a*) shift the tension–pCa relation to higher Ca^{2+} concentrations, and (*b*) reduce its slope. Results showed that the Ca^{2+} sensitivity of k_{tr} was unaltered by TnC extraction, thereby dissociating cross-bridge turnover kinetics and the shape and position of the tension–pCa relationship. However, our results do not exclude the complex possibility that TnC extraction had offsetting effects on apparent rate constants for formation and dissociation of the strongly bound state so that the observed rate (k_{tr}) was unchanged. That partial extraction of TnC has different effects on the Ca^{2+} sensitivities of tension and k_{tr} supports the idea that cooperative mechanisms of the thin filament regulatory system play an important role in determining the position and steepness of the tension–pCa relationship in skeletal muscle. However, dissociation of the Ca^{2+} dependence of tension and k_{tr} (Fig. 8) does not exclude the possibility that cross-bridge interaction kinetics contribute to steady-state tension. It seems

reasonable to propose that isometric tension is determined by two or more components: (a) an effect of Ca^{2+} to regulate thin filament activation via mechanisms that include cooperative interactions between regulatory proteins, and (b) an effect of Ca^{2+} to modulate the equilibrium constant for the transition(s) in the cross-bridge cycle that limits the rate of formation of force-bearing intermediates.

REFERENCES

Bagshaw, C. R., and G. H. Reed. 1977. The significance of the slow dissociation of divalent cations from myosin 'regulatory' light chains. *FEBS Letters.* 81:386–390.

Brandt, P. W., R. N. Cox, and M. Kawai. 1980. Can the binding of Ca^{2+} to two regulatory sites on troponin C determine the steep pCa/tension relationship of skeletal muscle? *Proceedings of the National Academy of Sciences, USA.* 77:4717–4720.

Brandt, P. W., M. S. Diamond, and F. H. Schachat. 1984. The thin filament of vertebrate skeletal muscle co-operatively activates as a unit. *Journal of Molecular Biology.* 180:379–384.

Brenner, B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. *Proceedings of the National Academy of Sciences, USA.* 85:3265–3269.

Brenner, B., and E. Eisenberg. 1986. Rate of force generation in muscle: correlation with actomyosin ATPase activity in solution. *Proceedings of the National Academy of Sciences, USA.* 83:3542–3546.

Brenner, B., M. A. Ferenczi, M. Irving, J. Kaplan, R. M. Simmons, and E. Towns-Andrews. 1989. Structural changes upon activation of single isolated muscle fibres of the frog by photolysis of 'caged-calcium' studied by time-resolved X-ray diffraction. *Journal of Physiology.* 418:P59. (Abstr.)

Cox, J. A., M. Comte, and E. A. Stein. 1981. Calmodulin-free skeletal-muscle troponin C prepared in the absence of urea. *Biochemical Journal.* 195:205–211.

Eisenberg, E., and T. L. Hill. 1985. Muscle contraction and free energy transduction in biological systems. *Science.* 227:999–1006.

Fabiato, A., and F. Fabiato. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal de Physiologie.* 75:463–505.

Godt, R. E., and R. D. Lindley. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *Journal of General Physiology.* 80:279–297.

Goldman, Y. E., and B. Brenner. 1987. Special topic: molecular mechanism of muscle contraction. *Annual Review of Physiology.* 49:629–636.

Hibberd, M. G., J. A. Dantzig, D. R. Trentham, and Y. E. Goldman. 1985. Phosphate release and force generation in skeletal muscle fibers. *Science.* 228:1317–1319.

Hibberd, M. G., and D. R. Trentham. 1986. Relationship between chemical and mechanical events during muscular contraction. *Annual Review of Biophysics and Biophysical Chemistry.* 15:119–161.

Homsher, E., and N. C. Millar. 1989. Tension transients induced by photolysis of caged phosphate in glycerinated rabbit soleus muscle fibres. *Journal of Physiology.* 418:P62. (Abstr.)

Huxley, A. F. 1957. Muscle structure and theories of muscle contraction. *Progress in Biophysics and Biophysical Chemistry.* 7:255–318.

Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism of force generation in striated muscle. *Nature.* 233:533–538.

Julian, F. J., and M. R. Sollins. 1975. Variations in muscle stiffness and force with increasing speeds of shortening. *Journal of General Physiology.* 66:287–302.

Metzger, J. M., M. L. Greaser, and R. L. Moss. 1989. Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. *Journal of General Physiology.* 93:855–883.

Metzger, J. M., and R. L. Moss. 1988. Thin filament regulation of shortening velocity in rat skinned skeletal muscle: effects of osmotic compression. *Journal of Physiology.* 398:165–175.

Metzger, J. M., and R. L. Moss. 1989. Further evidence that LC_2 modulates the calcium sensitivity of the rate of cross-bridge attachment in skinned skeletal muscle fibers. *Biophysical Journal.* 55:262a. (Abstr.)

Metzger, J. M., and R. L. Moss. 1990. Calcium-sensitive cross-bridge transitions in mammalian fast and slow skeletal muscle fibers. *Science.* 247:1088–1090.

Millar, N. C., and E. Homsher. 1990. The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibers. *The Journal of Biological Chemistry.* 265:20234–20240.

Moss, R. L. 1979. Sarcomere length-tension relations of frog skinned skeletal muscle fibres during calcium activation at short lengths. *Journal of Physiology.* 292:177–192.

Moss, R. L. 1986. Effects on shortening velocity of rabbit skeletal muscle due to variations in the level of thin-filament activation. *Journal of Physiology.* 377:487–505.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1985. The effects of partial extraction of TnC upon the tension-pCa relationship in rabbit skinned skeletal muscle fibers. *Journal of General Physiology.* 86:585–600.

Robertson, S. P., J. D. Johnson, and J. D. Potter. 1981. The time-course of Ca^{2+} exchange with calmodulin, troponin, parvalbumin, and myosin in response to transient increases in Ca^{2+}. *Biophysical Journal.* 34:559–569.

Rüegg, J. C., M. Schädler, G. J. Steiger, and G. Müller. 1971. Effects of inorganic phosphate on the contractile mechanism. *Pflügers Archiv.* 325:359–364.

Siemankowski, R. F., M. O. Wiseman, and H. D. White. 1985. ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proceedings of the National Academy of Sciences, USA.* 82:658–662.

Simmons, R. M., and A. G. Szent-Györgyi. 1978. Reversible loss of calcium control of tension in scallop striated muscle associated with the removal of regulatory light chain. *Nature.* 273:62–64.

Sleep, J. A., and S. J. Smith. 1981. Actomyosin and muscle contraction. *Current topics in Bioenergetics.* 11:239–286.

Szent-Györgyi, A. G., E. M. Szentkiralyi, and J. Kendrick-Jones. 1973. The light chains of scallop myosin as regulatory subunits. *Journal of Molecular Biology.* 74:179–203.

Taylor, E. W. 1979. Mechanism of actomyosin ATPase and the problem of muscle contraction. *Critical Reviews in Biochemistry.* 6:103–164.

Walker, J. W., Z. Lu, D. R. Swartz, and R. L. Moss. 1991. Thin filament modulation of cross-bridge transitions measured by photogeneration of P_i in skeletal muscle fibers. *Biophysical Journal.* 59:418a. (Abstr.)

Zot, H. G., and J. D. Potter. 1982. A structural role for the Ca^{2+}-Mg^{2+} sites on troponin-C in the regulation of muscle contraction. Preparation and properties of troponin-C depleted myofibrils. *Journal of Biological Chemistry.* 257:7678–7683.