Variations in Cross-Bridge Attachment Rate and Tension with Phosphorylation of Myosin in Mammalian Skinned Skeletal Muscle Fibers

Implications for Twitch Potentiation in Intact Muscle

JOSEPH M. METZGER, MARION L. GREASER, and RICHARD L. MOSS

From the Department of Physiology, School of Medicine and the Muscle Biology Laboratory, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT The Ca^{2+} sensitivities of the rate constant of tension redevelopment (k_{tr}; Brenner, B., and E. Eisenberg. 1986. *Proceedings of the National Academy of Sciences.* 83: 3542–3546) and isometric force during steady-state activation were examined as functions of myosin light chain 2 (LC_2) phosphorylation in skinned single fibers from rabbit and rat fast-twitch skeletal muscles. To measure k_{tr} the fiber was activated with Ca^{2+} and steady isometric tension was allowed to develop; subsequently, the fiber was rapidly (<1 ms) released to a shorter length and then reextended by ~200 nm per half sarcomere. This maneuver resulted in the complete dissociation of cross-bridges from actin, so that the subsequent redevelopment of tension was related to the rate of cross-bridge reattachment. The time course of tension redevelopment, which was recorded under sarcomere length control, was best fit by a first-order exponential equation (i.e., tension = $C(1 - e^{-kt})$ to obtain the value of k_{tr}. In control fibers, k_{tr} increased sigmoidally with increases in $[Ca^{2+}]$; maximum values of k_{tr} were obtained at pCa 4.5 and were significantly greater in rat superficial vastus lateralis fibers (26.1 ± 1.2 s^{-1} at 15°C) than in rabbit psoas fibers (18.7 ± 1.0 s^{-1}). Phosphorylation of LC_2 was accomplished by repeated Ca^{2+} activations (pCa 4.5) of the fibers in solutions containing 6 μM calmodulin and 0.5 μM myosin light chain kinase, a protocol that resulted in an increase in LC_2 phosphorylation from ~10% in the control fibers to >80% after treatment. After phosphorylation, k_{tr} was unchanged at maximum or very low levels of Ca^{2+} activation. However, at intermediate levels of Ca^{2+} activation, between pCa 5.5 and 6.2, there was a significant increase in k_{tr} such that this portion of the k_{tr}–pCa relationship was shifted to the left. The steady-state isometric tension–pCa relationship, which in control fibers was left shifted with respect to the k_{tr}–pCa relationship, was further left-shifted after LC_2 phosphorylation. Phosphorylation of LC_2 had no effect upon steady-state tension during maximum Ca^{2+} activation.

Address reprint requests to Dr. Joseph M. Metzger, Room 129 SMI, Department of Physiology, School of Medicine, University of Wisconsin, 1300 University Avenue, Madison, WI 53706.

In fibers from which troponin C was partially extracted to disrupt molecular cooperativity within the thin filament (Moss et al. 1985. *Journal of General Physiology.* 86:585–600), the effect of LC_2 phosphorylation to increase the Ca^{2+} sensitivity of steady-state isometric force was no longer evident, although the effect of phosphorylation to increase k_{tr} was unaffected by this maneuver. Readdition of purified troponin C to the extracted fibers restored the effect of phosphorylation to potentiate force at submaximal levels of Ca^{2+} activation. Thus, the mechanism of phosphorylation-dependent increases in steady-state isometric force appears to involve modulation of the effect of cross-bridges bound to actin to cooperatively enhance the Ca^{2+} activation of the thin filament. The observation of a phosphorylation-dependent increase in the Ca^{2+} sensitivity of k_{tr} likely has important implications in terms of dynamic contractile function in living muscle fibers and may provide a molecular basis for the phenomena of posttetanic twitch potentiation and treppe in vertebrate striated muscle.

INTRODUCTION

Beginning with the observation by Perrie et al. (1973) that the 20,000-dalton light chain (LC_2) subunit of vertebrate skeletal muscle myosin can be phosphorylated, there has been considerable interest in the role of phosphorylation in contraction. Barany and Barany (1977) subsequently demonstrated a marked increase in the phosphate content of LC_2 during a single tetanic contraction in frog skeletal muscle, and they proposed that LC_2 phosphorylation has a regulatory role in the interaction of actin and myosin. However, biochemical results indicated that the phosphorylation of LC_2 was not essential for actin-activated myosin adenosinetriphosphatase activity (Morgan et al., 1976), suggesting that the role of phosphorylation in skeletal muscle is modulatory rather than regulatory. More recently, a close temporal relationship between posttetanic twitch potentiation and LC_2 phosphate content in fast-twitch skeletal muscle was shown (Manning and Stull, 1979, 1982). Consistent with this observation, in slow-twitch muscle, which does not exhibit posttetanic potentiation, LC_2 was found to incorporate relatively small amounts of phosphate following tetanic stimulation (Manning and Stull, 1982). Furthermore, during treppe, potentiation of tension was correlated with increased phosphate content of LC_2 (Klug et al., 1982). Although these results are consistent with the idea that LC_2 phosphorylation underlies the phenomenon of twitch potentiation, a molecular basis for the effect has not been determined.

Studies on skinned fiber preparations, from which the sarcolemma has been chemically or mechanically removed to allow direct control of the solution bathing the contractile apparatus, have provided new information concerning the function of LC_2 phosphorylation in vertebrate muscle contraction. For example, steady-state isometric force at saturating concentrations of Ca^{2+} is unchanged by LC_2 phosphorylation, indicating that force per cross-bridge is unaffected by phosphorylation; however, at submaximal concentrations of Ca^{2+} isometric force increases after LC_2 phosphorylation (Persechini et al., 1985). Furthermore, studies on mammalian skinned skeletal muscle fibers have shown that maximum shortening velocity is unaltered after LC_2 phosphorylation (Persechini et al., 1985), suggesting that the detachment rate of negatively strained cross-bridges, g_2 in Huxley's (1957) model, is not modulated by LC_2 phosphorylation.

Although the finding that LC_2 phosphorylation results in an increase in isometric tension at submaximal levels of Ca^{2+} may ultimately prove to be a significant aspect of its physiological role, the magnitude of this effect is insufficient to explain post-stimulation twitch potentiation in which tensions can approach the tetanic value. In the present study, the effect of LC_2 phosphorylation upon cross-bridge interaction with actin was examined further. The specific question addressed in this work is whether the rate of cross-bridge attachment to actin binding sites is affected by LC_2 phosphorylation. By means of skinned single fibers, the rate of force redevelopment after a step release and reextension in overall muscle length was examined in control fibers and in the same fibers after LC_2 phosphorylation. Under sarcomere length control, the derived rate constant of force redevelopment relates to Huxley's (1957) parameter f, the rate constant of cross-bridge attachment (Brenner, 1986; Brenner and Eisenberg, 1986). Our results indicate that phosphorylation increases the rate of cross-bridge attachment at intermediate levels of activating Ca^{2+}. During the Ca^{2+} transient of a twitch, an increased rate of cross-bridge attachment would result in a greater rate of rise in force, thereby providing a basis for both posttetanic potentiation and treppe in vertebrate striated muscle.

A brief report of these findings has previously been made to the Biophysical Society (Metzger et al., 1988c).

METHODS

Skinned Fiber Preparation and Apparatus

Fast-twitch skeletal muscle fibers were obtained from psoas muscles of adult male New Zealand rabbits and from the superficial portion of vastus lateralis (svl) muscles (100% type IIB; Baldwin et al., 1972) of adult female Sprague-Dawley rats. Bundles of ~50 fibers were tied to glass capillary tubes with surgical silk, and stored at $-22°C$ for up to 3 wk in relaxing solution (described below) containing 50% (vol/vol) glycerol. Before each experiment, bundles were placed for 30 min in cold relaxing solution containing 0.5% Brij-58 to minimize the Ca^{2+} uptake capacity of the sarcoplasmic reticulum (Moss, 1979). In relaxing solution, individual fibers were pulled free from one end of the bundle and mounted between a force transducer (model 407; Cambridge Technology, Inc., Cambridge, MA; sensitivity, 2 V/g; 1–99% step response time, 100 μs; resonant frequency, ~5 kHz; noise level at the output equivalent to 1 mg-wt) and a DC torque motor (model 300s; Cambridge Technology, Inc.). Details of the experimental apparatus and mounting procedure have been reported elsewhere (Moss, 1979; Moss et al., 1983); however, the connector system has been modified (see *inset*, Fig. 3). The fiber was viewed through a model IM inverted microscope (Carl Zeiss, Inc., Thornwood, NY) and overall fiber length was adjusted to set resting sarcomere length by the use of a three-way positioner on which the motor was mounted. In the present study, average overall fiber length was 2.47 ± 0.07 mm ($\bar{x}$ ± SE; n = 27) with sarcomere length averaging 2.58 ± 0.02 μm in relaxing solution. During maximum isometric contraction mean sarcomere length was 2.52 ± 0.02 μm. Steady-state isometric force at pCa 4.5 averaged 56.4 ± 3.9 mg-wt (i.e., 107.1 ± 12.1 kN/m²).

Solutions

Relaxing and activating solutions contained, in mM: EGTA, 7.0; free Mg^{2+}, 1; ATP, 4.42; creatine phosphate, 14.5; imidazole, 20; and sufficient KCl to adjust ionic strength to 180 mM. Solution pH was adjusted to 7.00 with KOH. The pCa ($-\log$ free $[Ca^{2+}]$) of relaxing

solution was 9.0, whereas the pCa required for maximum isometric force was 4.5. The final concentrations of each metal, ligand, and metal-ligand complex in solution were determined using the computer program of Fabiato and Fabiato (1979) and the stability constants reported by Godt and Lindley (1982). The apparent stability constant for Ca^{2+}-EGTA was corrected for temperature (15°C), ionic strength, and pH (Fabiato and Fabiato, 1979).

Myosin Light Chain 2 Phosphorylation Protocol

The protocol to phosphorylate myosin light chain 2 consisted of 6–10 cycles of alternating between maximal Ca^{2+} activations of ~0.25–0.5 min in duration and relaxation of the fiber
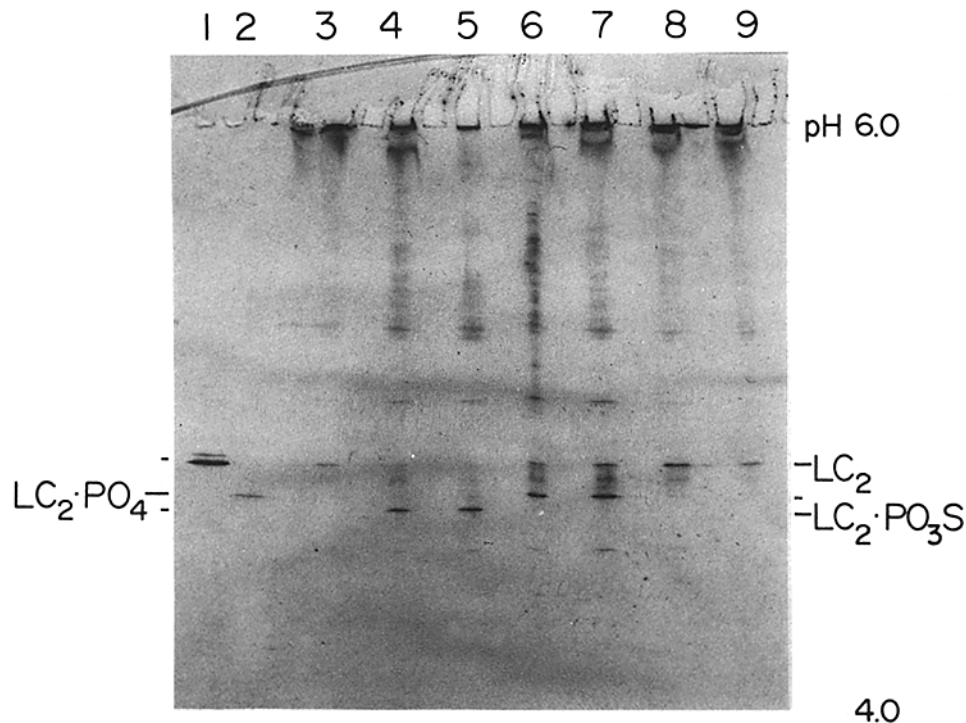

FIGURE 1.   Isoelectric focusing gel of muscle samples. Lanes *1* and *3*, rabbit LC_2 standard; lane *2*, rabbit phosphorylated LC_2 standard; lanes *4* and *5*, rabbit skinned fibers after protocol to thiophosphorylate LC_2 using ATPγS (not discussed in this paper); lanes *6* and *7*, rabbit skinned fibers after protocol to phosphorylate LC_2; lanes *8* and *9*, control skinned fibers.

segment in solutions (described above) containing 6 μM calmodulin and 0.5 μM myosin light chain kinase (MLCK). MLCK was purified by the method of Nagamoto and Yagi (1984) with the following modifications: (1) the soluble extract was chromatographed on DEAE cellulose DE52 using a 0–0.3 M NaCl gradient; (2) the MLCK fractions were mixed with 0.5 mM phenylmethylsulfonyl fluoride and 4 mg/liter of leupeptin before the addition of $CaCl_2$ and application to the calmodulin affinity column (no diisopropylflurophosphate was used). Calmodulin was purified from bull testes using the procedures of Dedman et al. (1977). The concentrations of MLCK and calmodulin used in the present study are similar to estimates of intracellular values in intact fast-twitch skeletal muscle fibers (Pires and Perry, 1977; Shenol-

ikar et al., 1979). The degree of phosphorylation was determined by high-voltage, vertical slab, isoelectric focusing (pH range of 4.0–6.0; Giulian et al., 1984) of fiber segments, 5–7 cm in total length, that were bathed in the same solutions as the experimental segments. The gels were subsequently stained with Coomassie R-250 Brilliant Blue, destained, dried to mylar backing (Giulian et al., 1984) and scanned using a laser light scanning densitometer (Biomed Instruments Inc., Chicago, IL). Percent phosphorylation of LC_2 was calculated as the area under the LC_2 phosphorylation peak on the scan divided by the sum of the areas under the LC_2 and LC_2 phosphorylation peaks. Isoelectric focusing gels showed that our protocol increased LC_2 phosphorylation from ~10% in controls to >80% after treatment (Fig. 1).

Rate Constant of Tension Redevelopment

The experimental procedure for measuring the rate constant of tension redevelopment (k_{tr}) was a modification of a multistep mechanical protocol developed by Brenner and Eisenberg (1986). This protocol resulted in a reduction to zero of the number of cross-bridge attachments to actin in a steadily activated fiber, so that the subsequent redevelopment of force reflected the rate of cross-bridge reattachment. The skinned fiber was first bathed in activating solution of pCa in the range 6.5–4.5 and steady isometric force was allowed to develop (Fig. 2). Subsequently, slack equivalent to 200–300 nm per half sarcomere was rapidly (within 1 ms) introduced at one end of the fiber, force abruptly fell to zero, and the fiber was permitted to shorten for 5–30 ms under unloaded conditions (i.e., at V_{max}). This maneuver reduces the proportion of available cross-bridges that are attached from ~80% in the isometric fiber to ~20% at V_{max} (Huxley, 1957; Julian and Sollins, 1975; Brenner, 1983). Dissociation of the remaining cross-bridges was accomplished by rapidly reextending overall muscle length to the initial value. Coincident with this restretch, force transiently increased because of positive straining of attached cross-bridges. These positively strained cross-bridges rapidly dissociated since the imposed length change (~200 nm per half sarcomere) was much greater than estimates of the working distance of a cross-bridge (~5 nm; Ford et al., 1977), and force dropped to zero. The redevelopment of force after this procedure is then a result of the reattachment of cross-bridges to actin binding sites along the thin filament.

Sarcomere Length Control System

To determine accurately the rate constant of tension redevelopment, it was necessary to maintain sarcomere length constant throughout the period of tension redevelopment, since in the absence of sarcomere length control k_{tr} is underestimated owing to compliance at the fiber-connector junction (Brenner and Eisenberg, 1986). To do this, a servo system was designed which effectively clamped sarcomere length to within 0.5 nm per sarcomere by controlling the position of the first-order line of the laser diffraction pattern from these fibers (Figs 2 and 3). As schematically shown in Fig. 3, the beam of a helium-neon laser (model 157; Spectra-Physics Inc., Mountain View, CA; wavelength, 632.8 nm; power equivalent to 3 mW; beam diameter, 800 μm) was directed to the fiber using a front-surfaced mirror. The first-order (1°) diffraction line, the position of which is inversely related to sarcomere length, was detected by a lateral effect photo diode (model LSC-5D; United Detector Technologies, Hawthorne, CA) positioned 6 cm above the preparation. Fiber orientation with respect to the laser beam was adjusted in the horizontal plane to maximize the 1° intensity at the photodiode. The position of the photodiode was also adjusted so that the 1° line was incident upon the midpoint of the detector. In our experimental setup (Fig. 3) the zero and second-order lines were positioned well away from the detector. Voltage signals corresponding to the intensity of the 1° line and its position relative to the midpoint on the detector were input to the state variable controller. In this study the total number of sarcomeres sampled by the laser averaged 320, which represented approximately one-third of the total number of sarcomeres
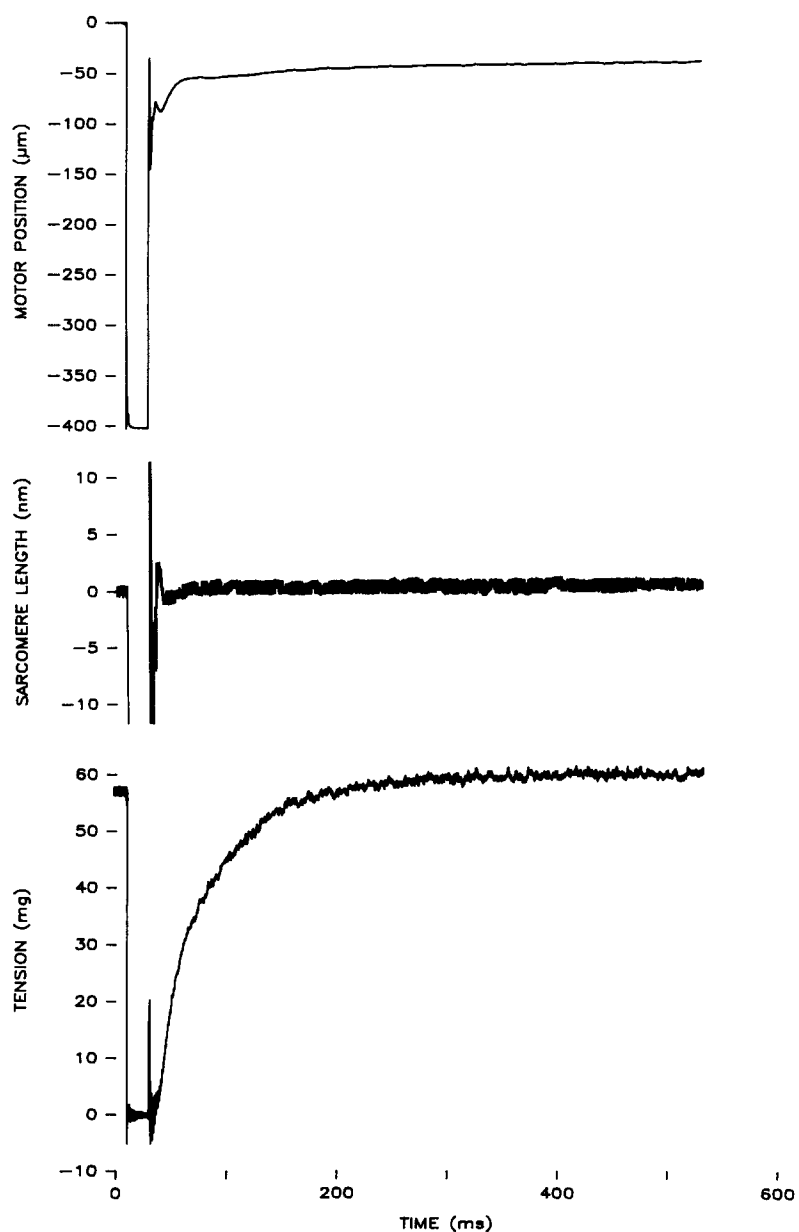
FIGURE 2. Original records of motor position (*upper trace*), sarcomere length (*middle trace*), and tension (*lower trace*) during the experimental protocol (described in text) to estimate the rate of cross-bridge attachment in skinned single fibers. The fiber (svl, No. 657) was maximally activated (pCa 4.5) and steady-state isometric force was allowed to develop; subsequently, the computer-initiated test sequence commenced with a step release of 400 μm in overall muscle length (*upper trace*), which reduced force to zero and the fiber shortened at its maximum unloaded velocity. Motor position was held constant for 20 ms and was then rapidly returned to its original position. This rapid restoration in muscle length represented an extension of 209 nm per half sarcomere, and resulted in a transient rise in force, owing to positive straining of attached cross-bridges, followed by a fall in force to zero due to dissociation of attached cross-bridges. The subsequent redevelopment of tension represents the rate at which cross-bridges reattach to binding sites along the thin filament. To assess accurately the rate constant of tension redevelopment it was necessary to insure that sarcomere length remained constant throughout the entire phase of tension redevelopment (see text). In our system sarcomere length was clamped to within 0.5 nm per sarcomere of the average value sampled during the 10 ms immediately preceding the initial step release in muscle length. In this experiment sarcomere length was maintained at 2.50 μm throughout the entire phase of tension redevelopment. The computer-determined rate constant (Fig 6) for the data shown was 23 s^{-1}. Muscle length was 2.39 mm.
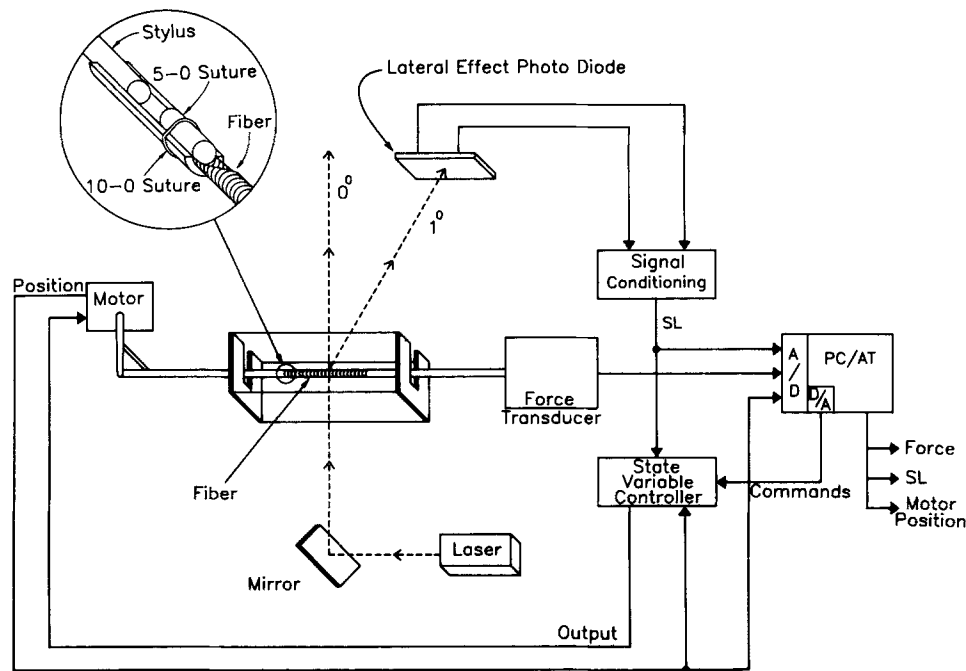
FIGURE 3.   Schematic representation of the experimental apparatus. The light from the He-Ne laser is reflected upward into the experimental chamber (Moss, 1979), so that it is incident upon the fiber segment. Lenses were not required to focus the laser light on the fiber. The position of the resultant 1° diffraction line is detected by a lateral effect photo diode situated above the experimental chamber. The voltage signal corresponding to the position of the 1° line (which is inversely related to sarcomere length) is input to the analog state variable controller. An IBM PC/AT computer monitors and records sarcomere length (*SL*), motor position, and force data. In an "open loop" experimental sequence, computer initiated commands are effected by the motor and sarcomere length is passively recorded; i.e., motor position is the ultimate control variable. In a "closed loop" experimental sequence, sarcomere length is the ultimate control variable; i.e. the system works in a null servo regulation mode. (*Inset*) Schematic diagram of the connector used to attach a skinned fiber to the apparatus, modified from an earlier design (Moss, 1979). A trough is fashioned from 29-gauge stainless steel tubing (Small Parts, Inc., Miami, FL) and is attached to a stylus from either the motor or force transducer using an epoxy resin (EPO-TEK 302; Epoxy Technology Inc., Billerica, MA). The end of the fiber is secured in the trough by overlaying a 0.5-mm section of 5–0 monofilament suture, which in turn is tied in place with 10–0 suture. This connector system minimizes the end compliance of the skinned preparation (Moss, 1986; Metzger and Moss, 1987; 1988*a*), which is critical for application of sarcomere length control.

in a particular preparation. This large sampling population makes it unlikely that there were significant changes in the population of sarcomeres sampled during the mechanical measurements. Depending on the objectives of an experiment, the state-variable controller was operated either in an open loop mode to servo-control overall muscle length and passively detect 1° line position or in a closed loop mode to servo-control sarcomere length. In the present experiments, servo-control of sarcomere length was initiated 3 ms after the reextension of overall muscle length and was maintained throughout the entire phase of tension redevelop-

ment. In all experiments, sarcomere length was clamped to within 0.5 nm of the mean value obtained by sampling sarcomere length for 10 ms just before the initial release step. Output signals from the state variable controller were used to drive the torque motor. During an experimental trial, force, motor position, and sarcomere length data were digitized at a rate of 10 kHz and then recorded and stored using an IBM PC/AT computer.

Calibration of the sarcomere length control system involved an initial demonstration of linearity between overall muscle length (i.e., motor position) and average sarcomere length. Fig. 4 shows command ramp lengthening and shortening of sarcomere length and the corresponding motor position, which is controlled as an intermediate variable to control sarcomere length. The relationship between sarcomere length and overall muscle length is linear.
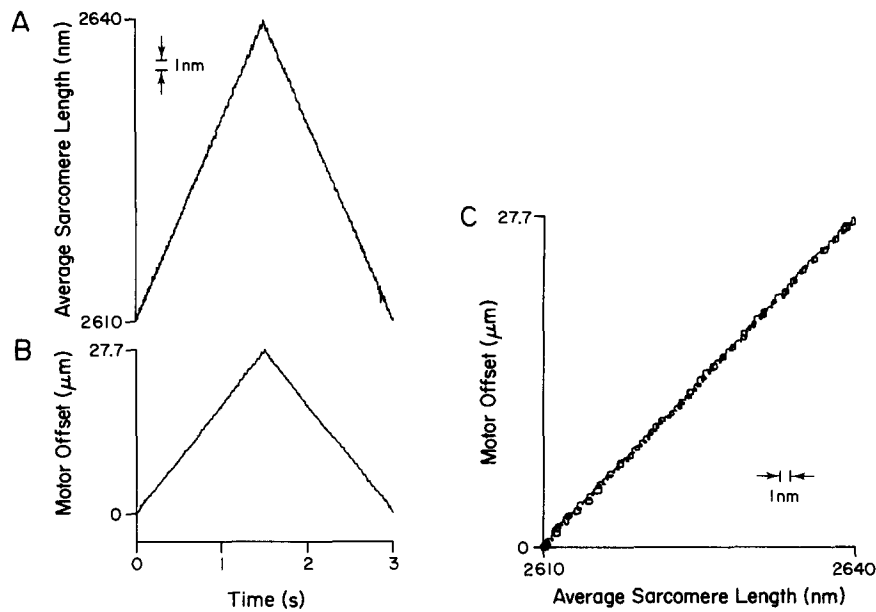

FIGURE 4.   Relationship of sarcomere length to muscle length. A ramp extension and shortening of 30 nm per sarcomere was accomplished by changes in overall muscle length (motor offset), and the resultant changes in (A) sarcomere length and (B) motor position were recorded. (C) Motor offset plotted against sarcomere length demonstrating linearity within this operating range with a resolution of 0.5 nm per sarcomere. Results are from a skinned single rabbit fiber in relaxing solution (pCa 9.0). Initial muscle length was 2.41 mm.

This linear relationship holds despite the fact that the detector is nonlinear since a linear or nearly linear relation exists for small excursions about an operating point. Results in Fig. 4 indicate that resolution on the detector is on the order of 3 μm, which is better than the absolute resolution of 10 μm stated by the manufacturer. A greater differential resolution is obtained here since we are operating at the midpoint of the detector's range and deviating small amounts about an operating point. The results presented in Fig. 4 show no evidence of stepwise sarcomere length changes within a resolution of 0.5 nm per sarcomere. This result differs from those of Pollack (1986) who reported stepwise changes in sarcomere length with ramp shortening of muscle length in unstimulated skeletal muscle fibers.

Prior to an experiment, two procedures were performed to calibrate the sarcomere length feedback system for each fiber. First, the voltage derived from the position of the 1° diffrac-

tion line was calibrated in terms of nanometers per sarcomere. To accomplish this, square wave commands were applied to release and extend overall muscle length by ± 10 μm. The total length change (20 μm) was divided by the number of sarcomeres in series and this value was then divided by the corresponding change in the peak-to-peak amplitude of the 1° position voltage obtained during the 20-μm excursion. Changes in sarcomere length, in nanometers per sarcomere, during an experiment could thus be obtained directly from the position of the 1° diffraction line. Calibration factors varied from fiber to fiber and were between 40 and 60 nm per sarcomere/V. The output range of the detector is $\sim \pm 6$ V, which translates to 2.4 V/mm on the detector. Next, the servo-response to a command step increase of $\sim$30 nm
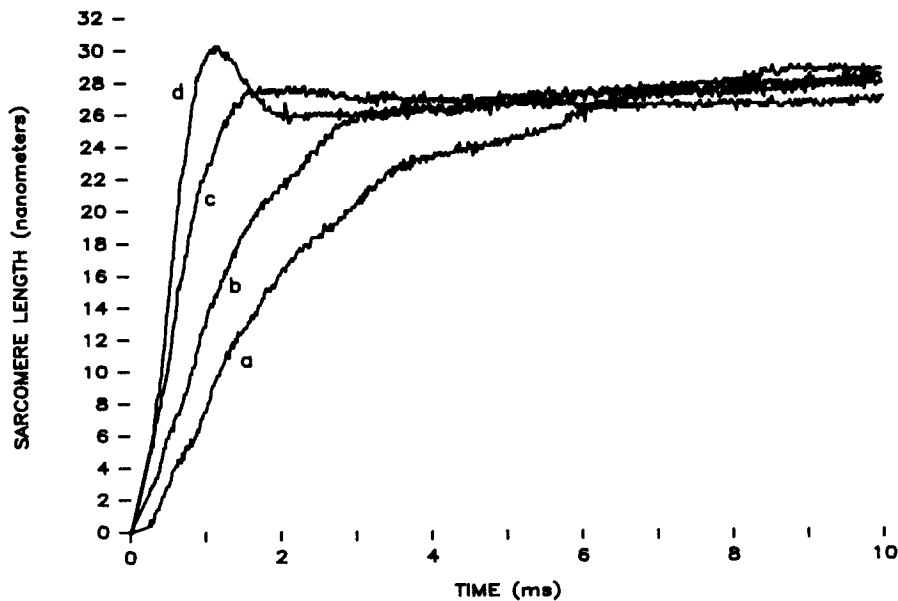

FIGURE 5.   Sarcomere length step-response test. A command step increase in sarcomere length of 54 nm per half sarcomere was introduced at zero time. (*a*) Record of sarcomere length as a function of time with low intergration gain. (*b–d*) Gain was sequentially increased to improve sarcomere length response time. For each preparation, the fastest response time possible without significant oscillatory excitation was used (e.g., record *c*). Rabbit psoas fiber No. 8128. All records obtained in relaxing solution; initial sarcomere length set at 2.50 μm.

per sarcomere was optimized by adjusting the controller integration gain. In our system, the 10–90% rise time averaged 1 ms (Fig. 5); however, in some preparations values as low as 0.5 ms were obtained.

Data Analysis/Curve Fitting

In all cases, the rate of force redevelopment was best described in terms of a first-order exponential equation:

$$f_t = F_{SS} (1 - e^{-k_{tr}t});$$

$$t_{1/2} = \ln (2)/k_{tr},$$

where F_{SS} is the final steady-state force, f is the force at time t, k_{tr} is the rate constant of

tension redevelopment, and $t_{1/2}$ is the time at which force was 0.5 F_{SS}. A computer algorithm employing a least squares fit was used to solve for k_{tr}. The curve fitting routine was initiated by estimating F_{SS} from a visual inspection of the data. The program then solved for k_{tr} and generated an exponential line which deviated from the actual data by a least squares–determined error of x. The F_{SS} value was then increased and decreased by 7% and new values of k_{tr}
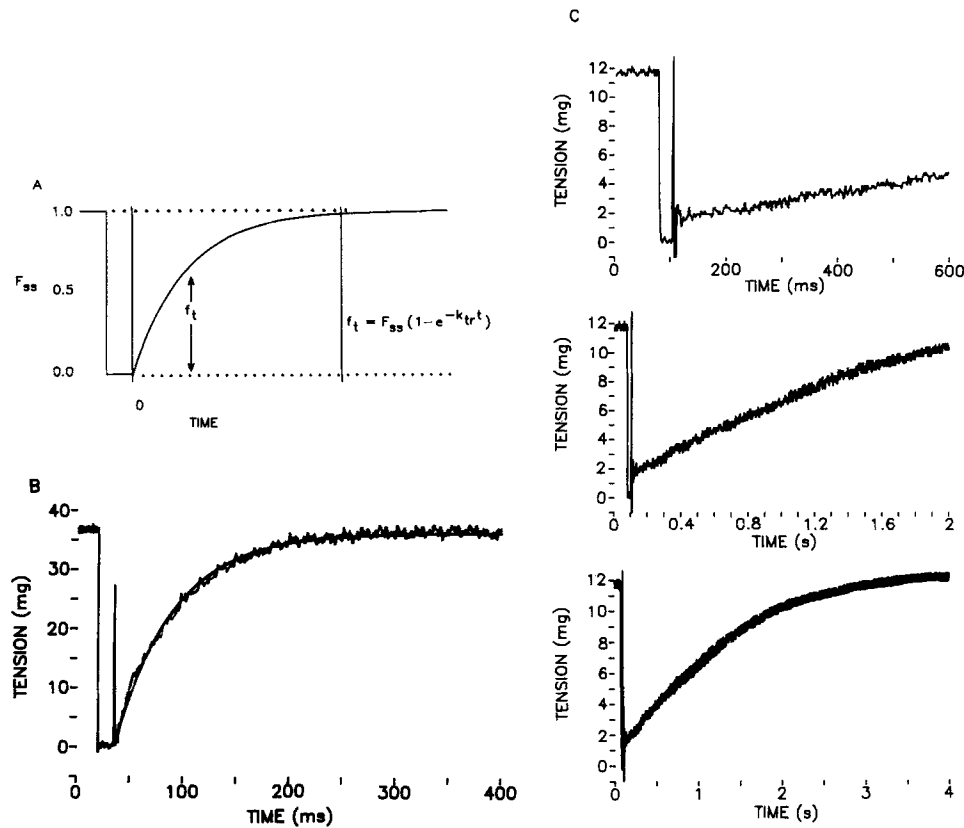

FIGURE 6. Computer data analysis for the determination of k_{tr}. (A) Schematic diagram of an experimental record showing the measured variables and the equation used for determining k_{tr}. F_{ss} is the final steady-state tension value, while f_t is the tension at time t. A computer-based binary search of the experimental data was performed using the least squares fit method (see text) to obtain k_{tr}. The portion of the curve to be fit is delineated by the solid and dotted lines. (B) Experimental record obtained during maximum Ca^{2+} activation (line noise on trace is introduced via the motor) along with the computer generated line fit to the data (smooth line). Experimental record was obtained from rabbit psoas fiber No. 688. k_{tr} in this example was 18 s^{-1}. Sarcomere length was maintained at 2.53 μm throughout the entire phase of tension redevelopment. Muscle length was 2.70 mm. (C) Results from a single experiment (psoas fiber No. 3218) at submaximal $[Ca^{2+}]$ (i.e., pCa 6.4) plotted on three different time scales. Lowest trace includes computer-derived best fit line (smooth line). k_{tr} was 0.9 s^{-1}. Results indicate that at all levels of Ca^{2+} activation the rate of tension redevelopment is well fit by a first-order exponential equation. Also, on a longer time scale, force is shown to re-develop to the prerelease value. Sarcomere length was maintained at 2.50 μm throughout the entire phase of tension redevelopment. Muscle length was 2.35 mm.

and x were derived. The computer-generated line with the least amount of error was kept in memory whereas the others were discarded. F_{SS} for the best line was then increased and decreased by 3.5%, again keeping the line which deviated the least from the experimental record. This binary search was terminated after four additional cycles (i.e., F_{SS} varied by $\pm 0.22\%$) and the resultant computer-generated line with the least amount of error was used to determine the values of k_{tr} reported in this study. In nearly all cases, the best-fit computer-generated line superimposed the experimental data (Fig. 6). In the few instances where the two results did not agree, the data were discarded. Such records were typically distorted as a result of large changes in the position of the motor necessary to achieve the reference sarcomere length in preparations that had higher than usual end compliance.

To facilitate comparisons between experimental records obtained under widely varying conditions, records have for the most part been plotted on the same time scale (i.e., 600 ms). For records obtained at low levels of Ca^{2+} activation this time base was too short to show complete force recovery. However, on a longer time scale it was evident that force completely recovered and the time course was well fit by a first-order exponential equation (Fig. 6 c).

Tension–pCa Relationship

At a particular pCa, total steady-state isometric force was determined as the difference in force immediately before the step release in muscle length and the force baseline subsequent

TABLE I

Assessment of Performance of Fibers from Which k_{tr} Data Was Obtained

	Relative tension	Total number of activations	Number of activations at pCa 4.5
Psoas	1.02 ± 0.02 (12)	24.5 ± 1.9 (12)	9.9 ± 0.5 (12)
svl	1.01 ± 0.02 (6)	20.0 ± 3.3 (6)	7.8 ± 1.4 (6)

Relative tension value obtained in each fiber by dividing the final pCa 4.5 value by initial pCa 4.5 value. Values expressed as mean $\pm$ SE (n). The table does not include fibers used in TnC extraction studies, which are described in Results and Fig. 16.

to the release. Active force was calculated by subtracting resting tension (force at pCa 9.0, which was typically <1 mg-wt) from total force. Steady-state isometric forces (P) at submaximal concentrations of Ca^{2+} were scaled relative to the maximum isometric froce (P_0) obtained in the same fiber at pCA 4.5. Every fourth contraction was performed at pCa 4.5 to assess any alterations in fiber performance (Moss, 1979). Force values after LC_2 phosphorylation were expressed as a fraction of the force developed by the same fiber at pCa 4.5 before phosphorylation. As summarized in Table I there was no significant change in tension at pCa 4.5 throughout the course of an experiment.

Troponin C Extraction/Readdition Protocols

Varying amounts of troponin C (TnC) were extracted from skinned single fibers using a solution containing 5 mM EDTA, 10 mM HEPES, and 500 μM trifluoperazine dihydrochloride (TFP; Smith, Kline and French Laboratories, Philadelphia, PA). TFP, an antagonist of Ca^{2+} binding to calmodulin, markedly reduced the extraction time necessary for TnC removal. The addition of TFP to the extracting solution was based upon (a) the structural homology between calmodulin and TnC (Watterson et al., 1980) and (b) an expectation that TFP would destabilize the binding of the C subunit to the troponin complex since binding of divalent cations to high-affinity binding sites is necessary for maintaining the structural integrity of

troponin (Zot and Potter, 1982). Extraction temperature was maintained at 15°C, and the duration was varied between 2 and 14 min in different fibers to achieve a maximum Ca^{2+} activated force of 0.46 ± 0.06 P_0 after extraction.

To subsequently restore full Ca^{2+} sensitivity, the TnC-extracted fibers were bathed in relaxing solution containing 0.3–0.5 mg/ml TnC purified from rabbit skeletal muscle (Moss et al., 1985). Fibers were exposed to this solution for short periods of time (10–20 s) and then returned to a relaxing solution with no TnC added. Fibers were cycled through this procedure an average of four times until the isometric force at pCa 4.5 was restored to control levels or until successive cycles did not further augment force. In this study, force at pCa 4.5 averaged 0.98 ± 0.01 P_0 after TnC recombination. Experiments in which TnC was extracted in the absence of TFP gave similar results.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

After each experiment a portion of the fiber segment was placed in a 0.5-ml microfuge tube containing SDS buffer (10 μl/mm of fiber length), and then stored at −80°C for subsequent analysis of the contractile and regulatory protein content by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as detailed elsewhere (Giulian et al., 1983; Moss et al., 1985). To determine the extent of TnC extraction, paired gels were run on segments obtained from each of these fibers before and after the TnC extraction procedure (Moss et al., 1985). Representative gels of control svl (Metzger and Moss, 1987) and psoas fibers (Moss et al., 1985) and TnC-extracted psoas fibers (Moss et al., 1985) have been published.

Statistical Analysis

A two-way analysis of variance (ANOVA) was used to test whether LC_2 phosphorylation and/ or $[Ca^{2+}]$ significantly affected either isometric force or k_{tr}. ANOVA was also used to determine if there was an interaction between the effects due to phosphorylation and those due to variations in $[Ca^{2+}]$. Where significant interactions were found, a Student's two-tailed t test was used to test for significant differences between two means. A level of $P < 0.05$ was chosen as indicating significance. All values are reported as mean ± standard error of the mean.

RESULTS

Effect of LC₂ Phosphorylation on Steady-State Isometric Tension

Original slow time-base recordings of isometric force obtained from a skinned psoas fiber at different levels of Ca^{2+} activation before and after phosphorylation of LC_2 are shown in Fig. 7. In agreement with earlier studies (Persechini et al., 1985; Sweeney and Kushmerick, 1985) increasing the phosphate content of LC_2 from ~10% to 80% had no effect on maximum force at saturating levels of Ca^{2+} (i.e., pCa 4.5); tensions of 154 kN/m² were obtained both before and after treatment. However, at submaximal levels of Ca^{2+} activation (i.e., pCa 6.1) steady isometric force after phosphorylation of LC_2 was 57.1 kN/m², which was markedly increased relative to the control value of 13.4 kN/m² obtained from the same fiber. Results summarizing the effect of LC_2 phosphorylation upon the relationship between steady-state isometric force and pCa in rabbit psoas fibers are shown in Fig. 8. Steady isometric force at pCa 4.5 averaged 1.03 ± 0.03 P_0 ($n = 9$) after phosphorylation. At submaximal concentrations of Ca^{2+}, which in control fibers resulted in isometric forces ≲0.60 P_0, relative tension was significantly increased by LC_2 phosphorylation.
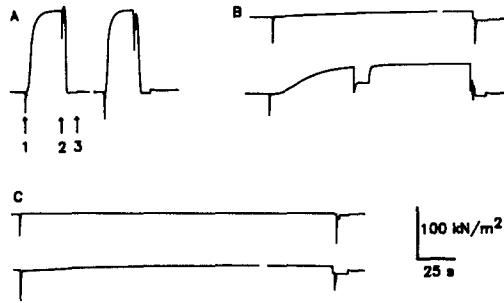
FIGURE 7. Original slow time-base recordings of isometric force at various levels of Ca^{2+} activation before and after LC_2 phosphorylation in a single psoas fiber (No. 1017). (A) Isometric force at maximal levels of Ca^{2+} activation (i.e., pCa 4.5) before (*left*) and after the protocol to phosphorylate LC_2 (*right*). Maximum isometric force was 154 kN/m^2 before and after LC_2 phosphorylation. (B) Isometric force at a submaximal concentration of Ca^{2+} (i.e., pCa 6.1) before (*top record*) and after phosphorylation of LC_2 (*lower record*). Steady isometric force was 13.4 kN/m^2 in control and increased to 57.1 kN/m^2 after phosphorylation. (C) Isometric force at a low level of Ca^{2+} activation (pCa 6.2) before (*top record*) and after phosphorylation of LC_2 (*lower record*). Steady isometric force was 1.26 kN/m^2 before and 8.8 kN/m^2 after phosphorylation. Force and time-base calibrations are shown at lower right. At the arrow marked 1, the fiber was transferred from relaxing solution to the calcium activating solution. At point 2, overall muscle length was rapidly (<1 ms) reduced by ~400 μm and force fell to zero (not seen on slow time base) so that total tension could be measured. The fiber was then returned to relaxing solution and muscle length was reextended to the prerelease value at point *3*. The gaps shown in the tension records at B (*top*) and C (*lower*) represent ~1 min.

For example, at pCa 6.3 the mean relative tensions were 0.27 ± 0.06 and 0.41 ± 0.05 (*n* = 4) in the control and phosphorylated fibers, respectively. Qualitatively similar results were obtained in rat svl fibers. At pCa 4.5, isometric force averaged 1.00 ± 0.02 P_0 (*n* = 4) after phosphorylation. At pCa 6.0, values scaled to each fiber's control force at pCa 4.5 were 0.66 ± 0.04 and 0.77 ± 0.03 (*n* = 6) in control and LC_2 phosphorylated fibers, respectively. Resting tension, which was measured at pCa 9.0, was unaffected by the state of phosphorylation of LC_2.
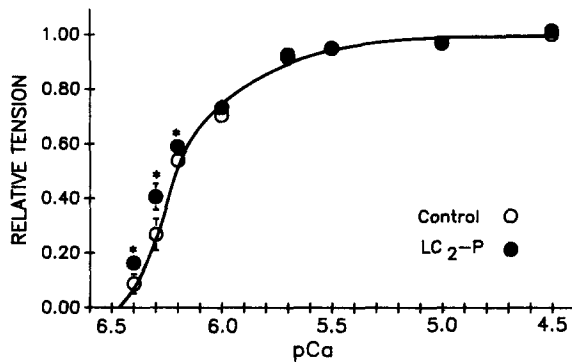

FIGURE 8. Summary of the effect of LC_2 phosphorylation upon the steady-state isometric tension–pCa relationship of rabbit psoas fibers. (O) Control data; (●) data from LC_2-phosphorylated fibers (LC_2-P). Tension has been scaled to the control tension measured in the same fiber at pCa 4.5 which in this study averaged 56 mg (107 kN/m^2). Values are mean ± SE. Average of six observations per value. Asterisks indicate that the LC_2-phosphorylated value is significantly greater than control value at the same pCa, $P < 0.05$.

Effect of LC₂ Phosphorylation on k_{tr}

Although there is a significant effect of phosphorylation upon steady isometric force at submaximal Ca^{2+}, the increases in force observed here and by others (Persechini et al., 1985; Sweeney and Kushmerick, 1985) are modest in comparison to the extent of twitch potentiation observed after a period of repetitive stimulation (Close and Hoh, 1968). To further investigate a possible causal relationship between phosphorylation and twitch potentiation we examined mechanical behavior corresponding to the rate constant of cross-bridge attachment. The rationale for these experiments was that phosphorylation-dependent alterations in the rate of cross-bridge attachment would affect the rate of force development and therefore the peak force during a twitch.

Records of tension redevelopment obtained from skinned fibers at maximal levels of Ca^{2+} activation (Fig. 9) showed that the rate constant of tension redevelopment
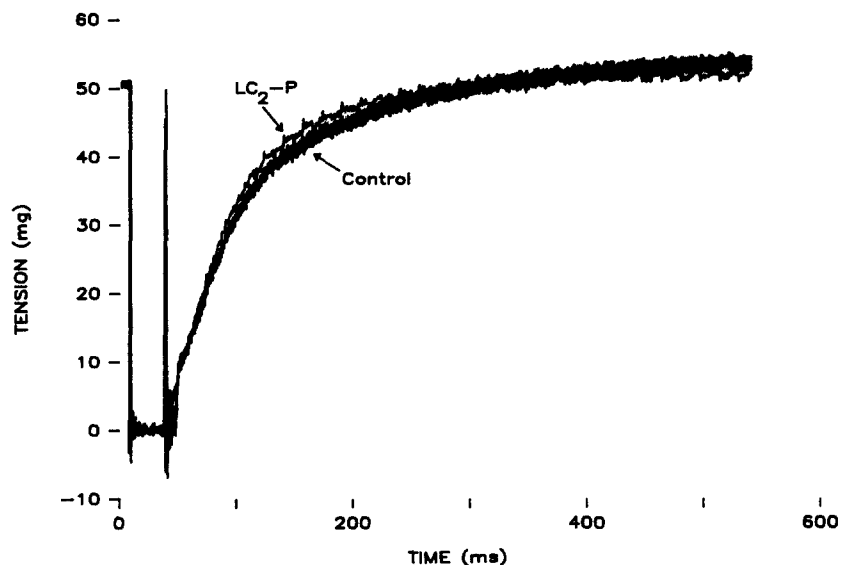

FIGURE 9. Effect of LC₂ phosphorylation upon the rate constant of tension redevelopment during maximal Ca^{2+} activation (pCa 4.5) of a rabbit skinned single psoas fiber. k_{tr} and corresponding $t_{½}$ values were 15 s⁻¹ and 46 ms in control fibers and 16 s⁻¹ and 43 ms after phosphorylation of LC₂ (LC₂-P). Psoas fiber No. 8207. Sarcomere length was maintained at 2.53 μm throughout the entire phase of tension redevelopment. Muscle length was 2.00 mm.

was unaffected by phosphorylation of LC₂. For rabbit psoas fibers, mean values of k_{tr} were 18.7 ± 1.0 s⁻¹ ($n = 11$) and 19 ± 1.0 s⁻¹ ($n = 5$), respectively, in control and LC₂-phosphorylated fibers. LC₂ phosphorylation was also without effect on k_{tr} in maximally Ca^{2+}-activated svl fibers, with values averaging 26.1 ± 1.2 s⁻¹ ($n = 6$) and 25.0 ± 2.5 s⁻¹ ($n = 2$), respectively, in control and LC₂-phosphorylated fibers.

In contrast, in all fibers studied, LC₂ phosphorylation resulted in a significant increase in the rate of tension redevelopment at intermediate levels of Ca^{2+} activation. For example, at pCa 6.3 in a skinned single psoas fiber k_{tr} increased from 0.7

to 1.8 s^{-1} after phosphorylation (Fig. 10). When scaled to the control value of k_{tr} at pCa 4.5, the rate of tension redevelopment in this psoas fiber increased from 0.03 to 0.08 after phosphorylation. As described previously, there was an elevation of steady isometric tension from 0.31 to 0.45 P_0 because of phosphorylation. Qualitatively similar findings were obtained in svl fibers. In the example in Fig. 11, k_{tr} at pCa 6.0 increased markedly to 20 s^{-1} due to LC$_2$ phosphorylation from a control value of 13 s^{-1}; that is, the half-time for tension redevelopment was reduced from 53.3 ms in the control to 34.7 ms after phosphorylation. Expressing these values relative to control at pCa 4.5, there was an increase in k_{tr} in this svl fiber from 0.55
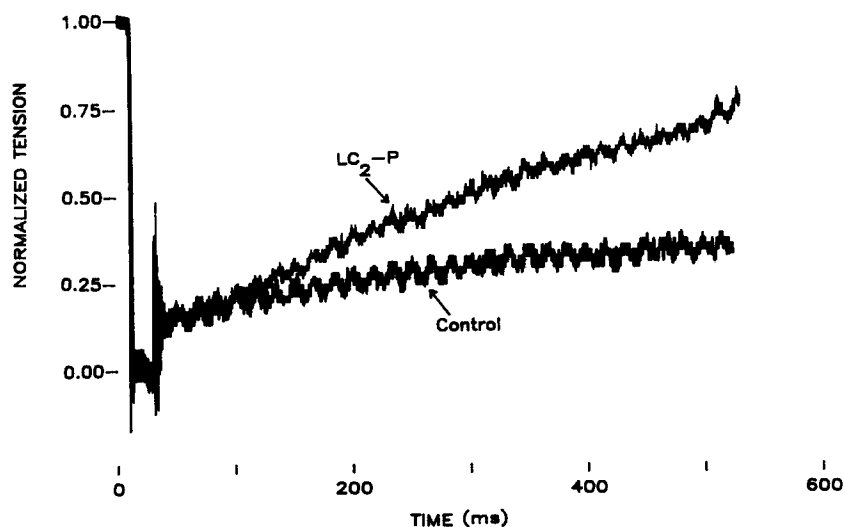

FIGURE 10.   Effect of LC$_2$ phosphorylation upon the rate constant of tension redevelopment at an intermediate level of Ca^{2+} activation (pCa 6.3) in a rabbit skinned psoas fiber. Records have been normalized to the maximum tension generated by the fiber under each experimental condition. k_{tr} was 0.7 s^{-1} in the control fiber and 1.8 s^{-1} after phosphorylation (LC$_2$-P). k_{tr} values, relative to control at pCa 4.5, were 0.03 in control and 0.08 after phosphorylation. Relative isometric tensions immediately before the release step were 0.31 P_0 in the control fiber and 0.45 P_0 after phosphorylation. P_0 was 46 mg. Rabbit psoas fiber No. 9247. Sarcomere length was clamped to 2.54 μm throughout the entire phase of tension redevelopment. Muscle length was 2.38 mm.

in control to 0.84 after phosphorylation. Again, isometric force was elevated from a control value of 0.69 to 0.80 P_0 after phosphorylation.

It was a consistent finding in both psoas and svl fibers that at low levels of Ca^{2+} activation, corresponding to relative isometric tensions less than about 0.20 P_0, k_{tr} was unaffected by LC$_2$ phosphorylation despite the increase in steady isometric tension. In the example shown in Fig. 12, k_{tr} at pCa 6.2 was 1 s^{-1} before and after phosphorylation whereas relative tension increased from 0.14 to 0.23 P_0.

The effects of LC$_2$ phosphorylation upon the k_{tr}–pCa and the isometric tension-pCa relationships in rabbit psoas fibers are summarized in Figs. 13 and 14. In Fig.

13, the results were scaled to the respective values measured in the same fiber at pCa 4.5 before phosphorylation. It is evident that at intermediate concentrations of Ca^{2+}, corresponding to a pCa range between 6.0 and 5.5, there was a marked effect of LC_2 phosphorylation to elevate the rate constant of tension redevelopment. For example, in psoas fibers at pCa 6.0, k_{tr} increased from a relative value of 0.26 ± 0.03 ($n = 9$) to 0.37 ± 0.04 ($n = 5$) with phosphorylation (Fig. 13). In svl fibers at pCa 6.0, k_{tr}, scaled to control values at pCa 4.5, increased from a control value of 0.38 ± 0.07 ($n = 6$) to 0.58 ± 0.07 ($n = 6$) after phosphorylation. In both psoas and svl fibers, k_{tr} was unchanged by phosphorylation at maximal levels of Ca^{2+} activation or at low concentrations of Ca^{2+}, corresponding to pCa's greater than ~6.2.
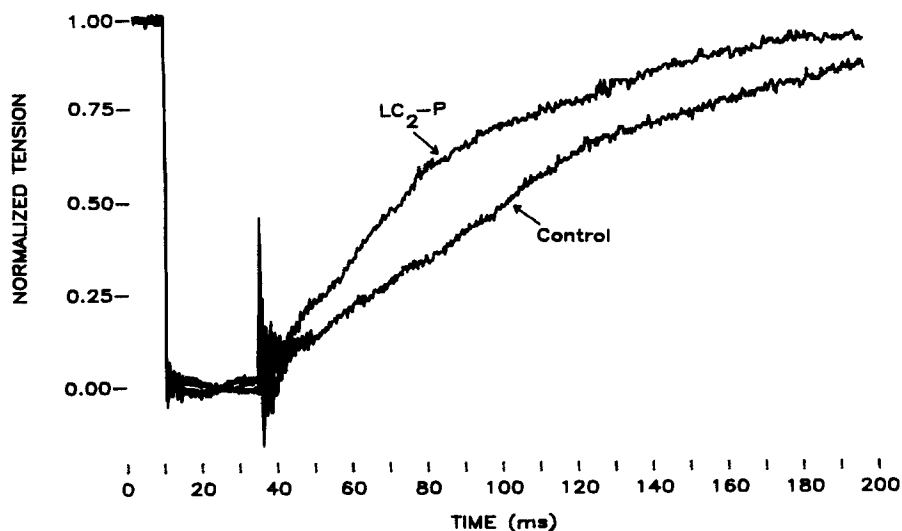

FIGURE 11. Effect of LC_2 phosphorylation upon the rate constant of tension redevelopment at an intermediate concentration of Ca^{2+} (pCa 6.0) in a rat skinned svl fiber. Records have been normalized to the maximum force generated by the fiber under each experimental condition. k_{tr} and $t_{1/2}$ values were 13 s^{-1} and 53 ms in control fiber and 20 s^{-1} and 35 ms after LC_2 phosphorylation (LC_2-P). k_{tr} values, relative to the control value at pCa 4.5, were 0.55 in the control and 0.84 after phosphorylation. Relative isometric tension immediately before release step was 0.69 P_0 in the control fiber and 0.80 P_0 after phosphorylation. P_0 was 41 mg. Fiber (svl) No. 9107. Sarcomere length clamped at 2.58 μm. Muscle length was 2.29 mm.

Also apparent from Fig. 13 are the different Ca^{2+} sensitivities of k_{tr} and steady-state isometric force, as shown previously (Brenner, 1986). The pCa's required for half-maximal activation (i.e., pCa_{50}) of k_{tr} were 5.72 and 5.93 in control and LC_2-phosphorylated psoas fibers, respectively. In svl fibers pCa_{50} values increased from 5.97 in controls to 6.09 after phosphorylation. In contrast, pCa_{50} values for isometric force were little changed by LC_2 phosphorylation, i.e., pCa 6.28 vs. 6.23 in control rabbit psoas fibers. However, comparison solely on the basis of the pCa_{50} is inappropriate since the effect of phosphorylation to increase tension was limited to Ca^{2+} concentrations at which control forces were ≤0.60 P_0. Hill coefficients describing the form of the tension–pCa and k_{tr}–pCa relations and pCa_{50} values are
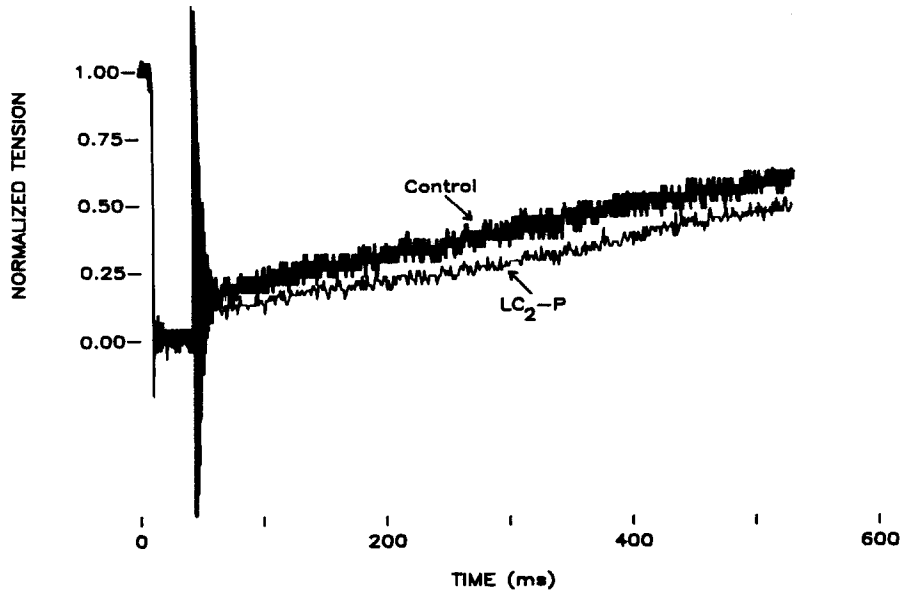
FIGURE 12. Effect of LC_2 phosphorylation upon the rate constant of tension redevelopment at a low level of Ca^{2+} activation (pCa 6.2) in a rabbit skinned psoas fiber. Records have been normalized to the maximum force developed by the fiber under each experimental condition. k_{tr} values were 1 s^{-1} before and after phosphorylation of LC_2 (LC_2-P). Relative isometric force immediately before the release step was 0.14 P_0 in the control fiber and 0.23 P_0 after phosphorylation. P_0 was 57 mg. Psoas fiber No. 1215. Sarcomere length was clamped to 2.59 μm. Muscle length was 2.10 mm.

summarized in Table II. The large Hill coefficients (n_2) obtained for $P/P_0 < 0.50$ have been discussed previously as indicating a high degree of molecular cooperativity during Ca^{2+} activation of the thin filament (Moss et al., 1985), whereas the values of n_1 approximate the number of low-affinity Ca^{2+} binding sites on TnC. The Ca^{2+} activation of k_{tr} showed apparently less cooperativity, but this increased after LC_2
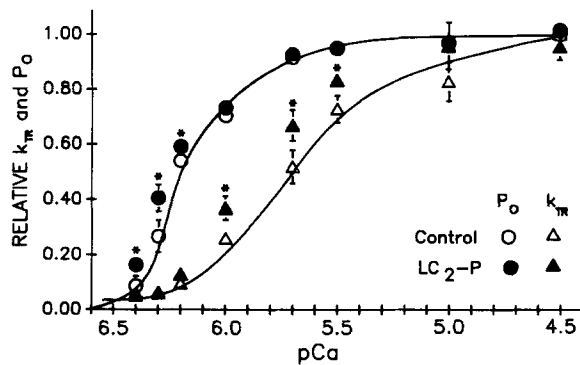

FIGURE 13. Summary of the effects of LC_2 phosphorylation upon relative tension (*circles*) and k_{tr} (*triangles*) vs. pCa in rabbit psoas fibers. (*Open symbols*) control data; (*filled symbols*) data from LC_2 phosphorylated fibers (LC_2-P). Values have been scaled relative to the control value at pCa 4.5 in the same fiber. Values are mean ± SE. Average of six observations per value. Asterisks indicate that the LC_2-phosphorylated value is significantly greater than control, $P < 0.05$.
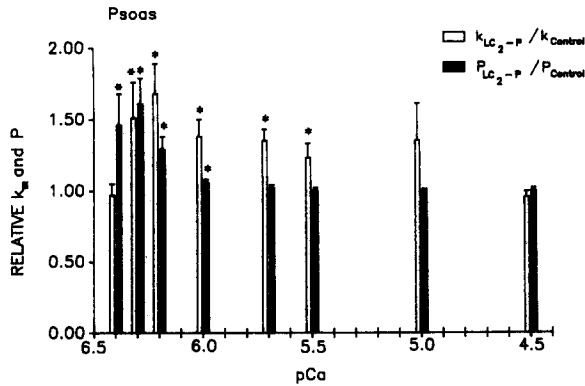
FIGURE 14. Summary of the effects of LC$_2$ phosphorylation upon k_{tr} (*open bars*) and force (*filled bars*) in rabbit psoas fibers at various pCa's. Values have been scaled to the control response of each fiber at the same pCa. Values are mean ± SE. Average of six observations per value. Asterisks indicate values are significantly greater than 1, $P < 0.05$.

phosphorylation as reflected in the increase in n_2 after phosphorylation. The values of n_1 for k_{tr} and n_1 and n_2 for tension decreased somewhat after LC$_2$ phosphorylation.

To facilitate comparisons of the relative effects on force and k_{tr} owing to LC$_2$ phosphorylation, results have been scaled to the corresponding control values obtained from the same fiber at the same pCa. In Fig. 14, which summarizes data for rabbit psoas fibers, it is clear that increases in relative force and k_{tr} due to LC$_2$ phosphorylation are not directly coupled. After phosphorylation, significant increases in force were observed at pCa 6.4–6.0. For example, at pCa 6.3, the mean ratio of forces in phosphorylated vs. control psoas fibers was 1.61 ± 0.18 ($n = 6$). In the pCa range 5.7–4.5, there was no effect of phosphorylation on isometric force. The range of Ca^{2+} concentrations in which k_{tr} was increased by phosphorylation differed from that for tension in that there was no change in k_{tr} at pCa 6.4. However, in the pCa range 6.3–5.5, k_{tr} was significantly elevated. The maximum increase in k_{tr} was obtained at pCa 6.2, at which the ratio of experimental to control values averaged 1.68 ± 0.21 ($n = 5$).

Results from rat svl fibers were qualitatively similar to those from rabbit psoas fibers (Fig. 15). Force was significantly increased in the pCa range 6.4–5.7; however, at pCa's <5.7, relative force values were not significantly different between control and phosphorylated fibers. At pCa 6.3, force after LC$_2$ phosphorylation increased

TABLE II

Summary of pCa$_{50}$ and Hill Coefficient Values for Tension–pCa and k$_{tr}$–pCa Data Obtained from Control and LC$_2$-phosphorylated Psoas Fibers

	Tension–pCa			k$_{tr}$–pCa		
	pCa$_{50}$	n_1	n_2	pCa$_{50}$	n_1	n_2
Control	6.23	1.84	5.70	5.72	2.00	2.50
LC$_2$-phosphorylated	6.28	1.75	5.60	5.93	1.51	3.21

Hill plots were obtained by expressing results as log [P_r or $k_r/(1 - P_r$ or $k_r)$] vs. −log [Ca^{2+}], where P_r is P/P_o and k_r is k_{tr}/maximum k_{tr}. As observed previously, Hill plots were best fit by two straight lines rather than one (Moss et al., 1985). In each case the slope of the data for relative values >0.50 is n_1, while n_2 is the slope of data for relative values <0.50. Values were obtained from the data shown in Figs. 8 and 13.
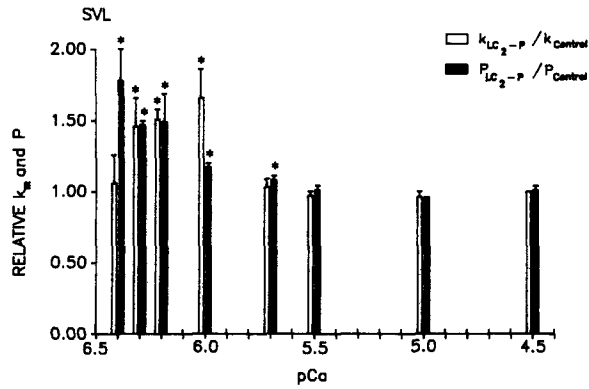
FIGURE 15. Summary of the effects of LC_2 phosphorylation upon k_{tr} (*open bars*) and force (*filled bars*) in svl fibers at various pCa's. Values scaled to the control value for each fiber at the same pCa. Values are mean ± SE. Average of five observations per value. Asterisks indicate values that are significantly greater than 1, $P < 0.05$.

to 1.47 ± 0.03 ($n = 3$) of control values. The values of k_{tr} were unchanged by phosphorylation at pCa 6.4 and at pCa's between 5.5 and 4.5; however, in the pCa range 6.3–6.0, k_{tr} was significantly increased. The greatest increase in k_{tr} was observed at pCa 6.0, where k_{tr} averaged 1.66 ± 0.20 ($n = 6$) of control values.

Effect of LC_2 Phosphorylation upon the Tension–pCa Relationship after Partial Extraction of TnC

In a separate series of experiments the effect of LC_2 phosphorylation to increase steady-state isometric tension at submaximal $[Ca^{2+}]$ was examined by extraction of varying amounts of TnC from rabbit psoas fibers (Fig. 16). If phosphorylation-induced increases in steady isometric force are related to the effects of cross-bridges to cooperatively activate the thin filament, then partial removal of TnC, which has been shown to disrupt cooperativity within the thin filament (Brandt et al., 1984; Moss et al., 1985), should abolish the effect. Alternatively, if increased isometric
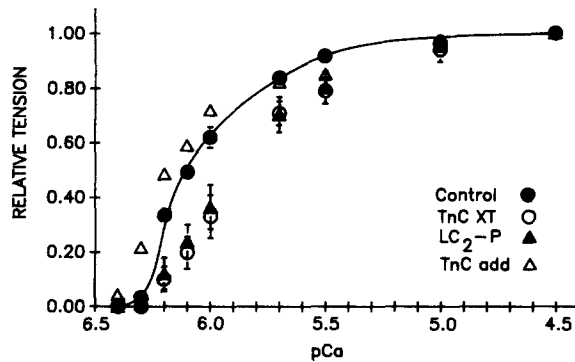

FIGURE 16. Effect of LC_2 phosphorylation upon the relative tension–pCa relationship after partial extraction of TnC from the thin filament. A control tension–pCa relationship was determined (●); subsequently, sufficient TnC was extracted so that maximum steady-state isometric tension was $0.46 \pm 0.06\ P_0$, and a second tension–pCa relation was determined (○). LC_2 was

then phosphorylated (LC_2-P) and a third tension–pCa relation was obtained (▲). Maximum isometric force under this condition was $0.46 \pm 0.06\ P_0$). Finally, troponin C was recombined into the fiber so that tension at pCa 4.5 was $0.98 \pm 0.01\ P_0$, and a fourth tension-pCa relation was determined (△). All values have been scaled relative to the tension developed at pCa 4.5 under each experimental condition. Values are mean ± SE, $n = 7$–10. Results were obtained from rabbit psoas fibers.

tension at submaximal activation is the result, for example, of increased force per attached cross-bridge, then TnC extraction should not alter the effect. In these experiments, a control tension–pCa relationship was first determined in each fiber. TnC was then partially extracted from the thin filament so that steady-state isometric tension at pCa 4.5 varied between 0.08 and 0.73 P_0 (average 0.46 ± 0.06 P_0) in the fibers studied. A second tension–pCa relationship was then determined. Fibers were subsequently cycled through the LC_2 phosphorylation protocol and a third tension–pCa relationship was determined. Finally, exogenous TnC purified from rabbit skeletal muscle was recombined into the fiber to fully restore maximum Ca^{2+}-activated tension at pCa 4.5 (average tension after TnC recombination was 0.98 ± 0.01 P_0) and a fourth force–pCa relationship was obtained.

In agreement with earlier reports (Brandt et al., 1984; Moss et al., 1985), TnC extraction produced a marked rightward shift in the relative tension-pCa relationship (Fig. 16). In this study, the pCa required for half-maximal activation of tension was 6.12 in controls and 5.91 after partial extraction of TnC. Most importantly, after partial extraction of TnC, there was no effect of LC_2 phosphorylation upon the tension–pCa relationship; however, when the thin filament was reconstituted with TnC, the elevation of steady-state isometric force observed previously at submaximal concentrations of Ca^{2+} (Figs. 7 and 8) was seen. As a control, tension–pCa relationships were determined as above except for the LC_2 phosphorylation procedure. In agreement with our previous findings (Moss et al., 1985), recombination of TnC into partially TnC-extracted fibers completely restored the form and position of the tension–pCa relation to that obtained in the control fiber.

DISCUSSION

The physiological significance of myosin LC_2 phosphorylation in vertebrate skeletal muscle contraction has been difficult to assess experimentally, although strong correlations have been reported between posttetanic twitch potentiation and LC_2 phosphorylation (Manning and Stull, 1979). Studies in which steady-state contractile parameters such as isometric force were measured have shown such phosphorylation to have rather modest effects (Persechini et al., 1985; Sweeney and Stull, 1986; present study). Whereas initial studies on living muscles showed an inverse relationship between maximum shortening velocity (V_{max}) and LC_2 phosphate content (Crow and Kushmerick, 1982), subsequent reports on living (Butler et al., 1983) and skinned fibers (Sweeney and Kushmerick, 1985) have shown V_{max} to be independent of the level of phosphorylation. Our main finding of a phosphorylation-dependent increase in the rate of tension redevelopment establishes a physiologically relevant effect of phosphorylation in vertebrate skeletal muscle. Based on our results, we propose a mechanism in which phosphorylation mediates an increased rate of cross-bridge attachment to binding sites along the thin filament. This possibility is envisioned schematically as a modulation by phosphorylation of the movement of cross-bridges away from the thick filament backbone and toward the thin filament (Fig. 17). To develop this idea in detail, it is useful to discuss the rate constant of tension redevelopment (k_{tr}) in terms of currently accepted models of muscle contraction. The observation that the rate of tension redevelopment is well fit by a first-order exponential equation allows discussion of the underlying processes in terms of a

simple two-step reaction; that is, going from a non–force-producing cross-bridge state (A), to a force-producing cross-bridge state (B) (Brenner, 1986). In relation to A.F. Huxley's (1957) model of muscle contraction the transition from A to B is determined by f, the rate constant of cross-bridge attachment while the reverse reaction, B to A, is limited by g_1 the rate constant of detachment for cross-bridges bearing positive force. In this scheme, k_{tr} is equal to the sum of f and g_1. Thus, our observation that k_{tr} is increased following phosphorylation could relate to increases

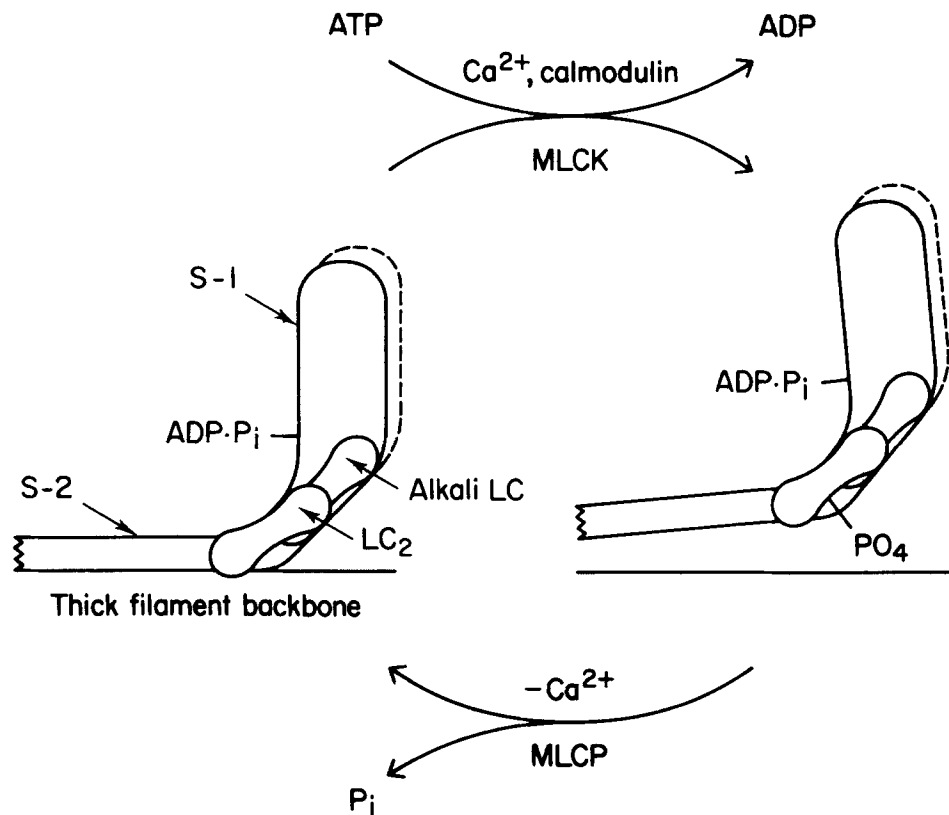FIGURE 17.   Schematic representation of cross-bridge orientation with respect to the thick filament backbone before and after phosphorylation of LC_2 which spans the hinge region between the S1 and S2 subunits of myosin (Winkelman and Lowey, 1984). Also shown is the nucleotide binding site on the myosin heavy chain. MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase.

in f or g_1 or some combination of both. In an attempt to distinguish between these possibilities, consider first that g_1 is proportional to the ratio of ATPase activity to isometric force (Huxley, 1957). Results concerning an effect of phosphorylation upon myosin ATPase activity vary widely. Myosin ATPase activity has been reported to decrease (Cooke et al., 1982; Kakol et al., 1982) remain unchanged (Morgan et al., 1976; Persichini and Stull, 1984) and to increase (Pemrick, 1980) after LC_2 phosphorylation, although most of these measurements were made in the absence

of the thin filament regulatory proteins and thus may not be applicable to intact muscles. Perhaps most relevant to this discussion is the finding that the concentration of calcium for half-maximal activation (i.e., pCa_{50})) of myosin ATPase activity in the presence of regulated actin was unchanged after phosphorylation (Pemrick, 1980). This, together with our findings of only small changes in the pCa_{50} of tension development and increased isometric force at submaximal $[Ca^{2+}]$, would suggest that the ATPase/isometric force ratio and therefore g_1 is either unchanged or reduced by LC_2 phosphorylation at this level of Ca^{2+} activation. This in turn suggests that the increases in k_{tr} observed in the present study relate to a phosphorylation-dependent increase in the rate constant of cross-bridge attachment, f. It must be noted, however, that the effect of phosphorylation on the Ca^{2+} sensitivities of force and ATPase have yet to be determined in the same preparation.[1]

The results of the present study fit well with recent biochemical evidence suggesting a phosphorylation-dependent alteration in the interaction between myosin cross-bridges and actin in vertebrate skeletal muscle. Barany et al. (1980) examined EDTA chelation of actin-bound Ca^{2+} after caffeine activation-induced increases in LC_2 phosphate content in frog skeletal myofibrils. After the binding of phosphorylated myosin to actin, the effect of EDTA to chelate actin-bound Ca^{2+} was reduced. It was suggested that the phosphoryl group on myosin formed a chelate with actin-bound Ca^{2+}, with the affinity constant of this bond being greater than that of EDTA for Ca^{2+}. Based on these results, it was hypothesized that LC_2 phosphorylation may result in an increased rate of combination of cross-bridges with actin-binding sites during muscular activity. In addition, it has been shown that the effect of pyrophosphate to dissociate the actomyosin complex is depressed after conversion of myosin to its phosphorylated form, suggesting an increased affinity of phosphorylated myosin for actin (Michnicka et al., 1982). Using synthetic myosin filaments from rabbit skeletal muscle, Mrakovcic and Reisler (1983) found that the rate of cross-linking between myosin subfragment S-2 and light meromyosin was reduced after LC_2 phosphorylation. This result was interpreted as evidence that LC_2 phosphorylation results in a partial release of cross-bridges away from the thick filament backbone (shown schematically in Fig. 17). The molecular basis for this possibility may involve phosphorylation-induced alterations in the net charge balance of myosin. Ueno and Harrington (1981) provided evidence that increases in the net negative charge of myosin owing to increases in solution pH results in the separation of cross-bridges from the filament backbone. This result, together with the idea that phosphorylation should result in the addition of two negative charges to the net charge on myosin (Barany et al., 1980), may provide a molecular basis for our observation of a phosphorylation-dependent increased rate of cross-bridge attachment.

Our findings indicate that the LC_2 phosphorylation-dependent increase in the rate constant of force development was restricted to a specific Ca^{2+} concentration

[1] Effects on isometric tension and ATPase activity due to LC_2 phosphorylation in skinned single psoas fibers have recently been reported (Sweeney and Stull, 1989). LC_2 phosphorylation-dependent increases in isometric tension were found to be linearly proportional to increases in ATPase activity, indicating that g_1 is unaffected by LC_2 phosphorylation. This result supports our hypothesis that phosphorylation-dependent increases in k_{tr} are directly related to increases in f, the rate constant of cross-bridge attachment.

range. The molecular basis for this effect is unknown but may involve the regulation of cation binding to sites on LC_2. Vertebrate skeletal muscle LC_2 contains a divalent cation binding site that is thought to be occupied by Mg^{2+} under physiological conditions (Bagshaw, 1980). Whether native LC_2 in vertebrate skeletal muscle contains a Ca^{2+}-specific binding site is at present unclear (e.g., Pulliam et al., 1983). The general finding in biochemical studies is that vertebrate skeletal muscle myosin lacks a Ca^{2+}-specific binding site; yet, the possibility that a such site is lost or damaged during preparation of myosin for binding studies cannot be excluded (Bagshaw, 1980). In this context, Lehman (1978), using vertebrate skeletal myofibrils in which the thin filament was devoid of endogenous regulatory proteins, found that the actin-myosin ATPase activity remained Ca^{2+} sensitive. Furthermore, there is a report that LC_2 obtained from vertebrate skeletal muscle may restore Ca^{2+} sensitivity to unregulated thick filaments in molluscan muscle which in the intact state require the binding of Ca^{2+} to myosin to regulate the contractile event (Kendrick-Jones, 1974). These results lend support to the possibility of myosin-linked Ca^{2+} regulation of vertebrate muscle contraction. Studies examining cation binding to isolated LC_2 have shown that its affinity for Ca^{2+} is decreased after phosphorylation (Alexis and Gratzer, 1978); however, others have reported no effect (Holroyde et al., 1979). Based on these results, further investigations into the possibility of Ca^{2+}-specific binding of LC_2 along with the potential interactive effect of phosphorylation and Ca^{2+} binding would be of great interest.

Mechanism of LC_2 Phosphorylation-induced Increased Ca^{2+} Sensitivity in Steady-State Isometric Tension

In agreement with others (Persechini et al., 1985; Sweeney and Kushmerick, 1985), LC_2 phosphorylation had no effect upon steady-state isometric force at saturating concentrations of Ca^{2+}. This provides evidence that force per attached cross-bridge is not altered by phosphorylation. However, as reported previously (Persechini et al., 1985; Sweeney and Stull, 1986), LC_2 phosphorylation induced a small but significant increase in steady-state isometric force at concentrations of Ca^{2+} below that necessary to elicit ~60% of maximum isometric force. This shifted the steady-state isometric force–pCa relationship to the left, indicating an increase in the sensitivity of the contractile apparatus to Ca^{2+}. As detailed elsewhere (Moss et al., 1986), a leftward shift in the tension–pCa relationship may involve enhanced cooperative activation of the thin filament resulting in an increased number of cross-bridge attachments and thus greater force at submaximal concentrations of Ca^{2+}. Similarly, our experimental findings indicate that the phosphorylation-induced shift in the tension–pCa relationship may be a result of an increase in the cooperative activation of the thin filament. Present results showed that when thin filament cooperativity was disrupted by partial extraction of troponin C (see Brandt et al., 1984; Moss et al., 1985; present study, Fig. 16), the effect of LC_2 phosphorylation to increase isometric force at submaximal concentrations of Ca^{2+} was no longer evident; however, when fibers were reconstituted with exogenous troponin C to restore Ca^{2+} sensitivity to the thin filament, the expected elevation in tension because of phosphorylation was observed.

Although the molecular mechanism of thin filament cooperativity is not com-

pletely understood, LC_2 phosphorylation may have one of several effects. The mammalian thin filament consists of a longitudinal array of 26 discrete functional groups (defined structurally as seven actin monomers, one troponin complex, and one tropomyosin) with partial overlap at the ends of the tropomyosins. In the thin filament, cooperation may exist between functional groups such that the state of activation, either by Ca^{2+} or bound cross-bridges, of one group could influence the activation of adjacent groups (Moss et al., 1985, 1986). This mechanism is suggested by the findings that both rigor (Bremel and Weber, 1972) and cycling (Güth and Potter, 1987) cross-bridges increase the Ca^{2+} affinity of TnC subunits of near-neighbor functional groups which have unoccupied low-affinity Ca^{2+}-binding sites. One possibility is that our finding of an increased Ca^{2+} sensitivity of steady-state isometric tension with phosphorylation is related to the increased rate of cross-bridge attachment; however, it is unlikely that this could provide a mechanism for increased thin-filament activation at all Ca^{2+} concentrations studied, since in some cases we found force to be elevated without a corresponding increase in the rate of attachment (Figs 12, 14, and 15). Alternatively, the increased Ca^{2+} sensitivity of steady-state isometric tension may be related to the phosphorylation-dependent increase in the affinity of myosin for actin (e.g., Michnicka et al., 1982), which in turn would enhance the cooperative activation of the thin filament. Consistent with our findings, an increase in Ca^{2+} sensitivity owing to phosphorylation would be most likely to occur at low levels of Ca^{2+} activation since there would be a greater probability of an activated functional group having nonactivated neighbors.

Ca^{2+} Sensitivity of Tension and k_{tr}

Our finding that k_{tr} is sensitive to variations in Ca^{2+}, but has a lower pCa_{50} than isometric tension, is consistent with an earlier report (Brenner, 1986). Brenner (1986) suggested that the position and shape of the force–pCa relation could be accounted for by Ca^{2+} modulation of the proportion of cross-bridges which are in a strongly bound force-producing state, i.e., $f/(f + g_1)$. According to this model, our observation of a leftward shift in the tension-pCa relationship owing to LC_2 phosphorylation would be accounted for by a leftward shift in the k_{tr}–pCa relation. Similarly, Brenner's model would predict that the rightward shift in the force–pCa relation after TnC extraction would be due to a rightward shift in the k_{tr}–pCa relation. Finally, the observed lack of an effect of LC_2 phosphorylation on force after partial extraction of TnC would be predicted if the effects of extraction negated the effect of LC_2 phosphorylation to increase k_{tr}. However, these possibilities are not borne out experimentally. As pointed out earlier, the increases in tension at the lower $[Ca^{2+}]$ studied occurred with no change in k_{tr}, whereas at intermediate $[Ca^{2+}]$ force was unchanged and k_{tr} was markedly elevated. Further, we have recently shown that the k_{tr}–pCa relationship is unchanged after partial TnC extraction (Metzger and Moss, 1988b). In other words, the shape and position of the force–pCa relationship was significantly altered without any effect upon the k_{tr}–pCa relation. Therefore, as has been discussed previously (Brandt et al., 1984; Moss et al., 1985, 1986) and above, alterations in the Ca^{2+} sensitivity of steady-state isometric force may best be related to effects of phosphorylated myosin on cooperative mechanisms within the thin filament.

Variations in the Ca^{2+} sensitivity of k_{tr} owing to LC_2 phosphorylation may be related to a direct effect upon f, the rate constant of cross-bridge attachment. We propose here that, at low and maximally activating levels of Ca^{2+}, f is unchanged because of LC_2 phosphorylation; however, throughout the intermediate range of pCa's, f is increased. Since f is apparently much greater than g_1 (Huxley, 1957), alterations in f would not be expected to greatly affect the proportion of available cross-bridges which are attached (i.e., $f/f + g_1$) and therefore the tension developed at a particular level of Ca^{2+} activation.

Implications for Twitch Potentiation in Intact Muscle

The phenomena of posttetanic twitch potentiation, which is a transient increase in twitch amplitude subsequent to a brief tetanic contraction, and treppe, a progressive increase in the amplitude of successive, closely spaced, twitches, are well known (see Lee, 1907); however, the molecular basis underlying these effects has yet to be established. Brown and von Euler (1938) found that posttetanic twitch potentiation could be elicited in mammalian skeletal muscles stimulated either directly in the presence of curare or indirectly through the motor nerve. They further demonstrated that evoked action potentials in these two muscles during twitch potentiation were similar to those observed in control muscles, indicating that posttetanic potentiation is related to mechanisms subsequent to membrane excitation. Consistent with this idea, caffeine-induced contractions result in maximum incorporation of phosphate into LC_2 (Barany et al., 1980). It is unlikely that posttetanic potentiation is a result of prolongation of the active state since twitch contraction and half-relaxation times are unchanged at a time when twitch amplitude is elevated (Close and Hoh, 1968). In fact, Blinks et al. (1978), using aequorin to record Ca^{2+} transients in frog skeletal fibers, found that during a twitch subsequent to a tetanus or low-frequency stimulation, aequorin luminescence was somewhat depressed relative to the control response. These results are consistent with the idea that posttetanic twitch potentiation and treppe involve direct modulation of cross-bridge interaction with actin.

Our main finding that the rate constant of tension redevelopment is increased at intermediate concentrations of Ca^{2+} after phosphorylation of LC_2 provides a mechanism for twitch potentiation in intact muscle. Under resting conditions in an intact skeletal muscle fiber the concentration of Ca^{2+} is ~0.06 μM, and this increases to a peak concentration of ~7 μM during a twitch (Blinks et al., 1978; Cannell and Allen, 1984). During the time course of a twitch, the myoplasmic Ca^{2+} transient precedes tension development such that the concentration of Ca^{2+} decreases toward baseline levels as tension approaches its peak value (Blinks et al., 1978). Our results predict that, throughout much of the Ca^{2+} transient of a posttetanic twitch, there would be a marked increase in the rate of rise of tension because of an increase in the rate of cross-bridge attachment. Although there is considerable variation among studies with regard to the extent of poststimulation twitch potentiation, results of parallel determinations of the level of LC_2 phosphorylation and the extent of twitch potentiation agree quite well with the present findings. For example, potentiation of twitch tension by 60% has been reported after stimulation-induced increases in phosphate incorporation from 10% to 70% in mammalian fast-twitch muscles (Klug

et al., 1982; Moore and Stull, 1984). In comparison, our findings showed that, at slightly higher levels of LC_2 phosphorylation and at concentrations of Ca^{2+} in the range expected during a twitch, the rate constant of tension redevelopment was elevated by 35–68%. Thus, our findings are consistent with the idea that twitch potentiation in intact muscle results from phosphorylation-dependent increases in the rate of cross-bridge attachment to actin-binding sites.

REFERENCES

Alexis, M. N., and W. B. Gratzer. 1978. Interaction of skeletal myosin light chains with calcium ions. *Biochemistry.* 17:2319–2325.

Bagshaw, C. R. 1980. Divalent metal ion binding and subunit interactions in myosin: a critical review. *Journal of Muscle Research and Cell Motility.* 1:255–277.

Baldwin, K. M., G. H. Klinkerfuss, R. L. Terjung, P. A. Mole, and J. O. Holloszy. 1972. Respiratory capacity of white, red, and intermediate muscle: adaptive response to exercise. *American Journal of Physiology.* 222:373–378.

Barany, K., and M. Barany. 1977. Phosphorylation of the 18,000-dalton light chain of myosin during a single tetanus of frog muscle. *Journal of Biological Chemistry.* 252:4752–4754.

Barany, K., M. Barany, J. M. Gilles, and M. J. Kushmerick. 1980. Myosin light chain phosphorylation during the contraction cycle of frog muscle. *Federation Proceedings.* 39:1547–1551.

Blinks, J. R., R. Rudel, and S. R. Taylor. 1978. Calcium transients in isolated amphibian skeletal muscle fibers: detection with aequorin. *Journal of Physiology.* 277:291–323.

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.

Bremel, R. D., and A. Weber. 1972. Cooperation within actin filament in vertebrate skeletal muscle. *Nature New Biology.* 238:97–101.

Brenner, B. 1983. Cross-bridge attachment during isotonic shortening in single skinned rabbit psoas fibers. *Biophysical Journal.* 41:33a. (Abstr.)

Brenner, B. 1986. The cross-bridge cycle in muscle. Mechanical, biochemical, and structural studies on single skinned rabbit psoas fibers to characterize cross-bridge kinetics in muscle for correlation with the actomyosin-ATPase in solution. *Basic Research in Cardiology.* 81:1–15.

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 Science.* 83:3542–3546.

Brown, G. L., and U. S. von Euler. 1938. The after-effects of a tetanus on mammalian muscle. *Journal of Physiology.* 93:39–60.

Butler, T. M., M. J. Siegman, S. U. Mooers, and R. J. Barsotti. 1983. Myosin light chain phosphorylation does not modulate cross-bridge cycling rate in mouse skeletal muscle. *Science.* 220:1167–1169.

Cannell, M. B., and D. G. Allen. 1984. Model of calcium movements during activation in the sarcomere of frog skeletal muscle. *Biophysical Journal.* 45:913–925.

Close, R., and J. F. Y. Hoh. 1968. The after-effects of repetitive stimulation on the isometric twitch contraction of rat fast skeletal muscle. *Journal of Physiology.* 197:461–477.

Cooke, R., K. Franks, and J. T. Stull. 1982. Myosin phosphorylation regulates the ATPase activity of permeable skeletal muscle fibers. *FEBS (Federation of European Biochemical Societies) Letters.* 144:33–37.

Crow, M. T., and M. J. Kushmerick. 1982. Phosphorylation of myosin light chains in mouse fast-twitch muscle associated with reduced actomyosin turnover rate. *Science.* 217:835–837.

Dedman, J. R., J. D. Potter, R. L. Jackson, J. D. Johnson and A. R. Means. 1977. Physiochemical properties of rat testis Ca^{2+}-dependent regulator protein of cyclic nucleotide phosphodiesterase. *Journal of Biological Chemistry.* 252:8415–8422.

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.

Ford, L. E., A. F. Huxley, and R. M. Simmons. 1977. Tension responses to sudden length change in stimulated frog muscle fibres near slack length. *Journal of Physiology.* 269:441–515.

Giulian, G. G., R. L. Moss, and M. L. Greaser. 1983. Improved methodology for the analysis and quantitation of proteins on one-dimensional silver-stained slab gels. *Analytical Biochemistry.* 129:277–287.

Giulian, G. G., R. L. Moss, and M. Greaser. 1984. Analytical isoelectric focusing using a high-voltage vertical slab polyacrylamide gel system. *Analytical Biochemistry.* 142:421–436.

Godt, R. E., and R. B. 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.

Güth, K., and J. D. Potter. 1987. Effect of rigor and cycling cross-bridges on the structure of troponin C and on the Ca^{2+} affinity of the Ca^{2+}-specific regulatory sites in skinned muscle rabbit psoas fibers. *Journal of Biological Chemistry.* 262:13627–13635.

Holroyde, M. J., J. D. Potter, and R. J. Solaro. 1979. The calcium binding properties of phosphorylated and unphosphorylated cardiac and skeletal myosins. *Journal of Biological Chemistry.* 254:6478–6482.

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

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

Kakol, I., K. Kasman, and M. Michnicka. 1982. The phosphorylation-dephosphorylation process as a myosin-linked regulation of super precipitation of fast skeletal muscle actomyosin. *Biochimica et Biophysica Acta.* 704:437–443.

Kendrick-Jones, J. 1974. Role of myosin light chains in calcium regulation. *Nature.* 249:631–634.

Klug, G. A., B. R. Botterman, and J. T. Stull. 1982. The effect of low frequency stimulation on myosin light chain phosphorylation in skeletal muscle. *Journal of Biological Chemistry.* 257:4688–4690.

Lee, F. S. 1907. The cause of the treppe. *American Journal of Physiology.* 18:267–282.

Lehman, W. 1978. Thick-filament-linked calcium regulation in vertebrate striated muscle. *Nature.* 274:80–81.

Manning, D. R., and J. T. Stull. 1979. Myosin light chain phosphorylation and phosphorylase A activity in rat extensor digitorum longus muscle. *Biochemical and Biophysical Research Communications.* 90:164–170.

Manning, D. R., and J. T. Stull. 1982. Myosin light chain phosphorylation–dephosphorylation in mammalian skeletal muscle. *American Journal of Physiology.* 242:C234–C241.

Metzger, J. M., and R. L. Moss. 1987. Greater hydrogen ion-induced depression of tension and velocity in skinned single fibres of rat fast than slow muscles. *Journal of Physiology.* 393:727–742.

Metzger, J. M., and R. L. Moss. 1988a. 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. 1988b. Evidence for myosin-linked Ca^{2+} regulation of contraction in vertebrate skeletal muscle. *Biophysical Journal.* 53:566a. (Abstr.)

Metzger, J. M., R. L. Moss, G. G. Giulian, and M. L. Greaser. 1988c. Increased rate of tension redevelopment at submaximal $[Ca^{2+}]$ in fast-twitch skeletal muscle following myosin light chain phosphorylation. *Biophysical Journal.* 53:23a. (Abstr.)

Michnicka, M., K. Kasman, and I. Kakol. 1982. The binding of actin to phosphorylated and dephosphorylated myosin. *Biochimica et Biophysica Acta.* 704:470–475.

Moore, R. L., and J. T Stull. 1984. Myosin light chain phosphorylation in fast and slow skeletal muscles in situ. *American Journal of Physiology.* 247:C462–C471.

Morgan, M., S. V. Perry, and J. Ottaway. 1976. Myosin light-chain phosphatase. *Biochemical Journal.* 157:687–697.

Moss, R. L. 1979. Sarcomere length-tension relations of frog skinned 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., J. D. Allen, and M. L. Greaser. 1986. Effects of partial extraction of troponin complex upon the tension–pCa relation in rabbit skeletal muscle. *Journal of General Physiology.* 87:761–774.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1983. Effects of EDTA treatment upon the protein subunit composition and mechanical properties of mammalian single skeletal muscle fibers. *Journal of Cell Biology.* 96:970–978.

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.

Mrakovcic-Zenic, A., and E. Reisler. 1983. Light-chain phosphorylation and cross-bridge conformation in myosin from vertebrate skeletal muscle. *Biochemistry.* 22:525–530.

Nagamoto, H., and K. Yagi. 1984. Properties of myosin light chain kinase prepared from rabbit skeletal muscle by an improved method. *Journal of Biochemistry.* 95:1119–1130.

Pemrick, S. M. 1980. The phosphorylated L_2 light chain of skeletal myosin is a modifier of the actomyosin ATPase. *Journal of Biological Chemistry.* 255:8836–8841.

Perrie, W. T., L. B. Smillie, and S. V. Perry. 1973. A phosphorylated light-chain component of myosin from skeletal muscle. *Biochemical Journal.* 135:151–164.

Persechini, A., and J. T. Stull. 1984. Phosphorylation kinetics of skeletal muscle myosin and the effect of phosphorylation on actomyosin adenosine-triphosphatase activity. *Biochemistry.* 23:4144–4150.

Persechini, A., J. T. Stull, and R. Cooke. 1985. The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibres. *Journal of Biological Chemistry.* 260:7951–7954.

Pires, E. M. V., and S. V. Perry. 1977. Purification and properties of myosin light-chain kinase from fast skeletal muscle. *Biochemical Journal.* 167:137–146.

Pollack, G. H. 1986. Quantal mechanisms in cardiac contraction. *Circulation Research.* 59:1–8.

Pulliam, D. L., V. Sawyna, and R. J. C. Levine. 1983. Calcium sensitivity of vertebrate skeletal muscle myosin. *Biochemistry.* 22:2324–2331.

Shenolikar, S., P. T. W. Cohen, P. Cohen, A. C. Nairn, and S. V. Perry. 1979. The role of calmodulin in the structure and regulation of phosphorylase kinase from rabbit skeletal muscle. *European Journal of Biochemistry.* 100:329–337.

Sweeney, H. L., and M. J. Kushmerick. 1985. Myosin phosphorylation in permeabilized rabbit psoas fibers. *American Journal of Physiology.* 249:C362–C365.

Sweeney, H. L., and J. T. Stull. 1986. Phosphorylation of myosin in permeabilized mammalian cardiac and skeletal muscle cells. *American Journal of Physiology.* 250:C657–C660.

Sweeney, H. L., and J. T. Stull. 1989. Effect of myosin light chain phosphorylation on mechanical and biochemical properties of skeletal muscle fibers. *Biophysical Journal.* 55:409a. (Abstr.)

Ueno, H., and W. F. Harrington. 1981. Cross-bridge movement and the conformational state of the myosin hinge in skeletal muscle. *Journal of Molecular Biology.* 149:619–640.

Watterson, D. M., F. Sharief, and T. C. Vanaman. 1980. The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. *Journal of Biological Chemistry.* 255:962–975.

Winkelman, D. A., S. Lowey, and J. L. Press. 1983. Monoclonal antibodies localize changes on myosin heavy chain isozymes during avian myogenesis. *Cell.* 34:295–306.

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