Effects of Phosphate and ADP on Shortening Velocity During Maximal and Submaximal Calcium Activation of the Thin Filament in Skeletal Muscle Fibers

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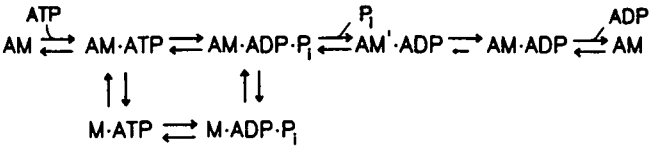
ABSTRACT The effects of added phosphate and MgADP on unloaded shortening velocity during maximal and submaximal Ca^{2+} activation of the thin filament were examined in skinned single skeletal fibers from rabbit psoas muscle. During maximal Ca^{2+} activation, added phosphate (10–30 mM) had no effect on unloaded shortening velocity as determined by the slack-test technique. In fibers activated at submaximal concentrations of Ca^{2+} in the absence of added phosphate, plots of slack length versus duration of unloaded shortening were biphasic, consisting of an initial high velocity phase of shortening and a subsequent low velocity phase of shortening. Interestingly, in the presence of added phosphate, biphasic slack-test plots were no longer apparent. This result was obtained in control fibers over a range of submaximal Ca^{2+} concentrations and in maximally Ca^{2+} activated fibers, which were first treated to partially extract troponin C. Thus, under conditions that favor the appearance of biphasic shortening (i.e., low $[Ca^{2+}]$, troponin C extraction), added phosphate eliminated the low velocity component. In contrast, in fibers activated in the presence of 5 mM added MgADP, biphasic slack-test plots were apparent even during maximal Ca^{2+} activation. The basis of biphasic shortening is not known but it may be due to the formation of axially compressed cross-bridges that become strained to bear a tension that opposes the relative sliding of the myofilaments. The present findings could be explained if added phosphate and MgADP bind to cross-bridges in a strain-dependent manner. In this case, the results suggest that phosphate inhibits the formation of cross-bridges that bear a compressive strain. Added MgADP, on the other hand, may be expected to detain cross-bridges in strong binding states, thus promoting an increase in the population of cross-bridges bearing a compressive strain. Alterations in the population of strained cross-bridges by added phosphate and MgADP would alter the internal load within the fiber and thus affect the speed of fiber shortening.

INTRODUCTION

Force development and shortening in muscle result from cyclical interactions between myosin cross-bridges and actin. The thermodynamic basis of the cross-bridge cycle comes from harnessing the chemical energy liberated by the hydrolysis of ATP and converting it into mechanical work. The hydrolysis of ATP by actin-myosin (AM) involves several intermediate steps that can be summarized as follows: 1) ATP mediated dissociation of the AM complex, 2) hydrolysis of ATP and reattachment of myosin to actin, and 3) release of the hydrolysis products inorganic phosphate followed by ADP (Scheme 1; Lymn and Taylor, 1971; Taylor, 1979; Eisenberg and Hill, 1985; Hibberd and Trentham, 1986; Goldman, 1987). The two product release steps appear to be physiologically significant in that they are thought to be closely associated with mechanical function in muscle fibers. For example, recent molecular models of the actin-myosin interface during contraction suggest that the phosphate release step permits a structural change in the myosin that could provide the basis of force generation in muscle (Rayment et al., 1993).



Scheme 1

The rate-limiting step of the ATP hydrolysis reaction determines the maximum speed at which the contractile filaments slide past one another during an unloaded contraction. There is good evidence to implicate the ADP release step, or an isomerization of the AM′.ADP state, as the slowest step in the ATP hydrolysis reaction during muscle contraction (Dantzig et al., 1991; Siemankowski et al., 1985). Using data from different muscles with widely different shortening speeds, Siemankowski et al. (1985) reported a strong correlation between the maximum shortening velocity and the rate of dissociation of ADP from actomyosin S1. Consistent with this finding, the addition of MgADP to skinned fiber activating solutions markedly slows the maximum shortening velocity (Cooke and Pate, 1985). This is thought to be due to a competition between ADP and ATP for the nucleotide binding domain of myosin (Cooke and Pate, 1985). A decrease in the rate of cross-

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bridge dissociation from actin at the end of the power stroke in actively shortening fibers is expected to increase internal load in the fiber and impede the rate of myofilament sliding.

Shortening velocity appears to be sensitive to the degree of thin filament activation in muscle fibers. In the absence of added phosphate or MgADP, slack-test plots appear biphasic under submaximal activating conditions in skinned fibers (Moss, 1986; Metzger and Moss, 1988; Farrow et al., 1988; Martyn et al., 1994). The biphasic slack-test plots consist of an initial linear phase of high velocity shortening followed by a linear phase of low velocity shortening. The basis of biphasic shortening during submaximal thin filament activation is not known but could arise due to the accumulation of cross-bridges at the end of the power stroke that are somehow detained in the attached state. It is hypothesized that with continued fiber shortening these cross-bridges would first become compressed axially, buckle, and eventually bear a tension that opposes myofilament shortening (Moss, 1986; Metzger and Moss, 1988).

Assuming that the mechanism of biphasic shortening resides in an increase in the population of cross-bridges bearing a compressive strain, it may be possible to modify experimental conditions to increase or decrease the fraction of cross-bridges bearing a compressive strain. The primary purpose of this study was to manipulate cross-bridge interactions with actin by varying the concentrations of phosphate and MgADP in chemically skinned single skeletal muscle fibers. There is evidence that phosphate may preferentially bind to those attached cross-bridges that bear the highest strain (Webb et al., 1986). If biphasic shortening results from an increase in the population of compressed cross-bridges, then it is possible that the addition of phosphate may affect the formation of these highly strained cross-bridges. In this case, added phosphate could alter the induction of the low velocity phase of shortening. It was also tested whether added MgADP could induce biphasic shortening during maximal Ca^{2+}activation by increasing the population of cross-bridges bearing a compressive strain that would oppose filament sliding.

MATERIALS AND METHODS

Skinned fiber preparations and experimental apparatus

Fast-twitch skeletal muscle fibers were obtained from the superficial portion of the vastus lateralis muscles of adult female Sprague-Dawley rats and from psoas muscles of adult male New Zealand rabbits. Results using psoas and vastus lateralis fibers were similar (only psoas 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 weeks at −20°C in relaxing solution containing 50% (v/v) glycerol. Individual fibers were carefully pulled free from one end of the fiber bundle and mounted between a force transducer (either model 407: sensitivity, 0.2 mV/μN; 1–99% response time, 100 μs; resonant frequency, ~5 kHz; or a model 400A: sensitivity, 0.2 mV/μN; 1–99% response time, 300 μs; resonant frequency, ~2 kHz; Cambridge Technology, Cambridge, MA) and galvanometer (model 300s or 6350; Cambridge Technology). The fiber was viewed through an inverted microscope, and its overall length was adjusted with a mechanical translator to set resting sarcomere length. Sarcomere length was set at 2.50–2.60 μm. Complete details of the mounting procedure and experimental setup have been reported elsewhere (Metzger et al., 1989).

Solutions

Relaxing and activating solutions contained (in mM): 7 EGTA, 1 free Mg^{2+}, 4 MgATP, 14.5 creatine phosphate, and 20 imidazole. Inorganic phosphate was added to solutions as KH_2PO_4 (pH 7.00) to yield final concentrations of 10 or 30 mM. KCl was added to yield a total ionic strength of 180 mM. Solution pH was adjusted to 7.00 with KOH. In solutions where no phosphate was added, the concentration of phosphate in fibers during Ca^{2+} activation is estimated to be ~0.7 mM because of contamination of added ATP and CP and formation of phosphate during active contraction (Pate and Cooke, 1989). In other experiments, the effects on velocity were determined in the absence and presence of 5 mM added MgADP. Standard solutions were used in these experiments (i.e., zero added phosphate, MgATP 4.0 mM, CP 14.5 mM) together with 100 μM diadenosine pentaphosphate (AP_5A) to inhibit endogenous myokinase activity (Pate and Cooke, 1989). During Ca^{2+} activation the MgADP generated by the myosin ATPase reaction in the fiber is estimated to be ~0.9 mM (Cooke and Pate, 1985). In the present experiments this production of MgADP is buffered in part by the creatine kinase reaction. Under conditions in which MgADP is not added to the experimental solutions, it is estimated that [MgADP] is ~0.5 mM in the center of the fiber with a gradient approaching 0.01 mM toward the surface of the fiber (Lu et al., 1993). 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 (1988) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex, employing 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, 1988).

Slack-test protocol

Shortening velocity was determined using the slack-test method (Edman, 1979). At each pCa, steady-state isometric tension was developed and length changes of varying amplitude (ΔL) were applied to one end of the fiber. These changes were of sufficient magnitude to reduce tension to zero. The fiber shortened against zero imposed load, and the time required for the fiber to take up the imposed slack was recorded (Δt). Generally, 5–10 points were collected to construct the Δl-Δt relationship during maximal Ca^{2+} activating conditions, whereas 8–14 data points were collected during submaximal activations as done previously (Moss, 1986; Metzger and Moss, 1988). Linear regression analysis was used to obtain the best fit straight-line. The slope of the Δl versus Δt relationship is taken as unloaded shortening velocity.

Protocol for extraction of troponin C

Troponin C 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 is based on Cox et al. (1981) and used here in modified form (Zot and Potter, 1982) and with the addition of TFP (Metzger et al., 1989). The temperature during extraction was 15°C. The time in extracting solution was varied, and greater amounts of troponin C were extracted as duration was increased from 2 to 30 minutes. The demonstration of functional reconstitution of extracted fibers upon add-back of purified troponin C (Moss et al., 1985; Metzger et al., 1989) indicates that the procedure is specific for removal of troponin C.

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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 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). Gels from control and troponin C extracted fibers have been published (Moss et al., 1985).

Statistics

Unless indicated otherwise data are presented as mean ± SEM (n). Student's two-tailed t-test was used to test for significant differences between two groups, with the confidence level set at $p < 0.05$.

RESULTS

Phosphate effects on velocity at varied Ca^{2+} concentrations

The effect of added phosphate on the velocity of unloaded shortening during maximal Ca^{2+} activation is shown in Fig. 1. In fibers activated at pCa 4.5, the addition of 10 mM phosphate markedly inhibited tension but had little effect on velocity of unloaded shortening. In this fiber, V_{max} was 3.3 and 3.4 muscle lengths (ML)/s in the absence and presence of 10 mM added phosphate, respectively. The mean V_{max} values in the absence (3.5 ± 0.5 ML/s (6)) and presence of 10 mM added phosphate (3.3 ± 0.3 ML/s (6)) were not significantly different ($p > 0.05$). This finding is in agreement with results from previous studies (Cooke and Pate, 1985; Cooke et al., 1988; Chase and Kushmerick, 1988).

Effects of added phosphate on velocity were also determined in fibers during submaximal Ca^{2+} activation. In agreement with earlier studies (Moss, 1986; Metzger and Moss, 1988), slack-test plots were biphasic during submaximal Ca^{2+} activation in the absence of added phosphate (Fig. 2). For extents of filament shortening of less than ~75 nm/half sarcomere (HS) velocity proceeded at a rate comparable with that obtained during full Ca^{2+} activation. This is referred to as the high velocity phase of shortening (Moss, 1986). After ~75 nm/HS of high velocity shortening, an abrupt decrease in velocity was observed that constitutes the low velocity phase of shortening. Both the high and low velocity phases of shortening were well fit by single straight lines ($r > 0.99$). By comparison, during submaximal Ca^{2+} activation in the presence of added phosphate, biphasic slack-test plots were no longer evident (Fig. 2). The main effect of added phosphate on velocity was an increase in the low velocity phase of shortening. Thus the slack-test plot was again best fit by a single straight line. Because added phosphate decreases maximum tension and shifts the tension-pCa relation to the right (Cooke and Pate, 1985; Chase

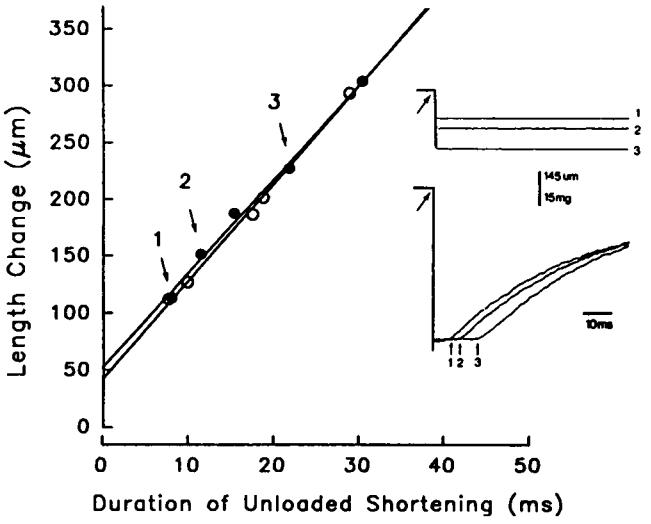

FIGURE 1 Effects on unloaded shortening velocity due to increased concentration of phosphate during maximal Ca^{2+} activation in a single psoas muscle fiber. Filled circles and best fit straight line are pCa 4.5 and zero added phosphate. Open circles and best fit straight line are pCa 4.5 and 10 mM added phosphate. Velocity of unloaded shortening was 3.3 ML/s for zero added phosphate and 3.4 ML/s for 10 mM added phosphate. Relative tension at 10 mM added phosphate was 0.61 P/P_o. (*Inset*) Records of fiber length (*upper records*) and tension (*lower records*) are shown for data obtained at zero added phosphate. The arrow in the upper left part of each set of records indicates the time point for initiation of the release in fiber length. Points marked 1, 2, and 3 in these records correspond to points 1–3 in the main figure. End-to-end fiber length was 2.50 mm. Sarcomere length was 2.50 μm. P_o was 133 kN/m^2.

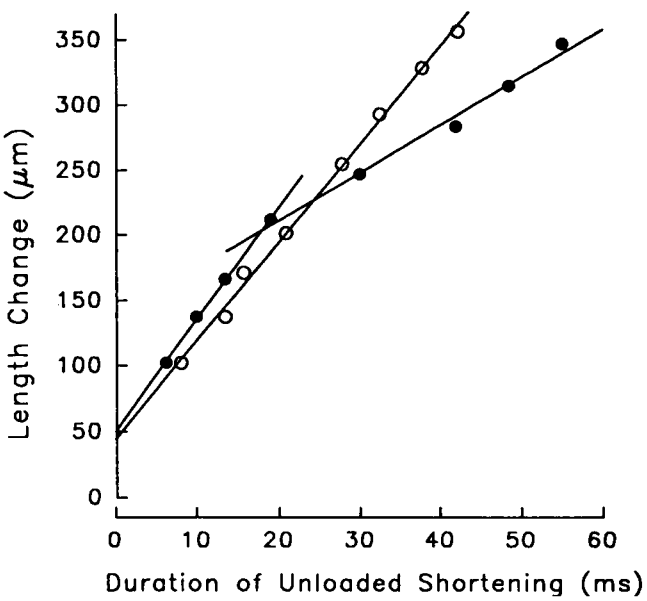

FIGURE 2 Effects on unloaded shortening velocity due to increased concentration of phosphate during submaximal Ca^{2+} activation of a psoas muscle fiber. Filled circles and best fit straight lines are pCa 6.2 and no added phosphate. Open circles and best fit straight line are pCa 5.8 and 30 mM added phosphate. Velocity of unloaded shortening was 3.4 ML/s and 1.4 ML/s for the high and low velocity phases of linear shortening at zero added phosphate, respectively. At 30 mM added phosphate velocity was 3.0 ML/s. P/P_o for zero added phosphate was 0.49, and 0.34 for 30 mM phosphate. End to end length was 2.53. Sarcomere length was 2.56 μm. P_o was 128 kN/m^2.

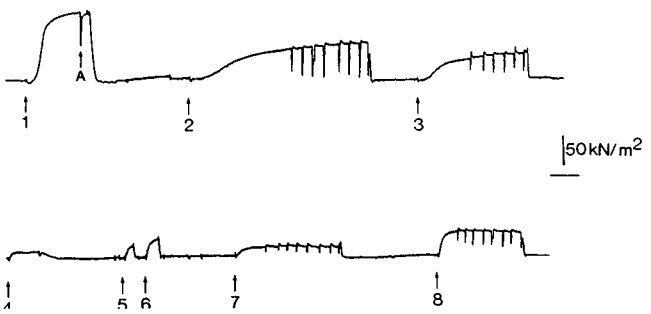

FIGURE 3 Slow time-base recordings of isometric tension at varied [Ca^{2+}] and [phosphate] before and after partial removal of troponin C in a single psoas fiber. The pCa and [phosphate] for each activation (*arrows*) were: 1) pCa 4.5, zero added phosphate, P_o 111 kN/m^2 (A indicates rapid release in fiber length); 2) pCa 6.2, zero added phosphate, 0.56 P_o; 3) pCa 5.8, 10 mM phosphate, 0.39 P_o; 4) partial troponin C extraction, pCa 4.5, zero added phosphate; 5–6) addition of exogenous troponin C to partially reconstitute fiber, pCa 4.5, zero added phosphate; 7) pCa 4.5, 10 mM phosphate, 0.17 P_o; and 8) pCa 4.5, zero added phosphate, 0.34 P_o. Horizontal bar is 10 s for pCa 4.5 activations, 50 s for submaximal Ca^{2+} activations. Relative tension scaled to the pCa 4.5, zero added phosphate value.

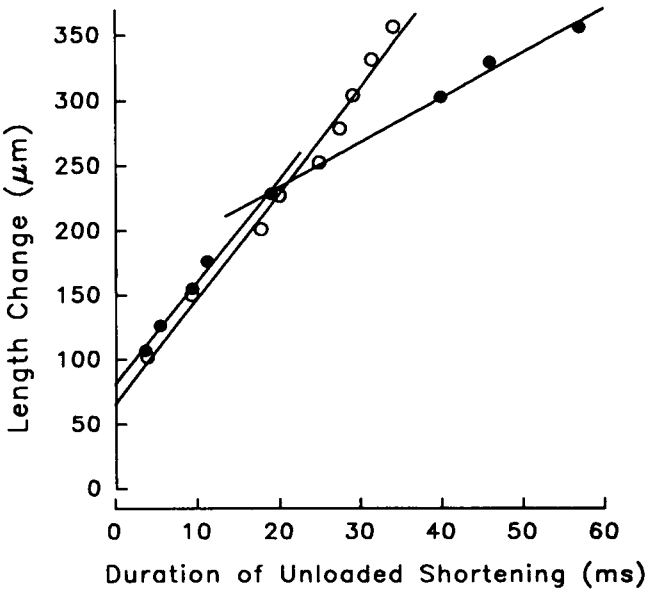

FIGURE 4 Effects on shortening velocity due to increased phosphate in a submaximally Ca^{2+} activated, and partially troponin C extracted psoas fiber. Open circles and best fit straight line are pCa 5.6, 10 mM phosphate. Tension was 0.28 P_o and V_{max} was 2.8 ML/s. Closed circles and best fit straight lines are pCa 6.1, zero added phosphate. Tension was 0.25 P_o, high velocity shortening was 2.9 ML/s and low velocity shortening was 1.2 ML/s. Relative tension at pCa 4.5 after partial extraction of troponin C was 0.90 P_o. End-to-end fiber length was 2.81 mm; sarcomere length was 2.52 μm.

and Kushmerick, 1988; Godt and Nosek, 1989; Millar and Homsher, 1990; Rüegg et al., 1971) velocities were compared at the same relative tensions rather than the same Ca^{2+}. This required that [Ca^{2+}] be increased in experiments performed during submaximal activation in the presence of added phosphate (Fig. 2). Thus, even at reduced relative tensions, biphasic slack-test plots were not detected in fibers activated in the presence of added phosphate (Table 1).

Phosphate effects on velocity in partially troponin C extracted fibers

To distinguish the effects on shortening due to altered [Ca^{2+}] as opposed to altered thin filament activation (as determined by relative tension) fibers were activated at a fixed [Ca^{2+}], whereas thin filament activation was varied by partial extraction of troponin C (Moss, 1986). Troponin C was partially extracted from fibers so that tension at pCa 4.5 approximated the tension obtained in the same fiber at submaximal [Ca^{2+}] before extraction (Fig. 3). In this study, maximum Ca^{2+} activated tension in the partially troponin C-extracted fibers was 0.63 ± 0.08 P_o (P_o is maximum tension in control fiber). As shown previously (Moss, 1986), partial extraction of troponin C induced biphasic shortening records during maximal Ca^{2+} activation. In a representative experiment during maximal Ca^{2+} activation after partial extraction of troponin C, high velocity shortening was 3.8 ML/s, and low velocity shortening was 1.4 ML/s (original data not shown; summary provided in Table 1). In the same fiber activated in the presence of 10 mM phosphate, slack-test data were best fit by a single straight line velocity, and velocity was 2.8 ML/s.

Added phosphate also abolished biphasic shortening in partially troponin C-extracted fibers that were activated at submaximal [Ca^{2+}]s (Fig. 4). Generally, the disparity in the

TABLE 1 Summary of effects on shortening velocity due to alterations in the concentrations of phosphate and Ca^{2+} in control and partially troponin C extracted psoas fibers

	Maximal Ca^{2+}		Submaximal Ca^{2+}		TnC-XT max-Ca		TnC-XT sub-Ca	
	$-P$	$+P$	$-P$	$+P$	$-P$	$+P$	$-P$	$+P$
HV	3.45 ± 0.45	3.33 ± 0.33	3.41 ± 0.17	3.20 ± 0.21	3.90 ± 0.34	3.00 ± 0.20	2.89	2.81
LV	–	–	1.17 ± 0.13	–	1.62 ± 0.28	–	1.2	–
P/P_o	1.00 ± 0.00	0.56 ± 0.03	0.45 ± 0.08	0.32 ± 0.04	0.63 ± 0.08	0.38 ± 0.06	0.25	0.28

Values are mean ± SEM, n = 6–8 (except for TnC-XT sub Ca where one fiber was studied). Velocity reported as ML/s. P/P_o is relative tension and is calculated by dividing the tension in each condition (e.g., max/sub max [Ca], ± P) by the tension at pCa 4.5 with no added phosphate in the same fiber. $-P$, no added phosphate; $+P$, 10 mM added phosphate; HV, high velocity phase of shortening; LV, low velocity phase of shortening; –, no LV observed. TnC-XT max Ca contains data obtained from partially troponin C-extracted fibers studied at pCa 4.5; TnC-XT sub Ca contains data after extraction at pCa 6.1 ($-P$) and pCa 5.6 ($+P$).

rates of high and low velocity shortening in a fiber are comparable at different reduced levels of thin filament activation, whether alterations in thin filament activation are accomplished by partial extraction of troponin C, reduced [Ca^{2+}], or both. As was the case for troponin C-replete fibers, added phosphate appeared to limit the induction of the low velocity phase of shortening such that slack-test data were best fit by a single straight line with velocity comparable with the high velocity shortening obtained in the same fiber in the absence of added phosphate.

Alterations in thin filament activation accomplished by either reducing activating Ca^{2+} in troponin C-replete fibers or by extracting troponin C at fixed [Ca^{2+}] are thought to be qualitatively similar. Evidence in support of this possibility comes from experiments in which the tension-stiffness relationships are obtained in control and troponin C-extracted fibers at varied [Ca^{2+}]s (Fig. 5). Results showed that tension-stiffness data obtained in troponin C extracted fibers fell on the line obtained from control fibers, which was constructed by varying [Ca^{2+}]. Assuming that stiffness provides a measure of the number of cross-bridge attachments to actin (Ford et al., 1977), this provides evidence that the troponin C extraction primarily affects the numbers of cross-bridge interactions rather than affecting some other process such as altering force per interaction.

A summary of the effects of phosphate on velocity is given in Table 1. Three experimental conditions resulted in biphasic slack-test plots in skinned psoas fibers: 1) submaximal Ca^{2+} activation in troponin C replete fibers, 2) maximal Ca^{2+} activation in partially troponin C extracted fibers, and 3) submaximal Ca^{2+} activation in partially troponin C extracted fibers. For each condition, the addition of phosphate to activating solutions reversed biphasic shortening so that the slack-test plots were linear and were generally comparable with those obtained during maximal Ca^{2+} activation in troponin C-replete fibers. Thus, the primary effect of phosphate was on the low velocity phase of shortening such that in the presence of added phosphate (10–30 mM) induction of low velocity shortening was not observed.

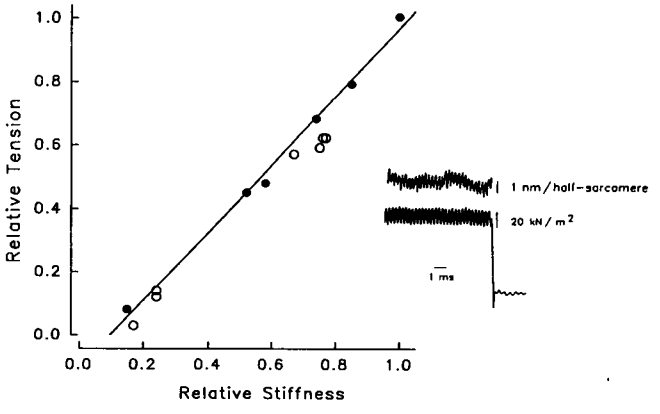

FIGURE 5 Isometric tension-stiffness relationship in control fibers (*filled circles with best fit straight line*) and after partial extraction of troponin C (*open circles*). Data were obtained at pCa 4.5 and at submaximal Ca^{2+} concentrations. (*Inset*) Records of sarcomere length (*upper record* obtained from the position of the first order laser diffraction pattern) and tension (*lower record*). The frequency of oscillation was 3.3 kHz and length change averaged 1 nm/HS.

MgADP effects on velocity at varied Ca^{2+} concentrations

Maximum Ca^{2+} activated tension was increased by ~20% upon addition of 5 mM MgADP to the activating solution (Fig. 6). Added MgADP also produced a leftward shift in the tension-pCa relationship. These effects of added MgADP on steady-state isometric tension are in general agreement with earlier findings (Cooke and Pate, 1985; Godt and Nosek, 1989).

During maximal Ca^{2+} activation slack-test plots became biphasic in the presence of 5 mM added MgADP (Fig. 7). In this fiber the high and low velocity phases of shortening were 2.9 ML/s and 1.5 ML/s, respectively, as compared with a velocity of 4.2 ML/s obtained in the absence of added MgADP. Overall, in fibers activated at pCa 4.5, added MgADP significantly ($p < 0.05$) reduced high velocity shortening (2.8 ± 0.5 ML/s (8)) in comparison with controls (4.5 ± 0.3 ML/s (9)). In control experiments it was found that the effects of added MgADP on altering shortening were reversible. Data collected from single fibers in the absence, presence, and again in the absence of added MgADP demonstrated a restoration in V_{max} (0.97 ± 0.11, $n = 5$, calculated as posttest V_{max} (no added MgADP)/ pretest V_{max} (no added MgADP) in the same fiber). Biphasic shortening was not observed in control fibers activated at pCa 4.5 in the absence of added MgADP.

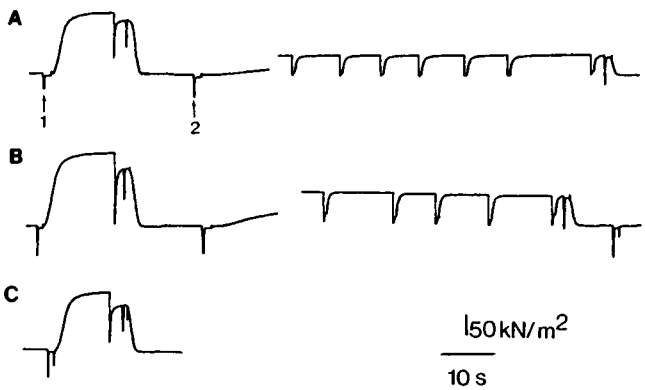

FIGURE 6 Slow time-base recordings of isometric tension at varied [Ca^{2+}] and [MgADP] in a single psoas fiber. (*A*) At point 1, the fiber was activated at pCa 4.5 with zero added MgADP. The fiber was relaxed and subsequently activated (point 2) at pCa 6.2 with zero added MgADP. Relative tension was 0.35 P_o. (*B*) The fiber was next activated at pCa 4.5 with 5 mM added MgADP (tension was 1.22 P_o), then relaxed and subsequently activated at pCa 6.2 with 5 mM added MgADP (tension was 0.46 P_o). (*C*) Fiber was then returned to nominal MgADP solution and activated at pCa 4.5 (tension was 1.00 P_o). Gaps in pCa 6.2 records represent 1–3 minutes. P_o was 150 kN/m^2. Fiber length was 1.48 mm and sarcomere length was 2.58 μm.

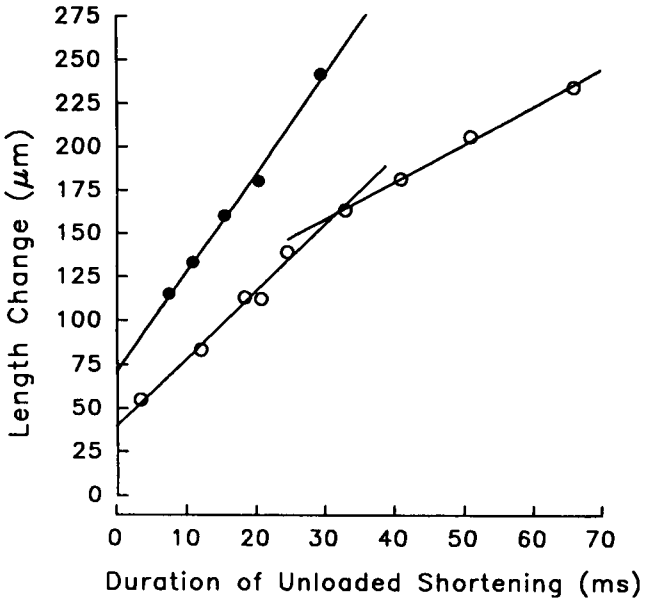

FIGURE 7 Effects on unloaded shortening velocity due to 5 mM added MgADP during maximal Ca^{2+} activation in a single psoas muscle fiber. Filled circles and best fit straight line are pCa 4.5 and zero added MgADP. Open circles and best fit straight lines are pCa 4.5 and 5 mM added MgADP. Velocity of unloaded shortening was 4.2 ML/s for zero added MgADP, and 2.9 ML/s and 1.5 ML/s for the high and low velocity phases of shortening, respectively, obtained in the presence of 5 mM MgADP. Relative tension at 5 mM added MgADP was 1.15 P_o. End-to-end fiber length was 1.36 mm. Sarcomere length was 2.58 μm. P_o was 188 kN/m^2.

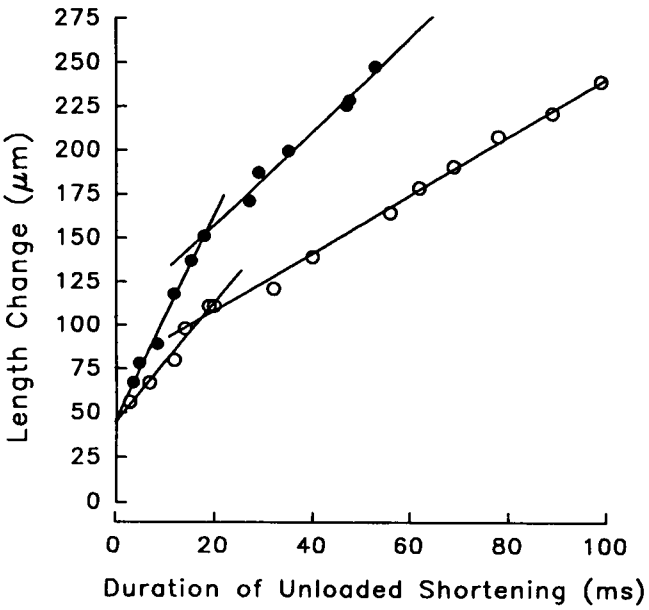

FIGURE 8 Effects on unloaded shortening velocity due to 5 mM MgADP during submaximal Ca^{2+} activation in a single psoas muscle fiber. Filled circles and best fit straight lines are pCa 6.3 and zero added MgADP. High and low velocity phases of shortening were 3.7 ML/s and 1.7 ML/s, respectively. Tension was 0.35 P_o. Open circles and best fit straight lines are pCa 6.3 and 5 mM added MgADP. High and low velocity phases of shortening were 2.1 ML/s and 1.1 ML/s, respectively. Tension was 0.57 P_o. Fiber length was 1.59 mm, sarcomere length was 2.58 μm.

TABLE 2 Summary of effects on shortening velocity due to alterations in the concentrations of MgADP and Ca^{2+} in psoas fibers

	Maximal Ca^{2+}		Submaximal Ca^{2+}	
	−MgADP	+MgADP	−MgADP	+MgADP
HV	1.00 ± 0.00	0.64 ± 0.11	0.62 ± 0.13	0.42 ± 0.06
LV	–	0.48 ± 0.04	0.30 ± 0.05	0.23 ± 0.01
P/P_o	1.00 ± 0.00	1.17 ± 0.02	0.31 ± 0.04	0.52 ± 0.06

All data are scaled to the pCa 4.5, zero added MgADP value obtained in each fiber. Values are mean ± SEM, average of six observations per group. −MgADP, no added MgADP; +MgADP, 5 mM added MgADP; HV, high velocity phase of shortening; LV, low velocity phase of shortening. Average velocity at maximum Ca^{2+} without added MgADP was 4.5 ± 0.3 ML/s (n = 9). No LV was apparent (−) during maximum Ca^{2+} activation in the absence of added MgADP. P/P_o is the relative tension and is calculated by dividing the tension in each condition (e.g., max/sub max [Ca], ± MgADP) by the tension at pCa 4.5 with no added MgADP in the same fiber.

Added MgADP also slowed unloaded shortening velocity in fibers during submaximal Ca^{2+} activation (Fig. 8). As before, in the absence of added MgADP, biphasic shortening was induced during submaximal Ca^{2+} activation. At the same submaximal [Ca^{2+}], 5 mM added MgADP had the effect of further reducing both the high and low velocity phases of shortening. In this fiber, isometric tension was increased from 0.35 P_o in control to 0.57 P_o with 5 mM added MgADP. Thus, during submaximal Ca^{2+} activation in the presence of added MgADP there was a dissociation of effects on velocity (decreased) and relative tension (increased).

The transition point between the initial high velocity phase of shortening and subsequent low velocity phase of shortening was calculated and found to be similar under the various conditions tested. For example, in fibers activated at pCa 4.5 in the presence of 5 mM MgADP the transition point between high and low phases of shortening occurred at 79 ± 16 nm/HS of filament shortening, which was comparable with values obtained in control fibers during submaximal Ca^{2+} activation (76 ± 10 nm/HS).

A summary of the effects of added MgADP on velocity and tension is presented in Table 2. A main effect of added MgADP was to decrease the high velocity phase of shortening in both maximally and submaximally Ca^{2+} activated psoas fibers. In addition, in contrast to controls, added MgADP induced biphasic shortening during maximal Ca^{2+} activation.

DISCUSSION

The main findings of this study are that 1) added phosphate eliminated biphasic slack-test plots during submaximal thin filament activation accomplished by reducing [Ca^{2+}] in activating solutions, the partial extraction of troponin C, or both; and 2) added MgADP induced biphasic shortening in fibers activated maximally by Ca^{2+}.

These findings can be discussed in the context of Huxley's (1957) model of contraction, in which the velocity of unloaded shortening is established by a dynamic balance between two types of attached cross-bridges: 1) those that bear positive strain and thus support the relative sliding of the myofilaments, and 2) those that are extended to regions of negative x, bear negative strain, and oppose myofilament sliding. In this model, the maximum velocity of unloaded shortening is controlled by the detachment rate constant for negatively strained cross-bridges (g_2) that oppose contraction. Thus, biochemical or mechanical factors that influence g_2 are predicted to alter the velocity of shortening in intact muscle fibers. Recent information suggests that the correlate to Huxley's (1957) g_2 in the ATP hydrolysis cycle is likely ADP release or an isomerization of the AM′.ADP intermediate (Dantzig et al., 1991; Siemankowski et al., 1985).

In the present study, biphasic shortening was apparent in psoas fibers activated maximally with Ca^{2+} in the presence of 5 mM added MgADP. The finding that added MgADP slowed the high velocity phase of shortening agrees with earlier reports (Cooke and Pate, 1985; Homsher et al., 1992). This effect may be attributed to competition between MgADP and MgATP for the nucleotide binding site on myosin (Cooke and Pate, 1985). In the presence of added MgADP, cross-bridges may be detained in the attached state so that they bear an increased compressive strain and hence provide a hindrance to the relative sliding of the myofilaments. This explanation, however, may not be sufficient to account for the low velocity phase of shortening apparent in fibers activated in the presence of added 5 mM MgADP. Although there have been several reports documenting the low velocity phase of shortening (Moss, 1986; Metzger and Moss, 1988; Farrow et al., 1988; Martyn et al., 1994), no consensus has been reached on a possible mechanism(s) underlying this behavior.

To account for biphasic shortening in skeletal fibers, Moss (1986) proposed that under conditions of reduced thin filament activation cross-bridges attached in regions between activated and inactivated functional groups (where a functional group is defined as 1 troponin complex, 1 tropomyosin, and 7 actin monomers; Bremel and Weber, 1972) may be physically constrained from detaching such that they become compressed axially, buckle, and bear a tension that opposes the relative sliding of the myofilaments. These long-lived cross-bridges must eventually detach to account for the observed steady rate of shortening. However, it is not readily apparent how this mechanism could be operating in fibers activated at pCa 4.5 in the presence of added MgADP, because under these conditions all functional groups should be activated. In the presence of added MgADP maximum isometric tension is increased and tension-pCa is shifted to the left (Cooke and Pate, 1985; Godt and Nosek, 1989; present study) indicating an overall enhancement of activation in the fiber.

One possible basis of biphasic shortening in maximally activated fibers in the presence of MgADP is that MgADP may bind to AM to form an AM.ADP cross-bridge state that is relatively long-lived with respect to the AM′.ADP state. The AM.ADP state is generally not considered to be part of the ATP hydrolysis pathway under normal physiological conditions. It is included in the ATP hydrolysis pathway (Scheme 1) to account for the finding that in fibers in rigor, added MgADP binds to AM to form an AM.ADP state. However, the AM.ADP state is probably different from the AM′.ADP state, which is obtained during an active contraction. One observation in support of this idea is that the addition of MgADP and then phosphate to a fiber in rigor has no effect on rigor tension (Hibberd et al., 1985). Thus, in the present study, added MgADP could be having two effects on the cross-bridge cycle: 1) altering the dissociation of AM by ATP, and 2) binding to AM to produce the AM.ADP state, which may be a long-lived state with respect to the AM′.ADP state. The basis of biphasic shortening in fibers activated at pCa 4.5 and in the presence of 5 mM MgADP may be different than that for fibers activated at submaximal $[Ca^{2+}]$s in the absence of added MgADP. This is so because the AM.ADP state does not appear to be a part of the normal ATP hydrolysis pathway in fibers (Sleep and Hutton, 1980).

An unexplained feature of the biphasic slack-test plots is the ~75 nm/HS of shortening that occurs before the transition to the slow velocity phase of shortening. The basis of this shortening distance is not known but could arise from the mechanical effects of a population of cross-bridges with reduced g_2, which would manifest as an internal load to the fiber (Moss, 1986; Metzger and Moss, 1988). However, this possibility seemingly requires that the working distance of the cross-bridge must be on the order of ≈75 nm, a value that is considerably larger than the estimated myosin step size of 8–10 nm as derived from quick release experiments on single muscle fibers (Huxley and Simmons, 1971). However, results obtained from in vitro motility assays provide evidence that during an unloaded contraction the working distance of an attached cross-bridge is at least 60 nm, and indeed may be as great as 200 nm (Yanagida et al., 1985; Harada et al., 1990). In skinned fiber experiments, the myosin-actin interaction distance per ATP molecule hydrolyzed has been measured during isotonic contractions (Higuchi and Goldman, 1991). It was found that as the external load decreased, the calculated interaction distance (D_i) increased, with the lower limit for D_i determined to be 61 nm. These findings raise the possibility that the transition point between the high and low velocity phases of shortening is a result of cross-bridges that have been detained in the attached state, and which travel a negative drag distance on the order of ≈75 nm before eventually detaching from the thin filament. It should be possible to test this hypothesis, because during unloaded shortening at submaximal activations, the structure of these highly negatively strained cross-bridge should be detectable in electron micrographs (Higuchi and Goldman, 1991).

Alternatively, it is possible that biphasic shortening could relate to thin filament inactivation as a result of reduced

numbers of attached cross-bridges during filament sliding. There is evidence that Ca^{2+} and attached cross-bridges influence the activation of the thin filament (Bremel and Weber, 1972; Gordon and Ridgway, 1990; Güth and Potter, 1987). Gordon and Ridgway (1990) observed an extra Ca^{2+} signal in single muscle fibers as a result of a step release in muscle length during the declining phase of the Ca^{2+} transient. This extra Ca^{2+} is thought to be released from the regulatory Ca^{2+} binding sites of troponin C due to a decrease in the number of attached cross-bridges during shortening, and is evidence of reciprocal interactions between Ca^{2+} binding to troponin C and cross-bridge binding to actin. During unloaded shortening there is a decrease in number of attached cross-bridges (Julian and Sollins, 1975) which, based on the above studies, could affect the level of thin filament activation. In fibers shortening during a submaximal activation, thin filament activation may be compromised because of the combined reduction in the activating ligands Ca^{2+} and cross-bridges. The distance of shortening to the break-point in the biphasic records could arise if the deactivation of the thin filament is not an instantaneous event, but rather occurs with some finite delay. In this case the break-point would be defined by the time required for the thin filament to deactivate after a step release in length rather than be related to the extent of translation of the myofilaments. One piece of evidence against this possibility, however, comes from earlier work in which biphasic shortening was examined in fibers treated with dextran to compress the myofilament lattice (Metzger and Moss, 1988). Results showed that the shortening distance to the break-point at a fixed submaximal [Ca^{2+}] was essentially unchanged in the presence of dextran, while the time to the break-point increased markedly from 50 to 140 ms (see Fig. 2 *C* of Metzger and Moss, 1988). This appears to be a direct result of dextran slowing cross-bridge cycling rather than an effect on the timing of thin filament deactivation. A second point to consider is that during an unloaded contraction, the Ca^{2+} present in the activating solutions may be sufficient to maintain the thin filament activated despite a reduction in the number of attached cross-bridges. Kress et al. (1986) used x-ray diffraction to resolve structural alterations in the position of tropomyosin during activation and relaxation of intact frog muscle fibers. In their analysis, the apparent alteration in the position of tropomyosin was the first detected structural event of muscle contraction, and the time course of this change in tropomyosin position was not significantly altered upon stretching the fibers so that the thick and thin filaments did not overlap. This is evidence that the activating effects of Ca^{2+} alone can lead to movement in tropomyosin. This raises the possibility that during an unloaded contraction, Ca^{2+} may be capable of sustaining the thin filament in the activated state despite the decrease in cross-bridge attachment. A final point is that if cross-bridge-mediated thin filament inactivation plays an important role in the mechanism of biphasic shortening, then it seems likely that biphasic records should be apparent to some degree at maximal Ca^{2+} activation. However, in control fibers activated at pCa 4.5, biphasic slack-test records are not observed. Taken together, these findings are evidence that the ≈75 nm/HS distance shortened, rather than the time required to shorten, is the critical parameter in defining biphasic slack-test plots.

An interesting finding of the present study was added phosphate prevented the induction of low velocity shortening in fibers. This was apparent under two experimental conditions which, in the absence of added phosphate, induce biphasic shortening, namely, submaximal Ca^{2+} activation and partial extraction of troponin C. If the induction of low velocity shortening is due to the accumulation of cross-bridges bearing a compressive strain with reduced g_2, then our finding could be explained if phosphate inhibits the formation of these highly strained cross-bridges. If this is the case then phosphate binding to cross-bridges should be strain-dependent. Consistent with this possibility, the affinity of AM′.ADP for phosphate is altered by strain such that the apparent phosphate association constant increases with increased strain of the AM′.ADP state (Webb et al., 1986). If this relationship holds for cross-bridges bearing a compressive strain, which oppose contraction, then the formation of these cross-bridges should be limited under high phosphate conditions. In this regard, one observation that requires comment is that during maximal Ca^{2+} activation there is no effect of added phosphate on the velocity of shortening (Cooke and Pate, 1985; Cooke et al., 1988; Chase and Kushmerick, 1988; present study), despite the hypothesized formation of negatively strained cross-bridges under these conditions (Huxley, 1957). Similarly, during submaximal activating conditions, added phosphate has no effect on the rate of filament sliding in the high velocity phase of shortening. One possible explanation for these findings is that there are qualitative differences in the negatively strained cross-bridges that arise during maximal compared with submaximal activation. In this case, phosphate would have to preferentially inhibit formation of the long-lived-type cross-bridges that are hypothesized to arise only during submaximal activation (Moss, 1986; Metzger and Moss, 1988).

In summary, this study focused on the basis of biphasic shortening that is induced during submaximal activation of the thin filament in skeletal muscle fibers. Biphasic shortening was prevented in fibers activated in the presence of added phosphate. By comparison, biphasic shortening was induced by MgADP during maximal Ca^{2+} activations. It has been proposed (Moss, 1986) that biphasic slack-test relations may be a manifestation of a shortening-induced increase in internal load due to the prolonged attachment of strained cross-bridges. The present findings could be explained if phosphate preferentially binds to, and specifically inhibits the formation of, cross-bridges that are strained to bear a tension that opposes contraction. Consistent with this idea, the induction of biphasic shortening during maximum Ca^{2+} activation by added MgADP could result from an increase in the population of long-lived cross-bridges that oppose the relative sliding of the myofilaments.

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