Suppression of Muscle Contraction by Vanadate

Mechanical and Ligand Binding Studies on Glycerol-extracted Rabbit Fibers

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ABSTRACT The suppression of tension development by orthovanadate (V_i) was studied in mechanical experiments and by measuring the binding of radioactive V_i and nucleotides to glycerol-extracted rabbit muscle fibers. During active contractions, V_i bound to the cross-bridges and suppressed tension with an apparent second-order rate constant of 1.34×10^5 $M^{-1}s^{-1}$. The half-saturation concentration for tension suppression was 94 μM V_i. The incubation of fibers in V_i relaxing or rigor solutions prior to initiation of active contractions had little effect on the initial rise of active tension. The addition of adenosine diphosphate (ADP) and V_i to fibers in rigor did not cause relaxation. Suppression of tension only developed during cross-bridge cycling. After slow relaxation from rigor in 1 mM V_i and low (50 μM) MgATP concentration (0 Ca^{2+}), radioactive V_i and ADP were trapped within the fiber. This finding indicated the formation of a stable myosin·ADP·V_i complex, as has been reported in biochemical experiments with isolated myosin. V_i and ADP trapped within the fibers were released only by subsequent cross-bridge attachment. V_i and ADP were preferentially trapped under conditions of cross-bridge cycling in the presence of ATP rather than in relaxed fibers or in rigor with ADP. These results indicate that in the normal cross-bridge cycle, inorganic phosphate (P_i) is released from actomyosin before ADP. The resulting actomyosin·ADP intermediate can bind V_i and P_i. This intermediate probably supports force. V_i behaves as a close analogue of P_i in muscle fibers, as it does with isolated actomyosin.

INTRODUCTION

A fundamental goal of muscle research has been to understand the relationship between the elementary biochemical reactions and the mechanical and structural states of the contractile machinery. Studies using isolated proteins have indicated that actomyosin hydrolyzes ATP via the following series of reactions (Lymn and

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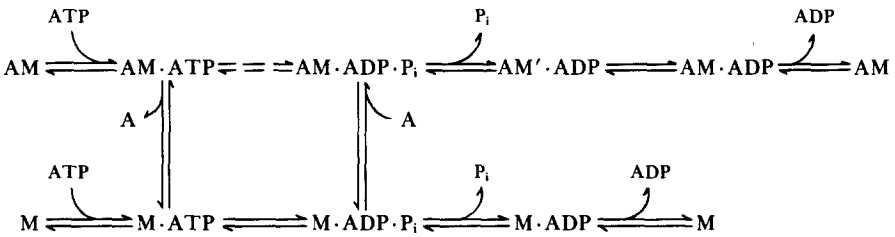
Taylor, 1971; Trentham et al., 1976; Taylor, 1979; Eisenberg and Greene, 1980; Sleep and Hutton, 1980; Mornet et al., 1981):



(1)

where A is actin, M is myosin, ATP is adenosine triphosphate, ADP is adenosine diphosphate, and P_i is inorganic phosphate.

It is generally presumed that the cyclic interaction between actin and myosin (cross-bridge cycle) during energy transduction by a muscle fiber corresponds to this biochemical scheme. The $M \cdot ADP \cdot P_i$ intermediate is relatively stable, until it binds to actin (cross-bridge attachment), which promotes the release of P_i. We have recently reported evidence that the P_i release step in muscle fibers is closely coupled to force generation in the cross-bridge cycle (Hibberd et al., 1985*a*) and that P_i release can be reversed by the binding of P_i to an $AM' \cdot ADP$ state (Webb et al., 1985).

One approach toward defining the relationship between muscle biochemistry and mechanics has been through mechanical and structural studies of well-defined but "frozen" biochemical states in muscle fibers (Reedy et al., 1965; Mannherz et al., 1973; Goody and Holmes, 1983). Several stable states of actomyosin that may correspond to intermediates of the cross-bridge cycle have been studied with the isolated proteins, but they have not yet been fully characterized in muscle fibers (Mornet et al., 1981; Wells and Yount, 1982). Among these is the stable complex formed by myosin, ADP, and orthovanadate (V_i).

In studies with isolated myosin and actomyosin, V_i acts as a tightly bound analogue of P_i. The dynein ATPase, Na,K-ATPase of red cell membranes, and Ca-ATPase of sarcoplasmic reticulum, as well as several other enzymes, are inhibited by the formation of a stable enzyme-V_i complex (Gibbons et al., 1978; Smith et al., 1980; Oritz et al., 1984, and references therein). The tight binding of V_i to the phosphate site of the proteins is a widely applicable model.

V_i blocks the myosin and actomyosin ATPase reactions by forming a stable $M \cdot ADP \cdot V_i$ complex (Goodno, 1979). This intermediate dissociates very slowly (rate constant $\approx 2.5 \times 10^{-6}\ s^{-1}$, Goodno, 1982), but binding to actin promotes V_i and ADP release (Goodno and Taylor, 1982). V_i also suppresses the contraction of skinned muscle fibers (Goody et al., 1980; Magid and Goodno, 1982). A more detailed mechanical and chemical characterization of muscle fibers suppressed by V_i would provide a comparison with the states of the isolated proteins.

We have measured the binding of radioactive ADP, ATP, and V_i to skinned muscle fibers to determine whether a stable $M \cdot ADP \cdot V_i$ complex forms as in solution. Cross-bridge attachment, force generation, and the kinetics of V_i binding were studied in mechanical experiments. The results indicate that the interactions between V_i and actomyosin in muscle fibers are closely analogous to

the corresponding behavior of the isolated proteins and that V_i binding and dissociation affect cross-bridge force production. Thus, V_i qualitatively behaves like P_i.

Preliminary results were presented to the Biophysical Society (Goldman et al., 1983).

METHODS

Glycerol-extracted single fibers from rabbit psoas muscle were prepared as described by Goldman et al. (1948*a*). T-shaped aluminum clips folded around the ends of the fiber held it horizontally in the apparatus (Goldman and Simmons, 1984). The fiber dimensions were determined by microscopy as described in Goldman and Simmons (1984).

Mechanical Apparatus

The method used for monitoring and recording tension and stiffness and the trough system for Figs. 1–6 were described in Goldman et al. (1984*a*). Briefly, tension was measured at one end of the fiber while a piezoelectric device imposed a low-amplitude (1 μm), 500-Hz sinusoidal length change. The steady tension and sinusoidal components of tension in phase and 90° out of phase with the length change were separated by a lock-in amplifier. The in-phase component (stiffness) is qualitatively related to the total number of cross-bridge attachments and the out-of-phase component (quadrature stiffness) indicates the presence of cross-bridges with quick stress-relaxation such as actively force-generating cross-bridges (Goldman et al., 1984*a*).

Stiffness was also determined by small step length changes (Fig. 7). In order to eliminate contributions of end compliance in those experiments, the sarcomere length change in a central region of the fibers was measured at high time resolution by a white-light diffraction method (Goldman, 1983).

When long incubation periods were required (Fig. 8), water evaporation and the consequent variation of the bathing constituents were prevented by covering the solution troughs with a layer of silicon oil. The solution exchange apparatus in this case has been described by Hibberd et al. (1985*b*).

Solutions

The constituents of the bathing solutions are listed in Table I. Vanadium pentoxide was purchased from Fisher Scientific Co., Pittsburgh, PA. A 5- or 10-mM stock of V_i was prepared and then heated to ~100°C for 1 h while maintaining the pH at 10 by the addition of KOH. The stock was cooled and then retitrated to pH 10 at room temperature. This procedure was used to minimize V_i polymerization (Pope and Dale, 1968; Goodno, 1979). Experimental solutions were made from the V_i stock a few hours before each experiment.

Initiation of Contraction

Ca-JUMP METHOD (FIGS. 1–3) The Ca^{2+} buffer gradient method of Moisescu (1976) was used to switch fibers rapidly from the relaxed to the actively contracting state. A fiber was first incubated for two periods of 2 min each in low-EGTA pre-activating solution to reduce the internal Ca^{2+} buffering capacity of the fiber. The fiber remained relaxed in the pre-activating solution. The fiber was then transferred into a high-CaEGTA activating solution, and tension rose quickly to a plateau value. The contraction was terminated after 5–60 s by transferring the fiber to a 0-Ca^{2+}, high-EGTA relaxing solution.

ATP-JUMP METHOD (FIG. 4) Fibers were also rapidly switched from rigor to active contractions by laser-pulse photolysis of caged ATP (Goldman et al., 1982, 1984*a*, *b*).

Fibers were transferred through the following series of solutions: (*a*) low-ATP relaxing solution (0.1 mM MgATP [Table I, *A*]); (*b*) rigor solution (0 Ca^{2+} [Table I, *A*]); (*c*) rigor solution with Ca^{2+} (without V_i [Table I, *A*] or with V_i [Table I, *B*]); and (*d*) rigor solution

TABLE I

Composition of Solutions

Solution name	Total $MgCl_2$	Final MgATP	Total Na_2ATP	Final MgADP	Total ADP	Total Ca	Total EGTA	Total HDTA	Total Na_2CP	Total V_i	Total CK
					(*A*) No-V_i solutions						
Activating	6.76	5.0	5.49	—	—	25.0	25.0	—	19.49	—	1.0
Pre-activating	6.93	5.0	5.45	—	—	*	0.10	24.9	19.49	—	1.0
Relaxing	7.70	5.0	5.44	—	—	*	25.0	—	19.11	—	1.0
Relaxing (low ATP)	2.66	0.1	0.12	—	—	*	30.0	—	21.56	—	1.0
Rigor	3.23	—	—	—	—	*	51.48	—	—	—	—
Rigor with Ca^{2+}	1.27	—	—	—	—	20.0	20.0	32.63	—	—	—
Rigor with ADP	4.07	—	—	2.0	4.80	—	—	—	—	—	—
					(*B*) V_i solutions						
Activating	6.55	5.0	5.48	—	—	30.0	30.0	—	13.96	1.0	1.0
Pre-activating	6.75	5.0	5.43	—	—	*	0.10	29.9	13.96	1.0	1.0
Relaxing	7.67	5.0	5.43	—	—	*	30.0	—	13.51	1.0	1.0
Rigor	2.50	—	—	—	—	*	30.8	21.30	—	1.0	—
Rigor with Ca^{2+}	1.17	—	—	—	—	30.0	30.0	23.66	—	1.0	—
Slow relaxing	2.5	0.05	0.05	—	—	—	30.8	21.30	—	1.0	—
Rigor with ADP	4.47	—	—	2.05	5.0	*	30.7	14.73	—	1.0	—
					(*C*) No-V_i photolysis solutions						
Rigor with CP	2.63	—	‡	—	—	*	20.0	13.58	20.0	—	1.0
Rigor with CP and Ca^{2+}	1.34	—	‡	—	—	20.0	20.0	12.49	20.0	—	1.0
					(*D*) V_i photolysis solutions						
Rigor with CP	2.63	—	‡	—	—	*	20.0	12.93	20.0	1.0	1.0
Rigor with CP and Ca^{2+}	1.87	—	‡	—	—	20.0	20.0	13.28	20.0	1.0	1.0
					(*E*) Radioactive binding solutions						
$^{48}V_i$ incubating	1.2	0.05	0.05	—	—	*	5.0	—	—	0.5–1.0	—

[^{14}C]ATP and [^{14}C]ADP incubating solutions were the same as V_i rigor (*B*) with labeled and unlabeled nucleotide added to above solution. 150 mM KCl was added to the $^{48}V_i$ incubating solution to maintain an ionic strength of 0.2 M.

* $Ca^{2+} < 10^{-8}$.

‡ All photolysis solutions contained 10 mM caged ATP.

All concentrations are in millimolar except creatine kinase (CK), which is given in milligrams per milliliter. All solutions contained 100 mM TES, 1 mM free Mg^{2+}, and 10 mM reduced glutathione (except $^{48}V_i$ incubating solution, which did not contain glutathione). pH was titrated to 7.1 with KOH at 20°C. The ionic strength was 0.2 M. HDTA: 1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; TES: *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; CP: creatine phosphate.

with Ca^{2+}, creatine phosphate, and 10 mM caged ATP (without V_i [Table I, *C*] or with V_i [Table I, *D*]). 2 min was alloted for the diffusion of caged ATP into the filament lattice. Then a 50-ns, 100–150-mJ, 347-nm pulse from a frequency-doubled ruby laser photolyzed 0.75–1.1 mM caged ATP to ATP and 2-nitrosoacetophenone. The ATP was liberated

exponentially with a time constant of ~10 ms, which led to a rapid contraction in the presence of Ca^{2+}.

Ligand Binding Experiments

The multi-trough system used in these experiments was similar to that described by Goldman and Simmons (1984). However, the troughs were smaller (volume ≈27 μl) and Teflon-coated to facilitate the collection of samples and washing of the troughs.

Vanadyl chloride ($^{48}VCl_5$) and the ammonium salts of [U-^{14}C]adenosine 5′-diphosphate ([^{14}C]ADP) and [U-^{14}C]adenosine 5′-triphosphate ([^{14}C]ATP) were purchased from Amersham Corp., Arlington Heights, IL. $^{48}V_i$ was added to an experimental solution containing 0.5–1 mM unlabeled V_i (Table I, *E*). The solution was then heated to ~100°C for 3 h at pH 7.1 to depolymerize the radioactive vanadate (Pope and Dale, 1968; Goodno, 1979). 50 μM ATP was added when the solution cooled to room temperature. Labeled ATP and ADP solutions were made by adding [^{14}C]ATP or [^{14}C]ADP to a solution with V_i and carrier nucleotides at the concentrations listed in the text. The final solutions were used within 1 wk.

A fiber was incubated in a solution with radioactive $^{48}V_i$, [^{14}C]ATP, or [^{14}C]ADP for 25 min. The fiber was then transferred to an unlabeled relaxing solution for ~1 s and then put through a series of unlabeled washout solutions for 1- or 3-min periods. Each 27-μl bath was collected into a scintillation bottle by a vacuum trap, followed by the rinsing of 1 ml of water through the trough into the scintillation vial. This procedure transferred at least 99.7% of the counts in a trough to the collection vial. The radioactivity was counted on an LS 7000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) after mixing with 15 ml of scintillation fluid. At the end of each experiment, the fiber was dissolved in 1 ml of 1 N NaOH and then treated as above to determine the counts remaining. The radioactivity within the fiber at a particular time in the washout period was calculated by summing the counts collected in the bathing media after that time point plus the final fiber radioactivity.

RESULTS

Mechanical Measurements

Ca-JUMP EXPERIMENTS Fig. 1*A* shows the tension and stiffness of a single glycerol-extracted rabbit fiber during an isometric contraction and relaxation cycle. The Ca-jump technique described in the Methods was used to rapidly increase the internal Ca^{2+} concentration. When the fiber was put into the activating solution, tension rose to a steady value of 161 ± 12 kN/m^2 (mean ± SEM, n = 10) with a half-time of <250 ms.

The addition of 1 mM V_i to the activation medium during the next contraction (Fig. 1*B*) caused the suppression of active force generation as previously reported (Goody et al., 1980; Magid and Goodno, 1982). Stiffness was also suppressed. Transferring the fiber to a trough containing activating solution without V_i (Fig. 1*C*) caused slow recovery to the fully active tension level. To ensure a thorough removal of V_i, the trough was exchanged twice again for fresh V_i-free activating solution during the recovery. The fiber was relaxed and a subsequent contraction and relaxation (Fig. 1*D*) showed that the fiber recovered fully from the suppressed condition. Thus, V_i binds and is released on the time scale of seconds during active contractions.

When V_i was added to the 0-Ca^{2+} relaxing and pre-activating solutions before a contraction, and the fiber was then activated in the same V_i concentration (Fig. 1*E*), tension and stiffness initially developed at normal rates but then decayed.

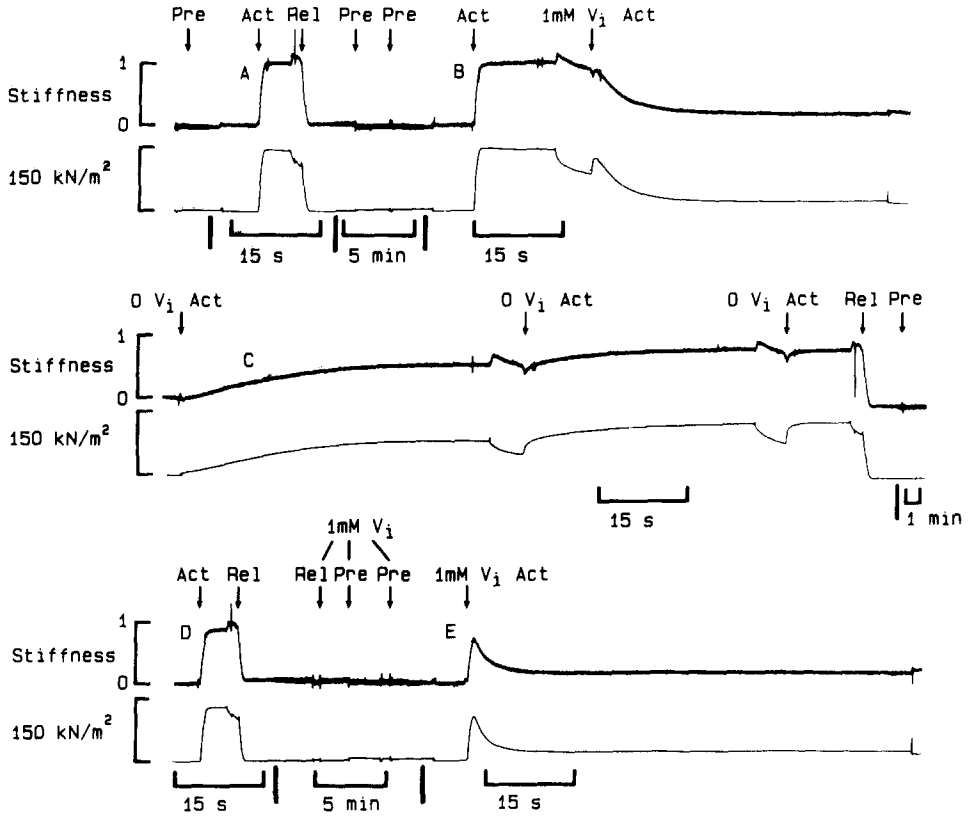


FIGURE 1. Suppression of active tension and stiffness by vanadate in a skinned muscle fiber. Continuous chart recording of tension (lower traces) and stiffness (500 Hz, in phase with a 1-μm sinusoidal length change) during a series of active contractions in the absence and presence of V_i. Before a contraction, the fiber was washed twice with low EGTA pre-activating solution (Pre). The fiber was then immersed in a high-CaEGTA activating solution (Act) and relaxed in a high-EGTA relaxing solution (Rel). (*A*) Control contraction in the absence of V_i. (*B*) The contraction was initiated as in *A*, but after 17 s the fiber was transferred to an activating solution containing 1 mM V_i. (*C*) Tension and stiffness recovery in 0-V_i Act. To ensure a thorough washout of V_i, the solution was exchanged for 0-V_i Act two more times. Transient decreases of tension immediately preceding each 0-V_i Act wash were due to cooling of the fiber in air during the solution exchanges. (*D*) Control contraction illustrating full recovery of tension and stiffness. After relaxation, the fiber was washed once in 1 mM V_i Rel and twice in 1 mM V_i Pre. (*E*) Activation in a steady 1 mM V_i concentration. Even though the V_i concentration was not changing, a transient contraction preceded suppression of tension. Sarcomere length, 2.12 μm; fiber dimensions, 2.29 mm × 7,133 μm^2; temperature, 21°C.

This transient contraction suggested that V_i does not bind until the fiber is activated. Since the V_i concentration in the fiber was constant during the transient contraction of Fig. 1*E*, the rate of suppression was not limited by V_i diffusion into the fiber. We took advantage of this characteristic to investigate the kinetic properties of V_i binding.

Fig. 2 shows tension recordings from control contractions in the absence of V_i (*A* and *C*) and Ca-jump activations in the presence of 125 μM (*B*) and 1 mM (*D*) steady V_i concentrations. In the presence of V_i, tension rose at a rate similar to that in the absence of V_i but then decayed to a lower steady level. As the V_i

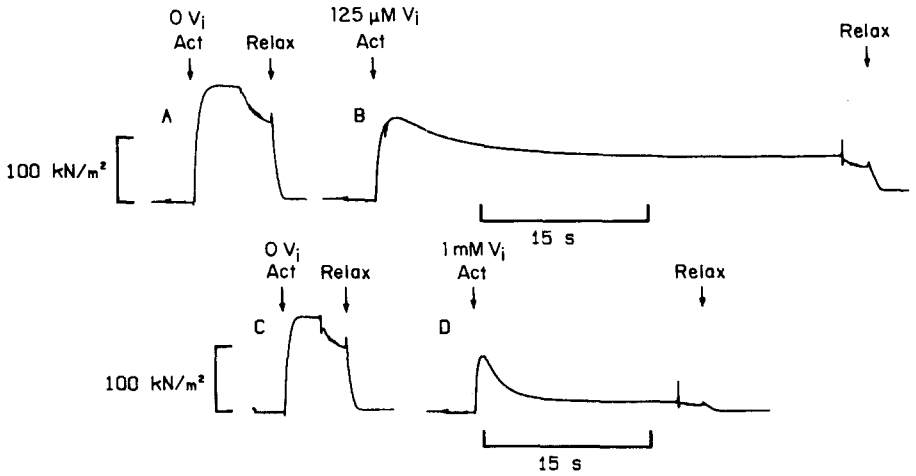


FIGURE 2. Suppression of active tension in various V_i concentrations. Conditions were the same as in Fig. 1. The fiber was activated at steady V_i concentrations: (*A*) 0, (*B*) 0.125 mM, (*C*) 0, and (*D*) 1 mM. After each contraction in the presence of V_i, tension recovered fully after the fiber contracted in the absence of V_i. Sarcomere length, 2.31 μm; fiber dimensions, 1.77 mm × 6,076 μm^2; temperature, 19–21°C.

concentration was raised from 50 μM to 1 mM, the final tension level decreased (Fig. 3*A*) and the observed rate of tension decay (obtained from semilogarithmic plots) increased (Fig. 3*B*). The V_i concentration leading to half-saturation of active tension suppression was 94 ± 23 μM (mean ± SD, n = 3). This is close to the values obtained by Solaro et al. (1980) and Herzig et al. (1981) in skinned cardiac fibers and by Magid and Goodno (1982) in skinned skeletal muscle fibers. The slope of the observed rate of suppression extrapolated to zero V_i concentration was 1.34 ± 0.30 × 10^5 $M^{-1}s^{-1}$ (mean ± SD, n = 3).

ATP-JUMP EXPERIMENTS The fibers could also be activated rapidly from rigor by the ATP-jump technique using laser photolysis of caged ATP (see Methods). Fig. 4 shows tension (bottom traces), in-phase stiffness (middle traces), and out-of-phase (quadrature) stiffness (upper traces) during activation of a fiber by caged ATP photolysis. The timing of the laser pulse is shown by the arrowheads. After liberation of ATP, tension decreased briefly and then increased to a relatively stable high level. These transients suggest that cross-bridges initially

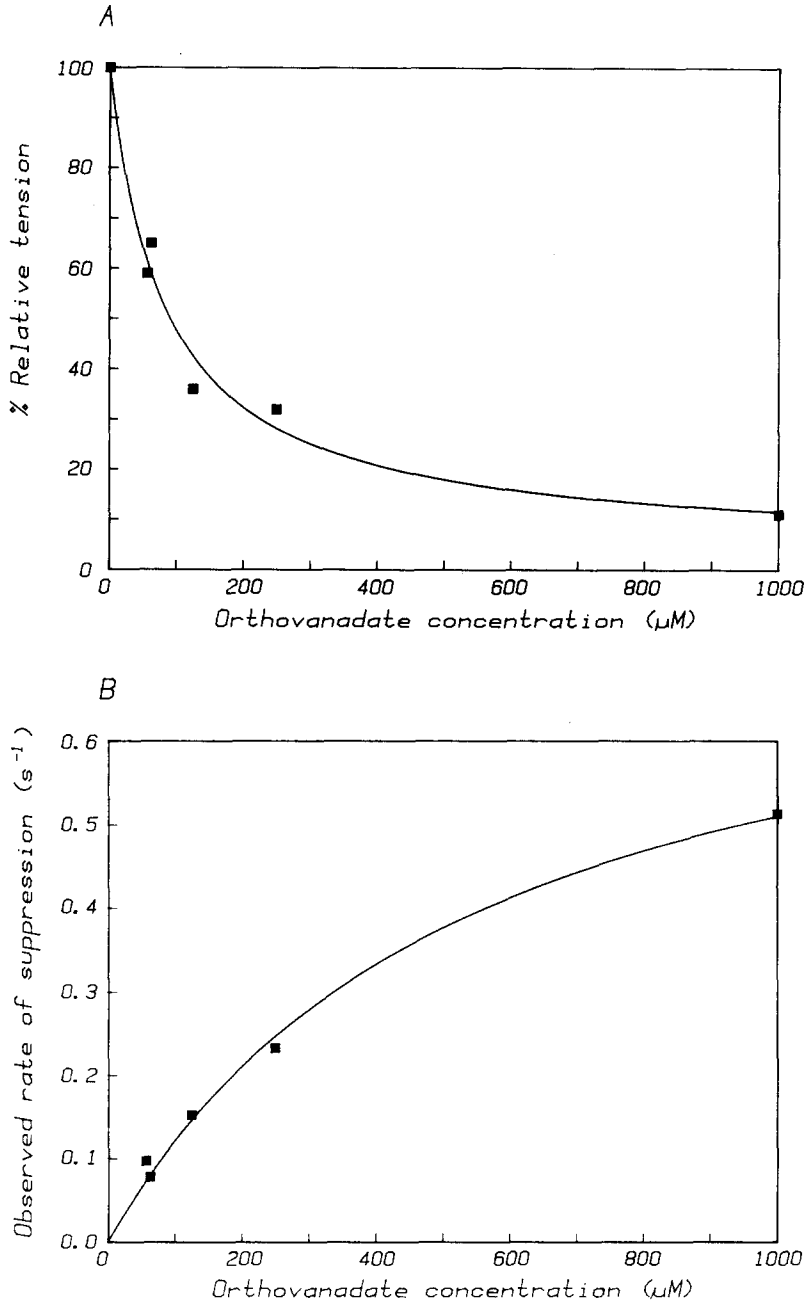


FIGURE 3. Dependence on V_i concentration of tension suppression (*A*) and the rate of tension decay (*B*). Data are from the experiment illustrated in Fig. 2. In *A*, the steady value of suppressed tension is plotted as a percentage of maximal active tension. The ordinate in graph *B* is the reciprocal of time constants obtained from semilogarithmic plots of tension recordings as in Fig. 2, *B* and *D*. Hyperbolic curves were fitted to the data in *A* and *B* by least-squares regression.

detach and then reattach to produce active force (Goldman et al., 1984*b*). When a steady 1-mM concentration of V_i was included in the medium, the initial tension dip and the first 20 ms of the tension rise were unaffected, but thereafter tension rose more slowly and then decayed. Panel *B* shows the same events on a slower time base. The exponential decay of tension in the presence of V_i was comparable to the tension suppression in the Ca-jump experiments (Figs. 1–3). These results

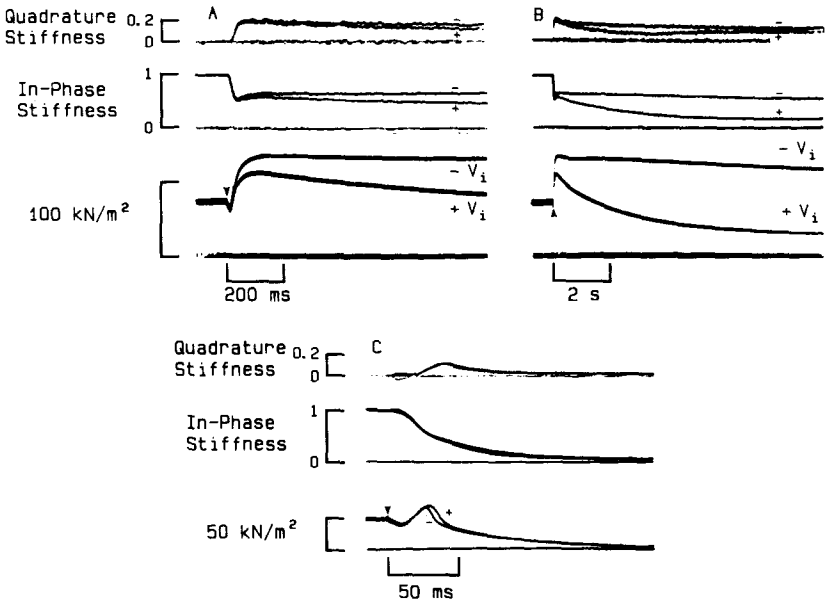


FIGURE 4. Stiffness and tension transients induced by photolysis of caged ATP within a muscle fiber. In each panel, the lower traces are tension, the middle traces are in-phase stiffness, and the upper traces are out-of-phase (quadrature) stiffness recordings in the absence (−) and presence (+) of V_i. Calibration bars for both stiffness signals are relative to in-phase rigor stiffness. (*A* and *B*) Fast and slow time base recordings of the same events. The arrowheads mark the time of the laser pulse that liberated 1.1 mM ATP in the presence of ~32 μM Ca^{2+}. Baseline tension and stiffness recordings were made after subsequent relaxation of the fiber in 5 mM ATP relaxing solution. Sarcomere length, 2.51 μm; fiber dimensions, 2.46 mm × 5,087 μm^2; temperature 21°C. (*C*) Similar recordings from another fiber in the absence of Ca^{2+} when 0.8 mM ATP was liberated. In this case, the baselines were recorded in the photolyzed solution after full relaxation. Sarcomere length, 2.19 μm; fiber dimensions, 1.78 mm × 7,965 μm^2; temperature, 20–22°C.

suggest that V_i does not bind to rigor cross-bridges. In the ATP-jump experiment, as in the Ca-jump experiment, V_i did not bind effectively until cross-bridge cycling was initiated.

In the presence of V_i, the in-phase stiffness decayed at approximately the same rate and to approximately the same extent as the tension, which qualitatively indicates that in the suppressed contractions, fewer cross-bridges were attached. The quadrature stiffness indicates the visco-elastic nature of the attached cross-

bridges (Goldman et al., 1984*a*, *b*). Quadrature stiffness was relatively well maintained after activation by caged ATP photolysis in the presence of V_i. This observation suggests that the remaining cross-bridges retain the quick stress-relaxation characteristic of normal, actively force-generating cross-bridges.

In the absence of Ca^{2+}, photolysis of caged ATP within a rigor fiber leads to relaxation with complex kinetics (Goldman et al., 1982, 1984*a*). V_i caused only a minor slowing of this relaxation (Fig. 4*C*).

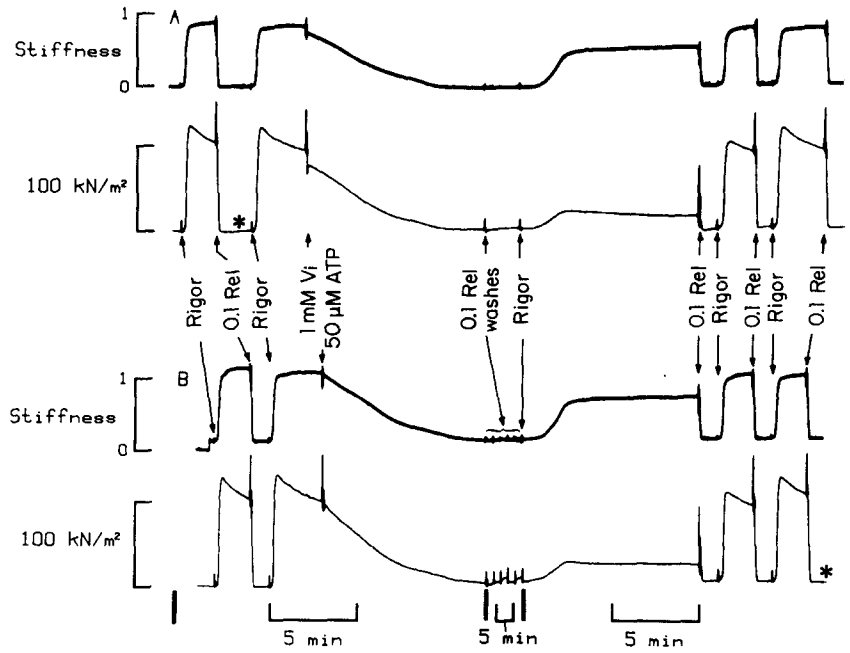


FIGURE 5. Suppression of tension and stiffness from rigor. Lower traces are tension; upper traces are (in-phase) stiffness. Each panel shows the following series of events: (1) a control rigor contraction and relaxation, (2) slow relaxation from rigor in the presence of 1 mM V_i and 50 μM MgATP, (3) a suppressed test rigor contraction, and (4, 5) two recovered contractions. In *B*, after the slow relaxation, the fiber was washed five times in V_i-free 0.1 mM MgATP relaxing solution before it was transferred it to the rigor solution. Note that the time base of the recording was slowed during these washes. All of panel *A* was recorded at the faster chart speed. In the experiment, panel *B* was recorded first. The equivalent time point is marked with a dingbat on both tension traces. Sarcomere length, 2.65 μm; fiber dimensions, 2.67 mm × 5,766 μm^2; temperature, 21°C.

RIGOR EXPERIMENTS Slow cross-bridge cycling can also be initiated in the absence of Ca^{2+} at low ATP concentrations because remaining rigor bonds may activate the thin filaments (Bremel and Weber, 1972; Moss and Haworth, 1984). The suppression of tension with V_i was also observed during the cross-bridge cycling induced by low ATP concentrations. In the experiment illustrated in Fig. 5*A*, the fiber was put into rigor and then relaxed fully in 0.1 mM MgATP with creatine phosphate and creatine kinase (low-ATP relaxing [Table I, *A*]). During

the subsequent rigor contraction, the fiber was transferred into a solution with 1 mM V_i and 50 μM ATP (slow relaxing [Table I, *B*]). In the absence of creatine phosphate and creatine kinase, this ATP concentration is not sufficient to relax the fiber but results in cross-bridge cycling. However, when V_i was included, tension and stiffness both decayed slowly to the relaxed level. The fiber remained in this solution for 9–10 min and was then transferred into the 0.1 mM MgATP relaxing solution without V_i. In the following rigor contraction, tension was markedly suppressed. Stiffness was suppressed, but less so than tension.

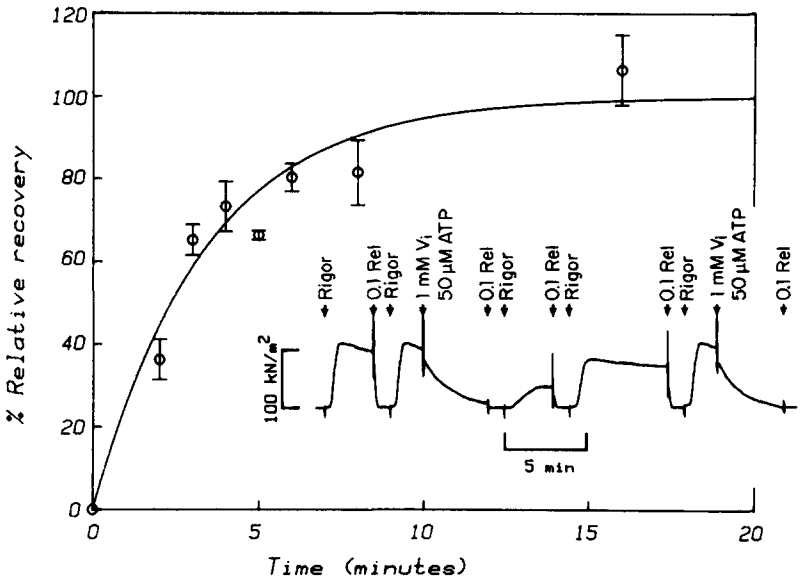


FIGURE 6. Time course of rigor tension recovery. Fibers were relaxed from rigor with 1 mM V_i and 50 μM ATP (inset). The fiber was washed in V_i-free 0.1 mM MgATP relaxing solution for 1 min and was then transferred to rigor solution for various durations. The extent of recovery was determined by the amplitude of a subsequent rigor contraction. The data plotted are means ± SEM determined from nine fibers. Inset: sarcomere length, 2.35 μm; fiber dimensions, 2.45 mm × 6,491 μm^2; temperature, 20°C.

The suppression of tension and stiffness was consistently observed in the rigor contraction after the incubation of the fiber in the V_i-containing, slow relaxing solution. Washing the fiber five times in V_i-free relaxing solution (0.1 mM MgATP; Fig. 5*B*) or allowing it to remain for up to 2 h in V_i-free relaxing solution (not shown) had no significant effect on this suppression. These findings suggest that after relaxing the fiber in the V_i-containing, slow relaxing solution, a stable intermediate was formed that produced less force in the subsequent rigor contraction. Recovery of tension and stiffness was observed in further active or rigor contractions (Fig. 5, *A* and *B*).

The extent of recovery from tension suppression increased with the duration of the rigor contraction. Fig. 6 (inset) shows the tension recording from an experiment in which the recovery of force development was tested after rigor

contractions of various durations. Full recovery was apparent after 10–20 min of rigor contraction (Figs. 5 and 6). However, a test rigor contraction was still 50% suppressed after a 2-min recovery (Fig. 6, graph). The recovery of rigor force seemed to depend on the total time in rigor rather than on the number of rigor contractions.

In the experiments of Figs. 1–5, the stiffness signals were reduced by mechanical compliance at the attachments between the fiber and the apparatus. To minimize the effects of end compliance, stiffness measurements were made under similar conditions, except that length changes were monitored in a central region of the fiber by a white-light diffraction method (Goldman, 1983). Step length changes were applied to a fiber at various stages of a V_i suppression and recovery protocol (Fig. 7): (*a*) in a normal rigor contraction, (*b*) in the low-tension (suppressed) contraction (Fig. 7*A*) after relaxation in 1 mM V_i, 50 μM ATP slow relaxing solution, and finally (*c*) in a recovered rigor contraction (Fig. 7*B*). The peak tension responses to the length steps are plotted against the observed sarcomere length changes in Fig. 7*C*. Stiffness, indicated by the slope of these force-extension curves, is decreased in the suppressed rigor contraction, but the tension indicated by the intercepts on the ordinate is decreased even more than stiffness. After a 10-min rigor contraction and relaxation, the subsequent rigor contraction had fully recovered tension and stiffness. These results are independent of compliances at the fiber attachments, since the length change was measured by light diffraction from a central region of the fiber. The experiment supports the data of Figs. 1–6, which indicate that tension and stiffness are suppressed by V_i. In rigor contractions, tension is suppressed more than stiffness.

Since our hypothesis derived from biochemical studies is that a stable M·ADP·V_i complex forms in the muscle fibers, we tested for tension suppression by adding V_i to rigor fibers pre-equilibrated in ADP (cross-bridge state AM·ADP). Fig. 8 shows a tension recording from this type of experiment. A control rigor contraction and relaxation were elicited first. The fiber was then transferred through the following series of bathing media: (*a*) rigor solution, (*b*) an extra rinse of rigor solution to remove traces of ATP, creatine phosphate, and creatine kinase, (*c*) rigor solution with 2 mM MgADP, a concentration sufficient to saturate the actomyosin binding sites with ADP (Marston, 1973; Dantzig et al., 1984), and (*d*) 2 mM MgADP, 1 mM V_i rigor solution. After 2 h of incubation in ADP and V_i, no significant relaxation of tension occurred. A small length step (ΔL) resulted in little tension recovery, which verified that the fiber was still in rigor. Relaxation in 5 mM ATP and then another rigor contraction showed that no appreciable tension suppression resulted from the procedure. This result required careful removal of ATP from the fiber and ADP stocks. Whenever cross-bridge cycling and active tension generation occurred, either from low levels of contaminant ATP or from intentionally added ATP (as in Figs. 1–7), V_i caused suppression of force.

Ligand Binding Studies

If V_i is a tightly bound analogue of P_i, a stable M·ADP·V_i complex would be formed in the fibers and the attachment of this complex to the thin filament

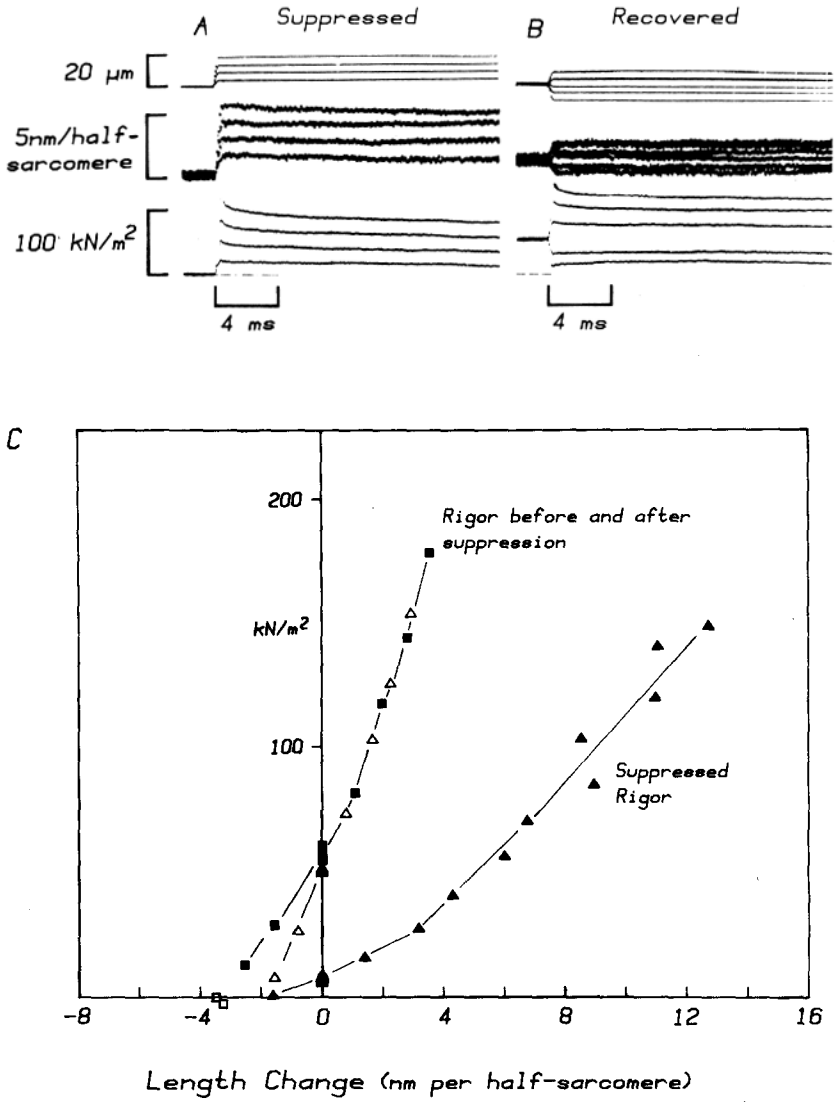


FIGURE 7. Tension and stiffness measurements using white-light diffraction to monitor the length change in a central region of the fiber. (*A* and *B*) Oscilloscope recordings of tension (lower traces), striation spacing (middle traces), and overall length (upper traces) during a series of step length changes. In *A*, rigor tension was suppressed after relaxation from rigor in 1 mM V_i, 50 μM ATP as shown in Figs. 5 and 6. The recordings of panel *B* were made in a recovered rigor contraction. Force-extension curves are plotted in *C* from an initial rigor contraction (sarcomere length, 2.68 μm [■]), the V_i-suppressed contraction corresponding to *A* (sarcomere length, 2.68 μm [▲]), and the recovered rigor contraction corresponding to *B* (sarcomere length, 2.59 μm [△]). The ordinate indicates the peak tension deflection after each length change. The abscissa indicates the change in striation spacing. Fiber dimensions, 2.24 mm × 5,222 μm^2; temperature, 19°C.

would promote V_i and ADP release. We tested this hypothesis by studying the binding of radioactively labeled V_i and nucleotides to fibers. Fig. 9*A* shows the time course of $^{48}V_i$ washout from a single fiber, which was first loaded with V_i by slowly relaxing it from rigor, in a solution containing 50 μM ATP, 1 mM V_i, and $^{48}V_i$ (Table I, *E*). After 25 min, the fiber was transferred to a new trough containing V_i-free, low-ATP relaxing solution for 1 s, and then to troughs containing fresh relaxing solution every few minutes for 1 h. The fiber was then put into a rigor solution (tension was suppressed) and the trough exchanges were continued every few minutes. The contents of these troughs were collected and the radioactivity that had diffused out of the fiber was determined by liquid scintillation counting as described in the Methods. In relaxing solution, free and/

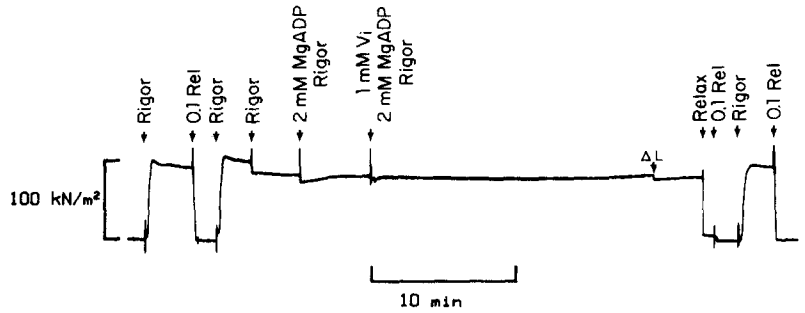


FIGURE 8. Lack of tension suppression by MgADP and V_i. Tension was recorded during a control rigor contraction and relaxation. The fiber was then transferred through the series of solutions labeled by arrows. After 2 h in 1 mM V_i, 2 mM MgADP solution, a 0.1% length release was imposed (ΔL) to test the mechanical condition of the fiber. The fiber was then relaxed in 5 mM MgATP relaxing solution. A subsequent rigor contraction showed no significant suppression of tension. Sarcomere length, 2.42 μm; fiber dimensions, 2.92 mm × 4,665 μm^2; temperature, 19.5°C.

or loosely bound V_i washed out of the fiber (Fig. 9*A*, open symbols). However, a component of V_i was trapped within the relaxed fiber and was released only when the fiber went into rigor (closed symbols). The burst of V_i released during the rigor contraction was equivalent to 164 ± 23 μmol V_i per liter fiber volume (mean ± SEM, $n = 7$). The fiber was relaxed again for 12 min and a subsequent rigor contraction indicated that tension was fully recovered. There was no significant further release of $^{48}V_i$.

After repeated washing for 1 h in V_i-free (low-ATP) relaxing solution, the V_i remaining in the fiber during the experiment of Fig. 9*A* could be trapped on a binding site with an extremely low desorption rate. Alternatively, it could be exchanging with free V_i, but the tight binding could restrict its diffusion out of the fiber. In order to distinguish between these possibilities, we performed the "cold chase" experiment illustrated in Fig. 9*B*. The fiber was loaded with V_i as in the experiment of Fig. 9*A*, by relaxing it from rigor in 50 μM ATP and 0.5 mM V_i labeled with $^{48}V_i$. After 25 min in the loading solution, the washout was determined by collecting 3-min effluent samples for 1 h in successive 0.1 mM

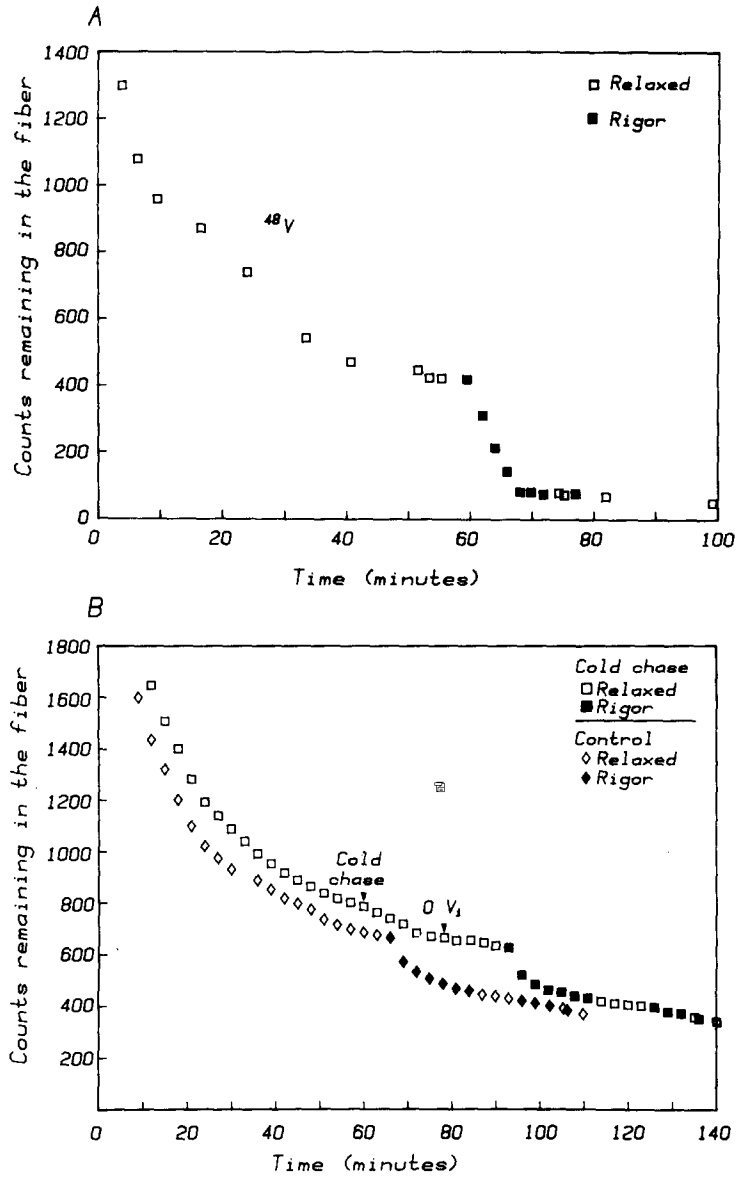


FIGURE 9. Washout of $^{48}V_i$ from a skinned muscle fiber. The fiber was loaded with $^{48}V_i$ by the slow relaxation protocol shown in Figs. 5 and 6. The data points represent $^{48}V_i$ counts remaining in the fiber at the times plotted. A data point is plotted for each solution exchange. Open symbols indicate 0.1 mM MgATP relaxing solution washes; closed symbols indicate rigor solutions. The solutions were V_i-free during the washout runs, except that there was 1 mM V_i present during the interval between the arrows marked "cold chase" and "0 V_i" on the upper curve in *B*. The final data point represents the number of counts that remained in the fiber. For *A*, the $^{48}V_i$ loading solution contained 1.0 mM carrier V_i. 150 μmol V_i per liter fiber volume was released in the rigor contraction. Sarcomere length, 2.18 μm; fiber volume, 15.7 nl; temperature, 20°C. For *B*, the $^{48}V_i$ loading solution contained 0.5 mM carrier V_i. 110 and 120 μmol V_i per liter fiber volume were released in the rigor contractions during the cold chase and control runs, respectively. Sarcomere length, 2.17 μm; fiber volume, 25.6 nl; temperature, 19°C.

MgATP, 0-V_i troughs. The fiber was then transferred into 1 mM V_i, 0.1 mM MgATP unlabeled solution (cold chase, Fig. 9*B*) for six 3-min washes. Little additional V_i washout occurred in the cold chase solution. The fiber was then transferred through several additional 0-V_i troughs and put into rigor (closed symbols). Tension was suppressed in this rigor contraction and $^{48}V_i$ was released. Another rigor-relaxation cycle did not release further $^{48}V_i$. In a control run on the same fiber without the cold chase (lower curve), an equivalent amount of V_i was trapped within the fiber again and released by another rigor contraction.

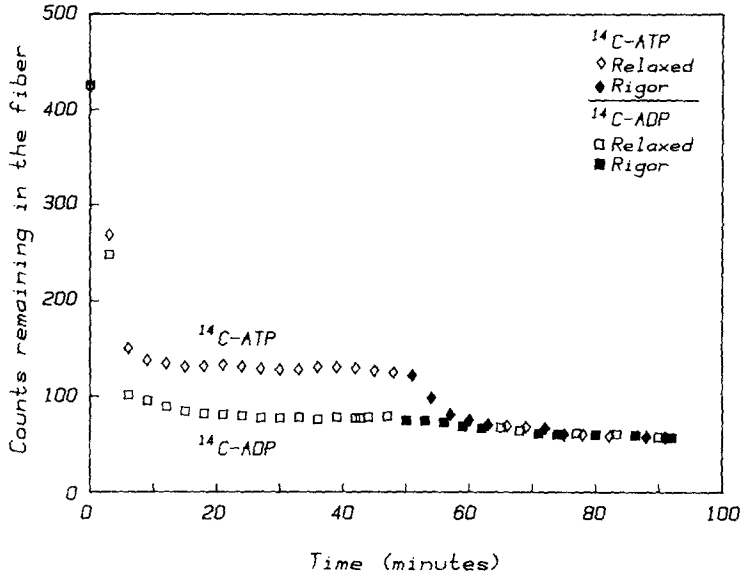


FIGURE 10. Trapping of nucleotide in a fiber. The fiber was loaded with [^{14}C]-ATP or [^{14}C]ADP by relaxing the fiber from rigor with 1 mM V_i, 200 μM ADP, and 75 μM ATP. Nucleotide was trapped (60 μmol per liter fiber volume) when the ATP was labeled (◊), but no significant amount of nucleotide was trapped when the ADP was labeled (□). Open symbols correspond to 0.1 mM MgATP relaxing solution baths and closed symbols correspond to rigor washes. The two sets of data were recorded from the same fiber. Sarcomere length, 2.33 μm; fiber volume, 20.3 nl; temperature, 20°C.

In the cold chase experiment, if the remaining radioactivity within the fiber could exchange with free V_i, the radioactivity would have been released as unlabeled V_i occupied the binding sites. However, little radioactivity was released until the fiber was contracted in a rigor solution (Fig. 9*B*, closed symbols). Thus, V_i appears to be trapped within the fiber because its dissociation rate from myosin is slow on the time scale of minutes to hours. The results indicate that a stable complex including V_i is formed in the fiber and this complex is dissociated by the formation of rigor cross-bridges.

Similar experiments with radioactively labeled ATP and ADP were conducted to test whether nucleotide is trapped as well as V_i. In the experiment illustrated in Fig. 10, a fiber was relaxed in 1 mM V_i, 200 μM ADP, and 75 μM ATP to

trap V_i. Two separate loading and washout runs were conducted on the same fiber and with equivalent solutions, except that the radioactive label was in the ADP during the first run and in the ATP during the second run. When the fiber was relaxed in the solution containing labeled ATP, a component of the label was trapped in the fiber, amounting to 125 ± 25 μmol nucleotide per liter fiber volume (mean ± SEM, n = 5). This trapped nucleotide was released when a

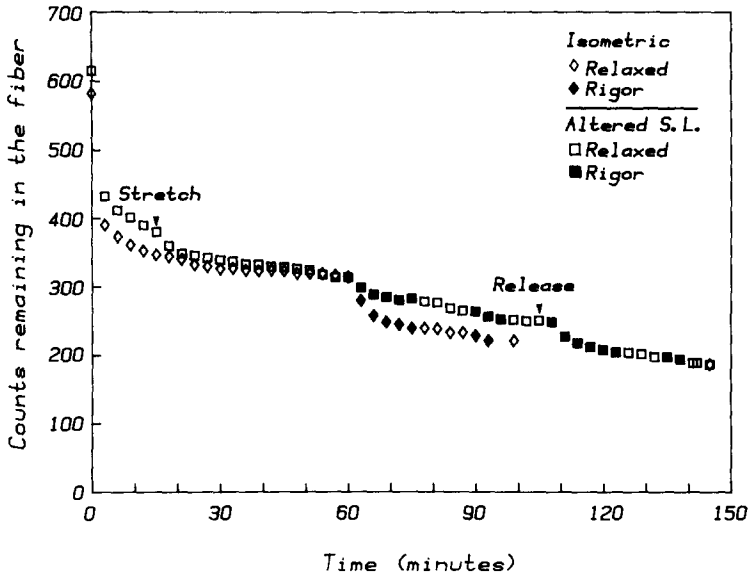


FIGURE 11. Dependence of trapped nucleotide release on cross-bridge attachment. Conditions were the same as in Figs. 9 and 10, except that the [^{14}C]ATP loading solution contained 1 mM V_i and 50 μM carrier ATP. Open symbols correspond to washes in 0.1 mM MgATP relaxing solution; closed symbols represent rigor washes. During the 1-h washout in unlabeled relaxing solution, the fiber was stretched from a sarcomere length of 2.31–3.39 μm at the time indicated by the arrowhead. Several rigor contractions were elicited and then the relaxed fiber was returned to the original sarcomere length at the time labeled "release" (second arrowhead). A control run at 2.31 μm sarcomere length was repeated on the same fiber. Fiber volume, 18.1 nl; temperature, 20°C.

(suppressed) rigor contraction was elicited (Fig. 10, closed symbols). When the label was located in ADP, no significant amount of label was trapped within the fiber or washed out in rigor, even though the mechanical record was similar. These results suggest that the stable complex leading to tension suppression is $M \cdot ADP \cdot V_i$ and that the trapped ADP is preferentially formed from ATP rather than from medium ADP. The experiment of Fig. 10 also corresponds to a cold chase situation in that unlabeled ATP was present during the first 45 min.

The release of trapped V_i and ADP during a rigor or active contraction is probably due to cross-bridge attachment. However, another possibility is that removal of ATP might lead directly to dissociation of the trapped ligands. To check this possibility, the test rigor contraction was performed with reduced

overlap between the thick and thin filaments. A fiber was loaded with ^{14}C-labeled nucleotide by relaxation from rigor in 1 mM V_i, 50 μM ATP, and [^{14}C]ATP. After a few washes, it was stretched to a sarcomere length of 3.39 μm in (low-ATP) relaxing solution. A rigor contraction then released a smaller quantity of trapped ADP than it did at full overlap (Fig. 11, closed symbols). The fiber was relaxed and a second rigor contraction at the long sarcomere length released little further radioactivity. The fiber was returned to the initial length and a rigor contraction at full overlap released a further quantity of labeled ADP. In a repeat run with the same fiber maintained isometric at the short sarcomere length, a rigor contraction released trapped nucleotide in an amount equivalent to the total released in the trials with the altered sarcomere length. This experiment indicates that myosin heads in the overlap zone of the sarcomere release the trapped nucleotide when they attach to actin, but outside the overlap zone they retain the tightly bound nucleotide even when ATP is removed. Similar results were obtained for the release of trapped V_i at a reduced filament overlap.

DISCUSSION

Our ligand binding experiments provide strong evidence that suppression of tension by V_i in skinned muscle fibers is associated with the trapping of V_i and nucleotide on myosin. When myosin heads with tightly bound V_i and nucleotide attach to actin, the trapped ligands are released. These aspects of tension suppression by V_i are entirely analogous to the formation of a stable $M \cdot ADP \cdot V_i$ complex (Goodno, 1979) that dissociates upon binding to actin in solution (Goodno and Taylor, 1982). In muscle fibers, the stable complex is also probably $M \cdot ADP \cdot V_i$ since ATP is hydrolyzed more rapidly (Ferenczi et al., 1984) than tension is suppressed by V_i (Figs. 1–4).[1] However, the ADP trapped in the fibers with V_i was preferentially derived from ATP (Figs. 8 and 10). The formation of a stable $M \cdot ADP \cdot V_i$ complex in muscle fibers was postulated on the basis of previous mechanical and X-ray diffraction experiments (Goody et al., 1980; Magid and Goodno, 1982). The present experiments are the first direct demonstration of the stable complex in fibers.

In order to evaluate the reaction pathway leading to the formation of stable $M \cdot ADP \cdot V_i$, it is useful to consider the normal ATPase reactions in the filament lattice. Myosin is thought to hydrolyze ATP slowly in a relaxed fiber via the bottom row of reactions of Scheme 1 (Marston, 1973). During active contraction, the major reaction flux for myosin is via the following series of steps:

(*a*) attachment of myosin to actin:

$$M \cdot ADP \cdot P_i \xrightarrow{A} AM \cdot ADP \cdot P_i$$

[1] Note added in proof: Further ligand binding experiments using [γ^{32}P]ATP and [8-^{3}H]ATP have shown that trapped nucleotide is ADP (Bamrungphol, Dantzig, and Goldman, unpublished observation).

(*b*) product release and force generation:

$$AM \cdot ADP \cdot P_i \xrightarrow{P_i} AM' \cdot ADP \rightarrow AM \cdot ADP \xrightarrow{ADP} AM$$

(*c*) detachment:

$$AM \xrightarrow{ATP} AM \cdot ATP \xrightarrow{A} M \cdot ATP \rightarrow M \cdot ADP \cdot P_i.$$

Many of these steps are probably reversible. In solution, the elementary ATP cleavage step occurs with dissociated myosin, $M \cdot ATP \rightarrow M \cdot ADP \cdot P_i$, and also directly with actomyosin, $AM \cdot ATP \rightarrow AM \cdot ADP \cdot P_i$. The contribution from the latter direct hydrolysis pathway in muscle fibers is not known. AM·ADP can be formed by binding ADP to the rigor complex (AM). During ATPase activity, the population of a different state, AM′·ADP, has been inferred from studies of P_i binding to isolated actomyosin (Sleep and Hutton, 1980) and to muscle fibers (Webb et al., 1985). P_i binds readily to AM′·ADP but not to AM·ADP.

In our muscle fiber experiments, V_i bound to cross-bridges during active cycling much more effectively than in relaxed or rigor conditions. Contractions were initiated by either a rapid increase of Ca^{2+} in a relaxed fiber (Figs. 1–3) or by a rapid increase of ATP concentration from rigor (with Ca^{2+}, Fig. 4). In either case, when V_i was present at a steady 1-mM concentration, the initial development of tension was similar to the contraction without V_i but then tension decreased at a relatively slow rate. Thus, V_i does not seem to bind tightly to AM or to the myosin states in a relaxed muscle fiber. V_i also does not bind tightly to AM·ADP since incubation of rigor fibers in ADP and V_i did not lead to relaxation or tension suppression of a subsequent contraction (Fig. 8) or to trapping of nucleotide in the fiber (Fig. 10, lower curve). AM·ATP is present only briefly during active cross-bridge cycling (Goldman et al., 1984*b*) and the active site of myosin is unlikely to accommodate ATP and V_i simultaneously. These arguments leave AM′·ADP as the sole intermediate of Scheme 1 to which V_i can readily bind to suppress tension. Therefore, our experiments suggest that stable M·ADP·V_i is formed by the following reaction:

$$AM' \cdot ADP \xrightarrow{V_i} AM \cdot ADP \cdot V_i \xrightarrow{A} M \cdot ADP \cdot V_i \qquad (2)$$

as suggested by Goody et al. (1980). Since the cross-bridge–nucleotide state capable of binding V_i (AM′·ADP) is present during normal cross-bridge cycling, our experiments provide evidence that P_i dissociated before ADP. There is evidence for this order of product dissociation from experiments on isolated heavy meromyosin (Trentham et al., 1972; Sleep and Hutton, 1980), but the present experiments are the first strong indication in muscle fibers for release of P_i before ADP.

The present results and previous studies agree that V_i binds readily to the AM′·ADP state populated in the actomyosin ATPase cycle. However, Goody et

al. (1980) and Goodno and Taylor (1982) observed slow binding of V_i to AM·ADP formed by adding ADP to AM, which we did not observe (Fig. 8). Possible explanations for this difference include the fiber types, the duration of the incubations (Goody et al., 1980: insect fibers, 12–24-h incubation; present experiment: rabbit fibers, 2-h incubations), the ionic conditions, or low-level ATP contamination. With regard to the biochemical study with actomyosin subfragment-1 (Goodno and Taylor, 1982), the ionic conditions were different (much lower ionic strength) and their AM·ADP intermediate was presumably in equilibrium with M·ADP, to which V_i could bind. However, this argument does not rule out the binding of V_i to actomyosin subfragment-1·ADP.

Since the M·ADP·V_i state is stable, V_i might be expected to bind to M·ADP in the relaxed muscle fiber. This did not occur (Figs. 1, 2, and 9). The M·ADP state may be present only briefly in the relaxed ATPase cycle if ADP dissociates rapidly and ATP binds immediately to myosin (Goodno, 1982). Alternatively, if the M·ADP state is significantly populated in relaxed fibers, it may be in a conformation that does not bind V_i tightly.

The kinetics of V_i binding to AM′·ADP from the present results can be compared to P_i binding to the same state. At a low V_i concentration, the apparent second-order rate constant for V_i binding and suppression of tension was $\sim 10^3$ $M^{-1}s^{-1}$. This value is close to the second-order rate constant, 5×10^3 $M^{-1}s^{-1}$, previously observed for tension reduction by P_i binding to AM′·ADP (Hibberd et al., 1984, 1985*a*). At higher V_i concentrations, the rate of suppression plateaued at rates in the range of 0.4–1 s^{-1}. This may indicate that M·ADP·V_i detachment from actin is slow, which would be consistent with the slower relaxation in 0-Ca^{2+}, caged ATP experiments (Fig. 4*C*). Alternatively, the plateau rate of V_i suppression could be limited by the overall cross-bridge cycling rate. Another possibility is that the free V_i concentration may not increase concomitantly with the total added V_i since it polymerizes (Pope and Dale, 1968) and is reduced to VO^{2+} by glutathione (Macara et al., 1980).

The radioactive binding experiments showed that the amount of V_i and nucleotide trapped was consistently lower than the expected myosin head concentration (≈200 μM). If the experimental conditions were not optimal, the concentrations of trapped ligands would be diminished. The above-mentioned uncertainty of the free V_i concentration may have caused an underestimation of the trapped ligand concentration. However, if all the myosin heads were arrested as M·ADP·V_i, it would be difficult to explain the development of rigor tension and the release of trapped ligands upon removal of ATP. Another possibility is that cooperativity between the two heads of a myosin molecule might prevent the trapping of V_i on one head if the other head has tightly bound ligands.

Can M·ADP·V_i attach to actin? The recovery of tension and the release of trapped ligands by active or rigor contractions suggests strongly that M·ADP·V_i can attach to actin. This cross-bridge attachment promotes V_i and ADP release, behavior similar to that postulated in the normal cross-bridge cycle for the M·ADP·P_i state. P_i release from M·ADP·P_i is relatively slow, but attachment to actin promotes P_i release (Lymn and Taylor, 1971; Hibberd et al., 1985*b*).

The force generation by cross-bridges formed from M·ADP·V_i is low (Figs. 1–7). If V_i release from AM·ADP·V_i is slower than P_i release from AM·ADP·

P_i, the equilibrium of the V_i dissociation step would be shifted toward AM·ADP·V_i. This hypothesis would be consistent with the observation that tension is suppressed more than stiffness in rigor contractions (Figs. 5 and 7). Alternatively, M·ADP·V_i attachment to actin may be slower than M·ADP·P_i attachment. Either of these kinetic differences would explain the reduced force of contractions in the presence of V_i if V_i and P_i release are closely linked to the power stroke of the cross-bridge cycle.

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