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Relaxation of muscle fibers with adenosine 5′-[γ-thio]triphosphate (ATP[γS]) and by laser photolysis of caged ATP[γS]: Evidence for Ca^{2+}-dependent affinity of rapidly detaching zero-force cross-bridges

(skinned muscle fibers/contraction/muscle regulation/caged molecules)

J. A. DANTZIG*, J. W. WALKER†, D. R. TRENTHAM†, AND Y. E. GOLDMAN*‡

*Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104; and †National Institute for Medical Research, London, England

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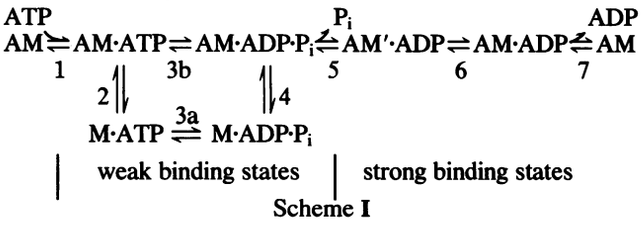
ABSTRACT **The relationship between the mechanical and biochemical states of the muscle cross-bridge cycle and the control of contraction were investigated by using the nucleotide analogs adenosine 5′-[γ-thio]triphosphate (ATP[γS]) and caged ATP[γS] {the *O*-1(2-nitrophenyl)ethyl P^3-ester of ATP[γS]}. ATP[γS] interacts with actomyosin in a manner similar to ATP but is hydrolyzed (by a factor of 500) more slowly. Generation of ATP[γS] by photolysis of caged ATP[γS] within a permeabilized fiber in rigor in the absence of Ca^{2+} relaxed tension and stiffness as occurs with ATP. The transient rise in tension prior to final relaxation observed with photolysis of caged ATP was absent with caged ATP[γS]. This result suggests that following detachment of a cross-bridge, ATP is normally hydrolyzed before force generation. In the presence of Ca^{2+}, photolysis of caged ATP[γS] within rigor fibers caused tension to relax fully but significant stiffness remained. Stiffness also developed without concomitant tension when Ca^{2+} concentration was raised from <1 nM to 30 μM in the presence of ATP[γS]. The amplitude of the tension response to ramp stretches in the presence of Ca^{2+} and ATP[γS] increased with ramp stretch velocity, suggesting that the cross-bridges have detachment rate constants extending into the 10^3 s^{-1} range. The results provide evidence that the Ca^{2+}-regulatory system can directly control attachment of cross-bridges into states before the power stroke.**

Contraction of vertebrate skeletal muscle is thought to be generated by a cyclic interaction between myosin in the thick filaments and actin in the thin filaments with hydrolysis of ATP as the energy source. This cross-bridge cycle is controlled by two regulatory components in the thin filaments: tropomyosin and troponin. Actomyosin ATPase activity and contraction occur when Ca^{2+} binds to troponin, relieving inhibition caused by the troponin–tropomyosin complex (1, 2).

Structural changes in the thin filament during activation of contraction led to the hypothesis that motion of tropomyosin into the groove of the actin filament removes a steric hindrance to myosin attachment (3–5) [reactions 2 and 4 in biochemical Scheme I, where A is actin and M is myosin (6–9)].



Scheme I

However, recent investigations on regulated actomyosin subfragment 1 and on skinned muscle fibers have demonstrated Ca^{2+} regulation of ATPase activity and contraction under conditions of low ionic strength, where Ca^{2+} does not affect myosin subfragment 1 association with actin (10, 11). Based on these experiments it was suggested that the transition from a weakly attached AM·ADP·P_i intermediate to a strongly attached AM′·ADP state (reaction 5) is controlled by troponin–tropomyosin rather than cross-bridge attachment.

In this paper, interactions between the cross-bridges and the Ca^{2+}-regulatory system are investigated by using photolabile (caged) analogs of ATP and adenosine 5′-[γ-thio]triphosphate (ATP[γS]). We have previously shown that release of ATP by laser photolysis of caged ATP within single glycerol-extracted rabbit muscle fibers causes rapid detachment of the cross-bridges and then reattachment into force-generating states when Ca^{2+} is present (12, 13). Liberation of ATP in the absence of Ca^{2+} relaxed rigor tension and stiffness as expected, but first there was a transient phase of active cross-bridge formation and tension development. This transient tension development was ascribed to a positive cooperativity of cross-bridge attachment whereby some cross-bridges, still attached during the initial phases of the relaxation, would maintain a switched-on condition of the thin filaments and allow further attachment, even in the absence of Ca^{2+} (14). However, the relationship between hydrolysis of the ATP and the mechanical steps of reattachment and force generation is unclear.

ATP[γS] is a nucleotide analog that binds to and dissociates actomyosin more slowly than ATP (by a factor of ≈10; $k = 3.8 \times 10^5$ $M^{-1} \cdot s^{-1}$; refs. 15 and 16), and the elementary cleavage step that produces protein-bound ADP and P_i also occurs more slowly (by a factor of ≈500; $k = 0.36$ s^{-1}; ref. 17). O-caged ATP[γS] {the *O*-1(2-nitrophenyl)ethyl P^3-ester of ATP[γS]} was synthesized by a method that is applicable to a number of nucleotides and biological signaling molecules (18). Caged ATP[γS] was used in laser pulse photolysis experiments to release ATP[γS] rapidly within the filament lattice of muscle fibers to compare the kinetics of the cross-bridge reactions with those induced by ATP.

We found that muscle fibers did not develop or maintain tension in solutions with ATP[γS] whether or not Ca^{2+} was present, but fiber stiffness was markedly sensitive to Ca^{2+} concentration. The cross-bridges contributing this stiffness are presumably in a state analogous to the nucleoside triphosphate state (AM·ATP in Scheme I) of the cross-bridge cycle, suggesting that direct control of cross-bridge attachment is an important factor in Ca^{2+} regulation. Some of the results have been presented in preliminary form (19).

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Abbreviation: ATP[γS], adenosine 5′-[γ-thio]triphosphate.

‡To whom reprint requests should be addressed.

METHODS

Preparation of Caged ATP[γS]. Caged ATP[γS] was synthesized as described in ref. 18. (Higher yields were obtained with a water/diethyl ether solvent in the alkylation step.) Oxygen and sulfur at the terminal phosphate of ATP[γS] are alkylated in approximately equal amounts to form O-caged ATP[γS] and S-caged ATP[γS], respectively. O-caged ATP[γS] was used here because it photolyzed more rapidly than S-caged ATP[γS]. O-caged ATP[γS] photolyzes with a quantum yield of 0.49 and under the experimental reaction conditions releases ATP[γS] at 140 s^{-1} (based on values of 0.63 and 118 s^{-1}, respectively, for caged ATP).

ATP[γS] used in the mechanical experiments was purified to remove ADP and other contaminating nucleotides by DEAE-cellulose chromatography as in ref. 18.

Muscle Fiber Mechanical Experiments. Single glycerol-extracted fibers from rabbit psoas muscle were mounted between a fast tension transducer and a piezo-electric length driver in a small volume (20 μl) trough (14). ATP[γS] or ATP was liberated from caged ATP[γS] or caged ATP within the fiber by a pulse of 347-nm radiation from a frequency-doubled ruby laser. Photolysis solutions contained (in mM) *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes buffer), 100; EGTA, 52; Mg^{2+}, 1–4; glutathione, 10; caged nucleotide, 10 (ionic strength, 200 mM; pH 7.1 and 20°C).

Tension and stiffness changes were recorded as the photogenerated nucleotides initiated detachment and further cross-bridge reactions (12–14, 20). Rapid activation of the fiber by Ca^{2+} was performed by using the Ca^{2+}-buffer gradient method of Moisescu (21). Recordings of stiffness were obtained by imposing a 1-μm, 500- to 2000-Hz sinusoidal length change on the fiber. The resulting sinusoidal tension signal was demodulated by a lock-in amplifier to produce in-phase and out-of-phase (quadrature) stiffness signals (13, 14). In-phase stiffness is primarily related to the number of attached cross-bridges.

The speed dependence of stiffness in low force states was evaluated by using ramp length changes with a wide range of velocities. The sarcomere length was monitored in these experiments by using white light diffraction as described (22). Speed dependence of stiffness observed by using ramp length changes and the out-of-phase stiffness signal with sinusoidal length changes are both sensitive to kinetic processes on the time scale of the applied length changes.

Hydrolysis of ATP[γS] by Muscle Fibers. Single muscle fibers were mounted in a solution changer and their volume was measured by compound microscopy. ATP was removed by transferring through at least two washes in ATP[γS] solutions. Fibers were then incubated in small (45 μl) temperature-regulated troughs in solutions of 5 mM ATP[γS], either with or without Ca^{2+}, for periods from 2 to 24 hr. The incubation media were covered by a layer of silicone oil to prevent evaporation. Following the incubation period the fibers were removed and the contents of the troughs were analyzed for ADP and ATP[γS] by anion-exchange HPLC. The amount of ADP formed in the trough above that formed by spontaneous hydrolysis in a control trough was converted to an apparent k_{cat} assuming the concentration of myosin heads to be 154 μmol per liter of fiber volume (23).

RESULTS

Photolysis of O-caged ATP[γS] and Caged ATP in Muscle Fibers. The tension and stiffness transients initiated when ATP[γS] or ATP was released by photolysis into single fibers in rigor in the absence of Ca^{2+} are shown in Fig. 1. Two trials are shown in each case: one in which the fiber was held isometric (i) and one in which the fiber was stretched (s) by 0.5% 1 s before triggering the laser pulse (arrowheads). The two traces in each panel follow different time courses until ≈100 ms after the laser pulse when they converge (vertical dashed lines) and almost superimpose during the final part of the relaxation. The time for convergence is taken as indicating the time for detachment of the original rigor cross-bridges (14). Approximately five times the concentration of ATP[γS] was required to cause the same detachment rate as ATP, which is compatible with the relative values of the second-order rate constants for ATP[γS]- and ATP-induced dissociation of actomyosin subfragment 1 (15).

With both caged nucleotides, photolysis resulted in full relaxation, but tension records following the release of 300 μM ATP initially showed a transient tension rise that peaked 80 ms after the laser pulse (Fig. 1*B*). As noted in the Introduction, we have interpreted this tension rise as indicating cooperative interactions in the thin filament that allow transient cross-bridge attachment and formation of force-generating AM′·ADP states (13, 14). If ATP[γS] hydrolysis in the fiber occurs at 0.24–0.36 s^{-1} (based on studies with isolated heavy meromyosin and subfragment 1; refs. 15, 17, 24), formation of AM′·ADP would not occur to a significant extent within 100 ms and the tension rise would be abolished (Fig. 1*A*). Even so, the tension and stiffness following release of ATP[γS] (Fig. 1*A*) do not decay in a single exponential phase, possibly indicating the influence of protein–protein cooperative effects on cross-bridge reattachment (see *Discussion*). Inclusion of 0.5 or 1.0 mM glucose, 0.5 mg of myokinase per ml, and 0.25 mg of hexokinase per ml in the photolysis medium in some experiments to reduce contaminant ADP levels had no appreciable effect on the records. This result suggests that slowing of cross-bridge detachment by ADP bound to the cross-bridges is not involved in the delay before relaxation shown in Fig. 1*A*.

Inclusion of 10 mM inorganic phosphate (P_i) also had no appreciable effect on the relaxation following photolysis of caged ATP[γS]. This is in marked contrast to the observation that the transient tension rise on photolysis of caged ATP is suppressed and the final relaxation is accelerated by P_i (20). These effects of P_i are thought to be due to binding of P_i to AM′·ADP. Inclusion of 10 mM thiophosphate in a 5 mM ATP

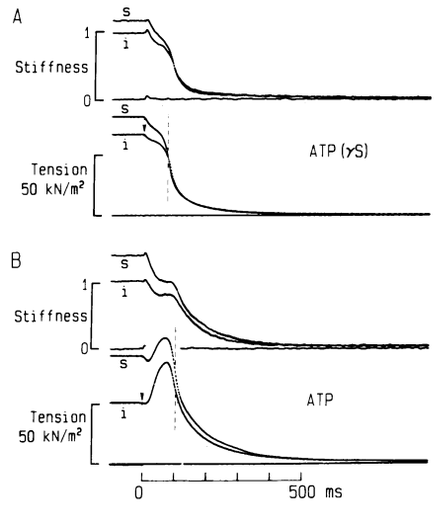

FIG. 1. Time course of relaxation from rigor by laser photolysis of O-caged ATP[γS] (*A*) and caged ATP (*B*) within a single glycerol-extracted psoas muscle fiber. Initial caged nucleotide concentration was 10 mM. Liberated nucleotide concentrations were 1.5 mM ATP[γS] in *A* and 300 μM ATP in *B*. Each panel shows superimposed tension and (in-phase) stiffness records from two successive trials, either isometric (i) or pre-stretched (s) 1 s prior to the laser pulse. Vertical dashed lines indicate the time at which the tension traces approximately converge. Baselines were recorded in the photolysis solution after relaxation was complete. Fiber dimensions: 7900 μm^2 × 2.3 mm; sarcomere length, 2.21 μm.

medium did not cause significant tension suppression of isometric tension in Ca^{2+}-activated muscle fibers (D. P. Vallette, J.W.W., J.A.D., and Y.E.G., unpublished observation), suggesting that thiophosphate does not bind to AM'·ADP as strongly as P_i. Thus the absence of effects of P_i on tension and stiffness following photolysis of caged ATP[γS] supports the hypothesis that the AM'·ADP state is not significantly populated.

When ATP[γS] is released into fibers in the presence of 30 μM free Ca^{2+}, tension also falls to the level of relaxed fibers (Fig. 2 *A* and *Inset* in *D*). However, in contrast to the Ca^{2+}-free situation, stiffness (at 500 Hz) does not decay completely but remains at a level 30–50% of that found during activation with ATP and Ca^{2+} (Fig. 2*B*). These results imply the presence of attached cross-bridges, presumably AM·ATP[γS], that generate zero net force.

The records obtained during photolysis of caged ATP (Fig. 2*B*) can be interpreted in terms of a two-step process in which cross-bridge detachment is followed by formation of the force-generating state, the rates of the two processes being controlled (*i*) by reactions 1 and 2 and (*ii*) by reactions 3a or 3b, 4, and 5 in Scheme I. The recordings with ATP[γS] are less straightforward to interpret. The initial fast drop of tension and stiffness (within 30 ms) is presumably related to cross-bridge detachment. The slower phase that requires several seconds may indicate a range of rate constants for detachment of M·ATP[γS] from actin. Fig. 2 *C* and *D* show comparisons of the tension relaxation in the presence and absence of Ca^{2+} following photolysis of caged ATP[γS]. The results suggest that in the absence of Ca^{2+}, the Ca^{2+}-regulatory system causes acceleration of relaxation about 100 ms following the laser pulse.

The onset of activation in the presence of ATP[γS] was also investigated by Ca^{2+}-jump perturbations in the presence of steady ATP or ATP[γS] concentrations. In the experiment of Fig. 3, the fiber was initially relaxed in a solution with low Ca^{2+}-buffering capacity and either 1 mM ATP or ATP[γS]. At the arrowhead, the fiber was activated by 30 μM free Ca^{2+} at the same nucleotide concentration. In the presence of ATP, tension and stiffness increased rapidly ($t_{1/2} \approx 250$ ms) to steady levels. In the presence of ATP[γS], however, tension remained at zero while stiffness increased to a value ≈30% of that in the activating solution with ATP. The attached zero-force cross-bridges in the presence of ATP[γS] and Ca^{2+} are similar whether the steady state was reached from the relaxed (Fig. 3) or rigor (Fig. 2*A*) condition.

Speed-Dependent Stiffness. Tension responses to ramp stretches with a range of durations have been used in previous studies to investigate the attachment–detachment kinetics of cross-bridges bearing little force (11, 25, 26). For quick stretches the tension response is expected to be defined by the cross-bridge elasticity, but with moderate speed ramp stretches, when the duration of the stretch is comparable to the cross-bridge detachment rate, the amplitude of the tension response will decrease. Thus the speed dependence of the apparent stiffness indicates the detachment rate and can be used to study the properties of the AM·ATP[γS] state.

We applied ramp length changes with a wide range of velocities to fibers in several conditions: (*i*) in ATP[γS] in the presence and (*ii*) absence of Ca^{2+}, (*iii*) in ATP in the absence of Ca^{2+}, (*iv*) in rigor, and (*v*) in the low ionic strength relaxing conditions (1 mM ATP, 5°C, 20 mM ionic strength, $[Ca^{2+}] < 1$ nM) reported to promote formation of weakly attached, zero-force cross-bridges (11, 26). The ionic strength in conditions *i–iv* was 200 mM. Fig. 4 shows sample recordings at high and moderate velocities in the ATP[γS] solutions at 200 mM ionic strength in the presence and absence of 30 μM Ca^{2+}. There is no appreciable stiffness (<4% of rigor stiffness) observed in the absence of Ca^{2+}. In the presence of Ca^{2+}, transient increases in tension during the stretches indicate an increased muscle fiber stiffness. The amplitude of the tension response increases markedly with the speed of the ramp stretch (compare *A* and *B*).

The tension change, termed chord stiffness, measured at a fixed stretch (20 μm), is plotted vs. the logarithm of the ramp stretch duration (t_d) in Fig. 4*E*. The data are corrected for mechanical end compliance by monitoring striation spacing away from the fiber attachment points using a white light diffraction technique (22) and for inertial lags in the recording system. In the absence of Ca^{2+} at 200 mM ionic strength, fibers show little chord stiffness in either 5 mM ATP[γS] (×) or ATP (○). Rigor cross-bridges are stiff, with little speed dependence of the tension response (□). In the presence of Ca^{2+} and ATP[γS] (+), there is a speed-dependent chord stiffness increasing from nearly zero at t_d = 1 s to almost the rigor value at t_d = 300 μs. As reported before (11, 26), cross-bridge attachments in low ionic strength relaxing conditions (△) also give rise to speed-dependent stiffness but in a more rapid range of stretches (t_d < 10 ms).

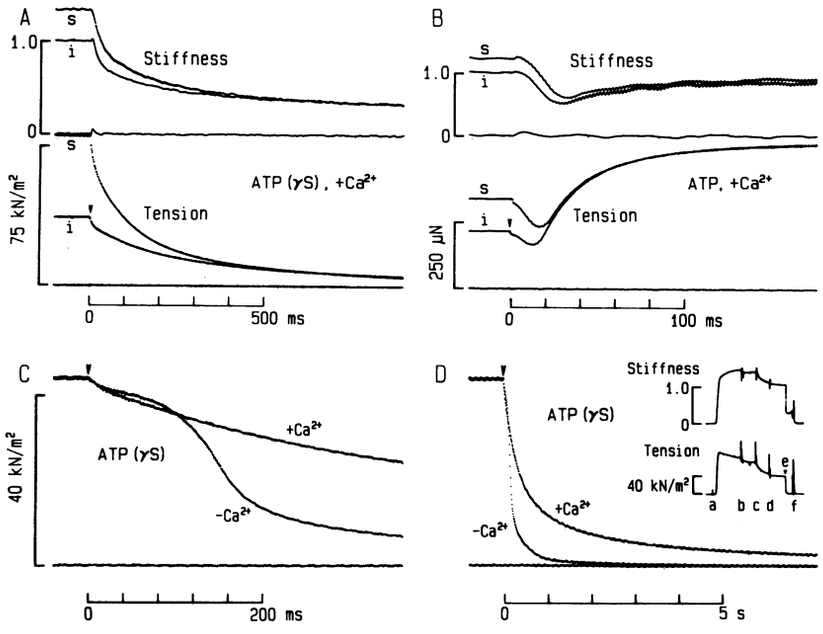

FIG. 2. Mechanical transients following photolysis of caged ATP[γS] (*A*) or caged ATP (*B*) in the presence of 30 μM Ca^{2+} and photolysis of caged ATP[γS] in the presence and absence of Ca^{2+} (*C* and *D*). Liberated nucleotide concentrations were 1.0 mM ATP[γS] in *A* and 850 μM ATP in *B*. Conditions otherwise were as in Fig. 1. Fiber dimensions: for *A*, 6950 μm^2 × 1.9 mm; sarcomere length, 2.42 μm. *C* and *D* are recordings of the same events at different time bases when 1.1 mM ATP[γS] was liberated in the presence and absence of Ca^{2+}. Baselines were recorded after relaxation in ATP. Fiber dimensions for *C* and *D*: 8070 μm^2 × 2.28 mm; sarcomere length, 2.10 μm. (*Inset*) Slow time base (strip chart) recording in the conditions of *A*. Tension (lower) and stiffness (upper) traces are shown as the fiber was put into (a) rigor, (b) Ca^{2+} rigor, (c and d) 10 mM caged ATP[γS] + Ca^{2+}, (e) full relaxation of tension by release of ATP[γS], and (f) relaxation of stiffness as well in ATP relaxing solution.

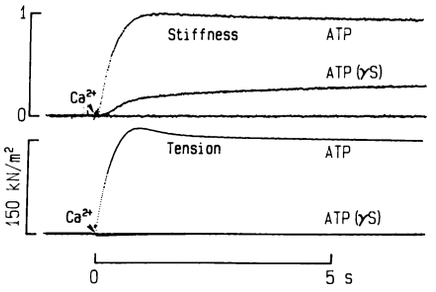

FIG. 3. Onset of tension and in-phase stiffness when the free Ca^{2+} concentration is increased from <1 nM to 30 μM. The fiber was relaxed in either 1 mM ATP[γS] or 1 mM ATP and then transferred through two successive 2-min washes in 0.1 mM EGTA. At the arrowheads, it was put into an activating solution with 20 mM CaEGTA and 30 μM free Ca^{2+}. Note development of stiffness without tension in ATP[γS]. Baselines were recorded with the fiber relaxed in ATP. Fiber dimensions: 6310 μm^2 × 2.06 mm; sarcomere length, 2.34 μm.

Hydrolysis of ATP[γS] by Muscle Fibers. The steady-state hydrolysis rates of ATP[γS] and ATP by fibers and actomyosin in solution are compared in Table 1. The rate of ATP[γS] hydrolysis per myosin head, in the fiber, was 0.16 ± 0.032 s^{-1} (mean ± SEM, n = 6) at 5 mM ATP[γS] in the absence of Ca^{2+} and 0.16 ± 0.061 s^{-1} (mean ± SEM, n = 6) in the presence of 30 μM Ca^{2+}. On the assumption that the reduced rate of ATP[γS] hydrolysis compared to ATP in the presence of Ca^{2+} is due solely to a slower elementary cleavage step (reactions 3a and 3b in Scheme I) and that the remainder of the cycle is limited by a step at 2.9 s^{-1} (27), ≈95% of the cross-bridges would populate the AM·ATP[γS] and M·ATP[γS] states. Verification of these assumptions would require direct chemical kinetic measurements.

DISCUSSION

Skinned muscle fibers were fully relaxed in solutions containing the nucleotide analog ATP[γS] in the absence of Ca^{2+}. When Ca^{2+} was added to the medium, force remained at the relaxed level, but mechanical stiffness measurements indicated that cross-bridges had formed. At 30 μM Ca^{2+}, 200 mM ionic strength with ATP[γS] as well as in 20 mM ionic strength relaxing conditions with ATP, the force is zero in the isometric state and returns to zero after a stretch. This behavior suggests that when these cross-bridges are put under mechanical strain, they detach from the thin filament and reattach to sites with zero average strain.

The speed dependence of stiffness shown in Fig. 4 is indicative of rapid attachment and detachment of such low-force cross-bridges. The mechanical component of free energy imparted to the cross-bridges by a ramp stretch is expected to decrease their affinity for actin, which might be expressed as variation of attachment and/or detachment rate. In a theoretical treatment, Schoenberg (25) showed that for the simplest case of a strain-independent cross-bridge detachment rate (strain-dependent attachment rate), the horizontal position of a chord stiffness vs. the logarithm of the t_d relation such as in Fig. 4*E* can be used to estimate the detachment rate. The stretch duration (t_d) at the 63% point (1 − 1/e) of the chord stiffness relation indicates the reciprocal of the rate constant for cross-bridge detachment. On this basis, the data indicate that cross-bridges in the presence of Ca^{2+} and ATP[γS] (+) detach at ≈100 s^{-1}. The data do not plateau even for ramp durations as short as 1 ms, suggesting a wide range of rate constants is involved and that some AM·ATP[γS] cross-bridges detach at rates on the order of 10^3 s^{-1}. The cross-bridges in low ionic strength relaxing solution (△) apparently have even higher detachment rates.

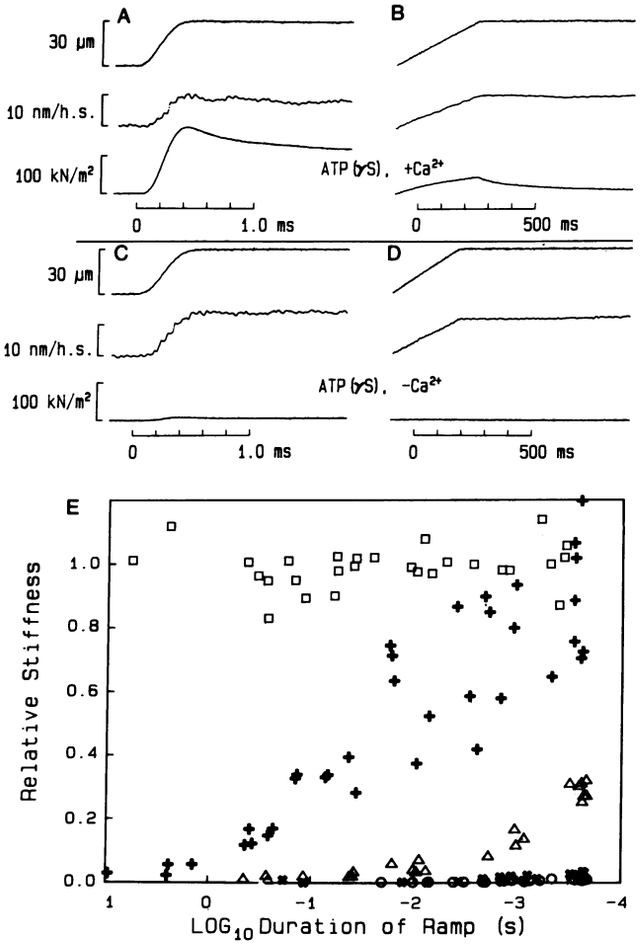

FIG. 4. Speed dependence of stiffness. Tension and striation spacing are shown during ramp stretches in 5 mM ATP[γS] in the absence (*C* and *D*) and presence (*A* and *B*) of 30 μM free Ca^{2+}. Tension (bottom), striation spacing (middle), and fiber length (top) were recorded during high-speed (*A* and *C*) and moderate-speed (*B* and *D*) ramp stretches. Typical single recordings are shown with no signal averaging. The steps apparent on the striation spacing signals (middle traces) in *A* and *C* are caused by the updating of a digital-to-analog convertor in the diffraction apparatus for monitoring striation spacing (22) and do not represent stepwise sarcomere elongation. Note that the tension response depends markedly on ramp velocity in the presence of ATP[γS] and Ca^{2+} (*A* and *B*). Fiber dimensions: 9780 μm^2 × 2.50 mm; sarcomere length, 2.45 μm. (*E*) Speed dependence of chord stiffness in the following conditions: rigor (□); 5 mM ATP[γS], 30 μM free Ca^{2+} (+); 5 mM ATP[γS], <1 nM [Ca^{2+}] (×); 5 mM ATP, <1 nM [Ca^{2+}] (○) (all at 20°C and 200 mM ionic strength); 1 mM ATP, <1 nM [Ca^{2+}] relaxing solution at 5°C and 20 mM ionic strength (△). The (×) and (○) symbols nearly overlap at the abscissa. Fiber stiffness was tested by applying 30-μm ramp stretches as in *A–D* except for the rigor condition, where smaller (6–10 μm) ramp releases were used. Chord stiffness was calculated as the ratio of tension change to striation spacing change, measured at fixed amplitude (20 μm; rigor, 6 μm) of length change and then normalized to the average chord stiffness in rigor for each fiber. The abscissa is the time (t_d) for each ramp stretch to reach 20 μm elongation, plotted on a logarithmic scale. Data represent four fibers, with lengths 1.74–2.53 mm.

Several mechanical and biochemical features of the cross-bridges in the presence of Ca^{2+} and ATP[γS] are analogous to the putative attachments before the power stroke of the cross-bridge cycle: (*i*) zero steady-state force, (*ii*) quick stress–relaxation following stretch, (*iii*) steep speed dependence of stiffness, and (*iv*) biochemical states after substrate binding but before product release. With ATP a structural change, probably coupled with release of inorganic phosphate (7, 9, 20, 30,

Table 1. Steady-state rates of ATP and ATP[γS] hydrolysis per myosin head

	k_{cat}, s^{-1}	
	ATP[γS]	ATP
Relaxed skinned psoas muscle fiber (− Ca^{2+})	0.16	0.08 (27)
Activated skinned psoas muscle fiber (+ Ca^{2+})	0.16	2.9 (27)
Subfragment 1	0.24 (15, 16, 24)	0.04 (28)
Actomyosin subfragment 1	0.36 (17)	40 (29)

Conditions were typically pH 7–8 and ionic strength 20–200 mM. For precise conditions, see references in parentheses.

31), transforms the transient attachment into a strongly bound state that generates relative sliding force between the two sets of filaments. There was no evidence of such a transition with the cross-bridges present in the ATP[γS] solutions, suggesting that hydrolysis of the β-γ phosphate bond is a prerequisite for the force-generating state.

The same conclusion follows from the transient tension response in photolysis experiments. Following detachment of cross-bridges on photolysis of caged ATP in the absence of Ca^{2+}, cross-bridge reattachment causes a transient redevelopment of tension (12–14). When ATP[γS] was released from caged ATP[γS] in the same Ca^{2+}-free conditions (Fig. 1*A*), tension decreased continuously to the baseline, suggesting that hydrolysis is required before active development of force in transient conditions as well as in the steady state.

The shape of the tension traces during relaxation after photolysis of caged ATP[γS], however, was more complex than expected for simple detachment of nucleotide-free (AM) cross-bridges by ATP[γS]. An initial rapid relaxation phase was followed by a plateau or delay of relaxation and then a more rapid final relaxation (Figs. 1 and 2*C*). One explanation for the plateau phase is that cross-bridges in the AM·ATP[γS] state as well as the rigor (AM) state can derepress the thin filament regulatory system. Overall detachment would then be a highly cooperative process, accelerating markedly when a critical number of cross-bridges have detached and the regulatory system then prevents further attachments. In contrast, the thin filament regulatory system remains in the derepressed condition in the presence of Ca^{2+}, so that the time course of approach to zero tension and the steady-state stiffness are smooth (Figs. 2 *A*, *C*, and *D*). The relatively slow relaxation is consistent with a wide range of detachment rate constants as discussed above.

When the affinity of actin for myosin is increased by reducing the ionic strength from the physiological level of 150–200 mM to 20 mM, experiments with isolated actomyosin subfragment 1 and with muscle fibers have shown that weakly binding myosin heads attach to regulated actin filaments whether or not Ca^{2+} is present (10, 11, 26). These results led to the hypothesis that the weak-to-strong transition of the cross-bridge cycle (AM·ADP·P_i to AM'·ADP, step 5 in Scheme I) is the predominant reaction controlled by the Ca^{2+}-regulatory proteins. In contrast to the steric hindrance theory (3–5), the results at low ionic strength suggested that the affinities of M·ATP and M·ADP·P_i for actin (steps 2 and 4) are not strongly influenced by troponin and tropomyosin.

At physiological ionic strength the available evidence suggests a direct regulatory effect on cross-bridge attachment. Few of the cross-bridges are attached in relaxed muscle, so if only a step following attachment is controlled by troponin–tropomyosin, during the onset of a twitch or tetanus the time course of net cross-bridge attachment should be concomitant with force generation. However, kinetic studies of cross-bridge attachment using the equatorial x-ray reflections (32) and mechanical stiffness of frog (33) and rabbit (34) muscle indicated that attachment precedes force generation by ≈5–15 ms. These results are consistent with direct blockage of cross-bridge attachment by the thin filament regulatory system, possibly by means of steric blocking of the myosin attachment site on actin (1–5). Upon activation, tropomyosin moves first and cross-bridges then attach and finally generate force. Our observation that the Ca^{2+}-regulatory system directly inhibits attachment of myosin states analogous to the initial weakly binding cross-bridge provides further support for the steric blocking model. However, the present data do not exclude additional roles of Ca^{2+}, such as mediating an isomerization of AM·ADP·P_i or promoting product release from the cross-bridges.

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