Relaxation from Rigor of Skinned Trabeculae of the Guinea Pig Induced by Laser Photolysis of Caged ATP

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ABSTRACT The kinetics of ATP-induced rigor cross-bridge detachment were studied by initiating relaxation in chemically skinned trabeculae of the guinea pig heart using photolytic release of ATP in the absence of calcium ions (pCa > 8). The time course of the fall in tension exhibited either an initial plateau phase of variable duration with little change in tension or a rise in tension, followed by a decrease to relaxed levels. The in-phase component of tissue stiffness initially decreased. The rate then slowed near the end of the tension plateau, indicating transient cross-bridge rebinding, before falling to relaxed levels. Estimates of the apparent second-order rate constant for ATP-induced detachment of rigor cross-bridges based on the half-time for relaxation or on the half-time to the convergence of tension records to a common time course were similar at 3×10^3 M^{-1} s^{-1}. Because the characteristics of the mechanical transients observed during relaxation from rigor were markedly similar to those reported from studies of rabbit *psoas* fibers in the presence of MgADP (Dantzig, J. A., M. G. Hibberd, D. R. Trentham, and Y. E. Goldman. 1991. Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibres. *J. Physiol.* 432:639–680), direct measurements of MgADP using [^{3}H]ATP in cardiac tissue in rigor were made. Results indicated that during rigor, nearly 18% of the cross-bridges in skinned trabeculae had [3H]MgADP bound. Incubation of the tissue during rigor with apyrase, an enzyme with both ADPase and ATPase activity, reduced the level of [3H]MgADP to that measured following a 2-min chase in a solution containing 5 mM unlabeled MgATP. Apyrase incubation also significantly reduced the tension and stiffness transients, so that both time courses became monotonic and could be fit with a simple model for cross-bridge detachment. The apparent second-order rate constant for ATP-induced rigor cross-bridge detachment measured in the apyrase treated tissue at 4×10^4 M^{-1} s^{-1} was faster than that measured in untreated tissue. Nevertheless, this rate was still over an order of magnitude slower than the analogous rate measured in previous studies of isolated cardiac actomyosin-S1. These results are consistent with the hypothesis that the presence of MgADP bound cross-bridges suppresses the inhibition normally imposed by the thin filament regulatory system in the absence of calcium ions and allows cross-bridge rebinding and force production during relaxation from rigor.

INTRODUCTION

It is generally believed that muscle contraction occurs by the cyclic attachment and detachment of the globular head of myosin in the thick filament with the actin subunits of the thin filament. The process is understood to be fueled by the concomitant hydrolysis of ATP by myosin. Biochemical studies of muscle contraction have provided a detailed description of how the chemical energy available from ATP hydrolysis is coupled to the interaction of actin and myosin (reviewed in Taylor, 1979; Eisenberg and Greene, 1980; Hibberd and Trentham, 1986; Goldman, 1987). These studies have assumed that the chemical intermediates formed during the interaction in vitro should correspond to particular cross-bridge states that are normally present during muscle contraction. The principal criticism of this description has been that in vitro, the steric constraints normally present on the proteins when arranged in the contractile filament lattice of muscle are lost. These constraints would be expected to alter the rate of transition from one cross-bridge state to another. With the synthesis of caged compounds (Kaplan et al., 1978) and the development of laser photolysis techniques (McCray et al., 1980), rapid changes of fixed concentrations of ATP or the products of its hydrolysis could be produced in complex macromolecular systems, thus circumventing normal diffusional delays. This approach has permitted estimates of the rates of the various steps of the cross-bridge cycle in skinned muscle fibers. Accordingly, we now can experiment using a model of the intact muscle in which the steric constraints imposed by the contractile filament lattice on cross-bridges are conserved.

To date, much of the work describing the non-steady-state kinetics of the acto-myosin interaction in skinned fibers has been carried out using the fast skeletal muscle fibers from rabbit *psoas* (Dantzig et al., 1988, 1991, 1992; Ferenczi et al., 1984, 1986; Goldman et al., 1982; Millar and Homsher, 1990; Walker et al., 1992). Recently, results from similar experiments on other muscle types have been reported (Arner et al., 1987; Somlyo et al., 1988; Horiuti et al., 1989; Steinen and Ferenczi, 1991; Yamakawa and Goldman, 1991; Millar and Homsher, 1992). Few data, however, are yet available from cardiac muscle (Barsotti and Ferenczi, 1988).

The rate constants of the transient reactions for a number of steps in the ATPase hydrolysis pathway measured in rabbit *psoas* fibers are near the limits of resolution of some of the

Received for publication 22 July 1993 and in final form 5 January 1994.
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This work was supported by grants from National Institutes of Health (HL40953) and the Whitaker Foundation.
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0006-3495/94/04/1115/14 $2.00

caged compounds currently available, e.g., caged ATP. In more slowly contracting muscle like cardiac muscle, however, it is expected that these rate constants would be slower, making them easier to measure.

The experiments in this study were designed to study the non-steady-state kinetics of rigor cross-bridge detachment in skinned cardiac muscle. The goals were twofold: (1) to understand the mechanism responsible for the tension transient previously reported after the photolytic release of ATP of skinned cardiac muscle (Barsotti and Ferenczi, 1988); and (2) to estimate the second-order rate constant for ATP dissociation of cross-bridges. Similar studies of different muscle types have reported a relatively complex time course of relaxation from rigor. This is inconsistent with a simple two-step detachment mechanism, involving the MgATP binding to myosin and the dissociation of cross-bridges from actin. Overall, the earlier studies reported several phases of the time course of the tension decline. In fast muscle types like rabbit *psoas*, a brief period of rapid tension decline occurs immediately after ATP release. Then, either the rate in the fall of tension slowed or the tension level increased transiently before declining to the relaxed level. Goldman and co-workers suggested that the transient change in the rate of tension and stiffness decline could be explained by a model in which there is cooperative cross-bridge binding to the thin filament. According to the model, after the photolytic release of ATP, cross-bridges detach, causing the initial tension decline. These cross-bridges would then reattach and generate force even in the absence of calcium ions because the binding of the remaining cross-bridges in rigor conformations suppresses the inhibition normally imposed by the thin filament regulatory system (Bremel and Weber, 1972). Because stiffness, a measure of the number of attached cross-bridges, falls continuously after the photolytic release of ATP, the number of reattaching cross-bridges must be less than the number detaching. When the number of attached cross-bridges drops below that required to suppress the thin filament regulatory system, tension declines to resting levels.

In slow muscle types like cardiac muscle, a large initial decline in tension usually has not been observed. Instead tension was reported to remain constant at the rigor level or even increase transiently (Barsotti and Ferenczi, 1988, Steinen and Ferenczi, 1991). In this study, we report that the relatively complex relaxation response after the photolytic release of ATP from caged ATP in absence of calcium ions is caused by the continued presence of MgADP in rigor. The MgADP is presumably bound to some cross-bridges. We reduced the tissue level of MgADP by incubation with apyrase—an enzyme possessing both ATPase and ADPase activities, before the release of ATP from caged ATP. We observed that the reduction was accompanied by significant changes in the time course of relaxation to one consistent with a relatively simple kinetic scheme. These results revealed that the complex time course of tension and stiffness during relaxation from rigor is in fact caused mainly by MgADP-bound cross-bridges. The reduction in the transient cross-bridge rebinding also allowed for a more direct estimate of the apparent second-order rate constant controlling rigor cross-bridge detachment in the absence of calcium ions. A preliminary report of some of the results was published previously (Martin and Barsotti, 1991).

MATERIALS AND METHODS

Tissue preparation and solutions

Chemically skinned cardiac trabeculae, <250 μm in diameter, were prepared from male Dunkin-Hartley guinea pigs (350–450 g) as described in Barsotti and Ferenczi (1988) and were stored in 50% glycerol at −15°C for no more than 4 days.

Unless otherwise indicated, all chemicals were of the highest purity and grade available, purchased from Sigma Chemical Co. (St. Louis, MO), and used without further purification. All other experimental solutions were prepared based on calculations using a computer program that employed the association constants for the various constituents from the literature. All solutions contained TES buffered at pH 7.1 and 21°C, 5 mM MgATP, 1 mM free Mg^{2+}. The ionic strength of all solutions was adjusted to 200 mM using 1,6-Diaminohexane-*N,N,N′,N′*-tetraacetic acid (HDTA, Aldrich Chemical Co., Milwaukee, WI). Relaxing and activating solutions contained 30 mM Ca^{2+}-EGTA (pCa 4.5) and EGTA (pCa > 8). In tissue not treated with apyrase, rigor was induced by incubation in a low MgATP-containing solution (0.1 mM, pCa > 8) followed by incubation in an identical solution that contained no MgATP. Before incubating the tissue in activating solutions, a pre-activating solution was used to lower the EGTA concentration to 0.1 mM. In the photolysis experiments, the solutions contained 10 mM caged ATP (pCa > 8) and 20 mM glutathione to bind 2-nitroacetophenone released upon photolysis of the caged compound. For the experiments using BDM (2,3-butanedione monoxime), a concentrated stock (100 mM) was made fresh on the day of each experiment, and the appropriate amount was diluted in the caged ATP solution.

Apyrase treatment was carried out in a low calcium (pCa > 8) rigor solution with the apyrase ADPase activity adjusted to 17.6 units/ml, and the apyrase ATPase:ADPase ratio was kept at roughly 3:1. The initial experiments using apyrase treatments employed a batch of apyrase (Sigma, Product A-6160, Grade VII, Lot # 7OH-7045) that is no longer available. Subsequently, we have approximated these conditions by mixing high ADPase apyrase (Sigma, Grade VII) and a less ADPase-enriched grade (Sigma, Grade VIII, approximately 10:1 ATPase:ADPase) to produce a treatment solution with the same total ADPase activity and a comparable ATPase:ADPase ratio. Apyrase treatments with these solutions have given results identical to those of the earlier studies. Apyrase was solubilized in low calcium rigor solution, and stored on ice in a refrigerator, and used within 3 days of preparation. In some instances the stability of the enzyme during storage was assayed using *p*-nitrophenyl phosphate as a substrate.

Apyrase was also used to eliminate low levels of contaminating MgADP and ATP from caged-ATP solutions. Before some experiments, the caged-ATP solution was incubated with 17.6 units/ml for 1 h, similar to the method described in Sleep and Burton (1990). The apyrase was removed by centrifugal filtration (Ultra Free-MC, 10 kDa NMWL, Millipore, Bedford, MA) for 45 min at 20°C in a JA-20 rotor (Beckman Instruments Inc., Palo Alto, CA) at 5000 rpm. In control experiments to test the resistance of caged ATP to hydrolysis by apyrase, we found no detectable change in the concentration of caged ATP as measured by HPLC after a 6-h incubation with the enzyme at room temperature.

Apparatus

T-shaped aluminum foil clips were crimped around the ends of the skinned trabeculae and mounted in an apparatus similar to that in Barsotti and Ferenczi (1988). The tissue was stretched 1.2 times above slack, resulting in an average sarcomere length of 2.23 ± 0.01 mm (mean ± SEM, $n = 67$). The transducer was a semiconductor element of a silicon strain gauge (Akers 801, SensoNor, Norway) and was mounted as described in Goldman and Simmons (1984). The resonance frequency with connections was 4 kHz.

Either small (<0.5% of total length) sinusoidal or step (~2%) length changes were made using a piezoelectric device (P-840.40, Physik Instrumente, Polytec Optronics Inc., Costa Mesa, CA) driven by a custom built low-voltage amplifier. A two-phase lock-in amplifier equipped with a sine wave oscillator (Model 3961B, Ithaco, Ithaca, NY) supplied the sine wave signal used to drive the low-voltage amplifier. The signal from the strain gauge was pre-amplified and distributed to a four-channel digital oscilloscope (Model 4094C, Nicolet, Madison, WI), the lock-in amplifier, and a custom built notch filter that removed the small sinusoidal signal for some of the tension records. The lock-in amplifier isolated the carrier component of the tension change from the imposed sinusoidal oscillation and performed real-time demodulation of the oscillation into components in-phase and 90° out-of-phase with the imposed length oscillation. The in-phase component of the tension oscillation is a measure of the stiffness of the fiber and is an index of the number of attached cross-bridges; increases in the 90° out-of-phase component indicate a phase lead of the tension signal over the imposed length oscillation. The phase lead is primarily due to changes in the viscoelastic properties of the fiber and is thought to be due the presence of actively cycling cross-bridges (Kawai and Brandt, 1980; Goldman et al., 1984a, b). Demodulation of the sinusoidal oscillation in the tension recordings was also carried out using a PC-based program that emulates a lock-in amplifier. Length and tension data were collected at 5 kHz and stored on disk either by the digital oscilloscope or a data acquisition board (MetraByte, DAS-16f, Taunton, MA) installed in a 386 25 MHz IBM clone.

Caged ATP was photolyzed by a 50 ns, 50- to 300-mJ pulse of 347-nm light produced using an angle-tuned, temperature-stabilized potassium dideuterium phosphate (KDP) crystal that frequency-doubled the output of a Q-switched ruby laser (Laser Applications, Winter Park, FL). The primary beam (λ = 694 nm) was separated from the 347-nm pulse by a Brewster stack polarizer and a UG-11 filter placed between the KDP crystal and the tissue. The beam was focused on the specimen by a cylindrical lens and truncated in the horizontal plane by an adjustable slit placed between the lens and the specimen trough. To minimize mechanical artifacts in the tension records, a low energy continuous helium-neon laser was positioned on the axis of the ruby laser and used in setting the slits to mask the hooks from the high energy laser pulse. During an experimental trial, step length changes, if required by the protocol, charging and firing the laser, and triggering of data collection on the digital oscilloscope and the A/D board was controlled by a custom built programmable timer based on a Z8 microprocessor (Model 16 MPG, Bell Associates, Philadelphia, PA).

Nucleotide content of skinned tissue

Skinned trabeculae were held at their in vivo length between small split wire clips at either end of the fiber. The clips were mounted on small cylindrical caps, machined from plexiglass, so that the fibers could be manually transferred between 350-μl wells in a 96 well tissue culture plate (Linbro, Flow Laboratories, McLean, VA). The wells were filled with 200 μl of experimental solution. Fibers were labeled by incubation for 2 min in activating solution: pCa = 4.5, 5 mM MgATP, containing [^{3}H]ATP (1 mCi/ml) and [^{14}C]-sucrose (24 μCi/ml) as a volume marker. Labeled tissue was transferred to either low calcium rigor solution (pCa > 8) or apyrase treatment solution (low calcium rigor solution with added apyrase) containing [^{14}C]--sucrose at the same specific activity as the activating solution, but with no labeled ATP. The fibers were incubated for 15 min with three changes of solution. Some fibers then again were transferred to relaxing solution (pCa > 8, 5 mM MgATP), containing [^{14}C]-sucrose but not [^{3}H]ATP, in order to displace any bound radiolabeled nucleotide from the tissue. At the end of the incubations, nucleotides were extracted in ice-cold 0.5 N $HClO_4$ and then neutralized with KOH/BES solution containing 1 mM unlabeled AMP, ADP, and ATP. The nucleotides in the extract were separated by HPLC on a SAX column using a 5 min linear gradient from 100 to 700 mM $NH_4H_2PO_4$, 10% methanol at pH 4.2 at a flow rate of 2 ml/min. Fractions corresponding to the void volume ([^{14}C]-sucrose) and nucleotide peaks were collected for quantification by liquid scintillation counting (Beckman Model LS3801, Beckman Instruments, Inc., Fullerton, CA). The ratio of the disintegrations/min (dpm) measured for [^{3}H]ADP to that for [^{14}C]-sucrose from the tissue extract was compared with the same ratio measured from a similar procedure performed on the initial incubation medium.

Synthesis and estimate of the extent of caged ATP photolysis

Caged ATP was synthesized according to the method described in Walker et al. (1988). The only change was a substitution of a water/diethylether solvent alkylation step for the water/chloroform step.

Total caged ATP concentration was determined by UV spectroscopy in unphotolyzed samples assuming an extinction coefficient of 19.4 mM^{-1} cm^{-1} at 260 nm. After photolysis, bath samples were analyzed by HPLC using a paired ion method on a C18 column (nucleotide-nucleoside column, 10 μm particle size; Alltech Associates, Deerfield IL). The nucleotides and the caged ATP were eluted using a linear gradient of 5 mM KH_2PO_4, 1.5 mM tetrabutylammoniumphosphate, pH 3.5 from 15 to 50% methanol over 20 min at a flow rate of 1 ml/min. The nucleotides were eluted using a Perkin-Elmer pump (Model Series 3, Norwalk, CT) and detected by a diode-array detector (Model LC 235, Perkin-Elmer) by monitoring at 260 nm. The concentration of ATP produced by photolysis was calculated from the product of the initial concentration of caged ATP and the ratio of the area of the caged ATP peak to that of released nucleotides. The areas were determined by integration using Perkin-Elmer Omega 235 software running on an IBM PC clone.

A single laser pulse (100 mJ, 50 s) typically photolyzed 10% of 10 mM caged ATP in rigor buffer containing low (pCa > 8) calcium. The rate of release of ATP was assumed to be 100^{-1} s (Goldman et al., 1984a).

During the equilibration period, when caged ATP was allowed to permeate the tissue, some of the apyrase apparently would diffuse from the tissue into the trough. The apyrase would hydrolyze a significant portion of the ATP released by photolysis during handling of the bath sample before final storage in −70°C. The enzyme has both ATPase and ADPase activity, but was found also to breakdown AMP to adenosine. In these samples, the concentration of ATP produced was estimated from the product of the initial concentration of the caged ATP and the ratio of the area of the caged ATP peak to that of adenosine, AMP, ADP, and ATP. The amount of adenosine in the trough samples was corrected by substraction of the amount present in an aliquot of the unphotolyzed caged ATP solution used for those series of experiments.

Fibers incubated in apyrase treatment solution for 15 min at room temperature fully relaxed after the photolytic production of ATP in the absence of calcium ions. Because some apyrase was retained by the tissue, tension began to rise approximately 10 s after photolysis. Treated fibers consistently redeveloped the same level of rigor tension. Thus, after the photolysis trial, rather than be returned to a bath containing relaxing solution (as was done with untreated fibers), they were transferred to a rigor solution containing added apyrase.

During a photolysis trial, part of the bath volume was not exposed to the laser pulse: the ends of the fiber that are masked by mechanical slits (see above). There was no caged ATP photolysis in the masked region, and this masked bath volume diluted the photolysis products produced in the unmasked region. A geometric factor based on the width of the trough and fiber length (the fiber length is equivalent to the unmasked, illuminated region of the trough) was applied to the calculated concentration of ATP produced in the bath to determine the final concentration of ATP released within the fiber.

Data analysis

The time to the convergence of tension records during relaxation from rigor was used to estimate the initial rate of cross-bridge detachment (Goldman et al., 1984a). Convergence in this study describes the point at which the decay in rigor tension in the same trabeculae, either stretched or held isometrically, began to follow the same time course after the photolytic production of ATP. The difference between the tension traces from pre-stretched (s) and isometric (i) traces was calculated at each time point in the digital recording, producing a difference trace (s–i) for that trabeculae at similar final ATP concentrations. It was not possible to produce the same concentration of ATP in each of the trials used in the comparison. The ATP concentration was determined in both trials, and the average value was used for comparisons. The ATP concentration used to construct a difference trace did not vary by more than 5%.

The relaxation time course of apyrase-treated fibers was fit to the sum of two exponential terms that is described by the following equation:

$$\text{tension} = b_1 + c_1 e^{(-k_f t)} + c_2 e^{(-k_s t)} \quad (1)$$

The data were fit by the nonlinear least-squares routine of Levenberg-Marquardt (1988). Approximately, the first 5 ms after the laser pulse were not included in the fit. These few milliseconds reflect the rate of ATP release from caged ATP after photolysis, 100 s^{-1}. (Goldman et al., 1984a)

Regression lines were calculated by the method of least squares. Data are expressed as means ± SEM.

RESULTS

Relaxation from rigor after the photolytic release of ATP

One goal of these experiments was to measure the second-order rate constant for ATP-induced rigor cross-bridge detachment. The approach was to measure the dependence of the rate of relaxation from rigor in the absence of calcium ions (pCa > 8) upon the concentration of ATP photolytically produced by laser photolysis of caged ATP.

Fig. 1 illustrates the mechanical responses of two different skinned trabeculae in rigor to the photolytic release of ATP. The production of ATP did not produce an immediate decrease in tension (upper traces in panel *A* and *B*). Instead, there was a variable interval of several hundred milliseconds during which the tension remained at the rigor level (Fig. 1, panel *A*) or increased (panel *B*) before finally decaying to relaxed levels. We have previously reported similar tension responses of the same preparation to the photolytic release of ATP in the absence of calcium ions at 12°C (Barsotti and Ferenczi, 1988). The tension transients were almost identical to those reported for rabbit soleus fibers (Yamakawa et al., 1986; Poole et al., 1988) and are similar to those reported for rabbit *psoas* fibers (Goldman et al., 1984), smooth muscle (Arner et al., 1987; Somlyo et al., 1988), and insect flight muscle (Rapp et al., 1986; Yamakawa and Goldman, 1991). Goldman et al. (1984a) have suggested that the tension rise results from the transient rebinding of cross-bridges that have bound the ATP, detached from the thin filament, and then reattached and generated force, like active cross-bridges. Cross-bridge rebinding in the absence of calcium ions is thought to proceed because the remaining rigor cross-bridges suppress the inhibition imposed by the thin filament regulatory system (Bremel et al., 1973). There is a net decrease in fiber stiffness throughout relaxation in this muscle, indicating that the number of detaching cross-bridges exceeds the number of reattaching cross-bridges. When the number of attached cross-bridges drops below that necessary to relieve the inhibition of the thin filament regulatory system, cross-bridge reattachment ceases, and tension declines to the relaxed level.

After the release of ATP, the in-phase component of stiffness, which is an estimate of the number of attached cross-bridges, continually declined (middle traces in both panels of Fig. 1). However, the rate of the decline slowed either near the end of the tension plateau (panel *A*) or during the transient

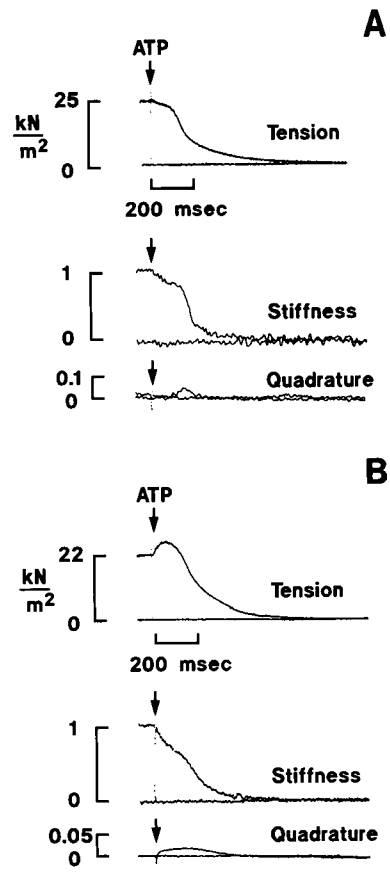

FIGURE 1 Examples of mechanical transients elicited by photolysis of caged ATP in skinned trabeculae in the absence of calcium. At the arrow, trabeculae in rigor in the presence of 10 mM caged ATP were relaxed by photolytic production of approximately 1 mM ATP. Each panel shows the transient response and relaxed levels of tension, stiffness, and quadrature. Panel A: final concentration of ATP = 970 μM, fiber dimensions: 1120 × 212 μm (length × diameter), sarcomere length: 2.18 μm. Panel B: final concentration of ATP = 1020 μM, fiber dimensions: 600 × 118 μm sarcomere length: 2.35 μm.

rise in tension (panel *B*). The peak of the quadrature signal also occurred about this time. These results suggest two things: (1) that cross-bridge reattachment occurred during the end of the tension plateau, and (2) that upon reattachment, cross-bridges enter conformations similar to those of active cross-bridges. After the transient rise in tension, the time course of the decline in the in-phase component of stiffness approximated that of tension. During this same period, the quadrature signal decayed back to the resting level. In general, the rate of the decline in stiffness during the period immediately following release of ATP was greater than the rate of decline of tension, suggesting that the remaining attached cross-bridges bore relatively more stress.

The rate of the decline in both stiffness and tension increased with increasing concentrations of ATP produced upon photolysis. The increase in the observed rates was mainly due to a reduction in the duration of the tension plateau and a relatively smaller increase in the rate of relaxation, as illustrated by the records in Fig. 2. Compare traces labeled

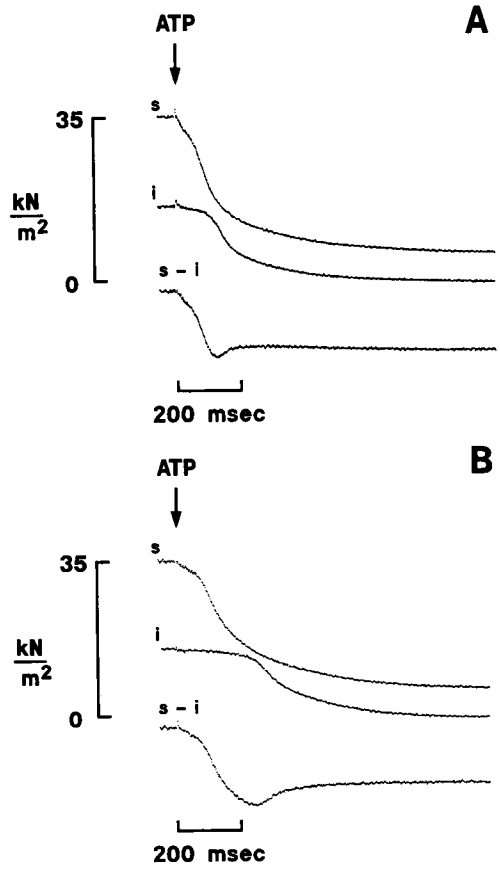

FIGURE 2 ATP dependence of difference traces for trabeculae relaxed from rigor in the absence of calcium ions. Each panel shows the tension transient during relaxation for fibers relaxed from the isometric rigor length (i) or 1 s after a small stretch (s), as well as a difference trace ($s - i$) constructed from the algebraic difference between the stretched and isometric tensions at each point. Final concentration of ATP = 2085 μM (*panel A*), 480 μM (*panel B*). Fiber dimensions: 700 × 147 μm, sarcomere length: 2.28 μm.

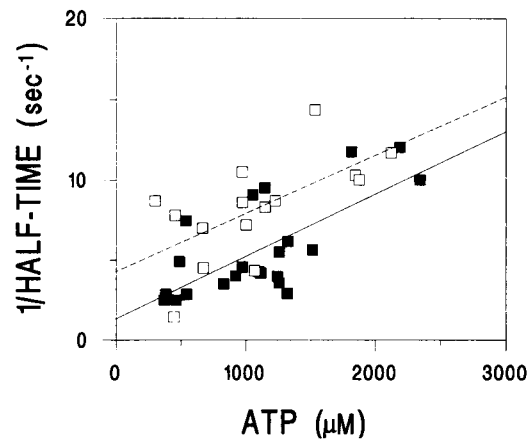

FIGURE 3 Apparent rate of cross-bridge detachment as a function of ATP concentration. The detachment rate was estimated from the overall half-time for full relaxation from rigor (■) of fibers held isometrically (23 experimental trials from 12 different trabeculae) or from the half-time for the decay of the difference in tension between a fiber held at its isometric length and the same fiber pre-stretched before release of ATP (□) (16 experimental pairs from 8 different fibers). The solid line is a linear least-squares fit to the data from the overall half-time for the relaxation of fibers held at their isometric length and is described by the equation: $y = 1.352 + 0.00389x$, $r = 0.69$. The dashed line is a linear least-squares fit to the difference data and is described by the equation: $y = 4.306 + 0.00361x$, $r = 0.63$.

i in each of the panels A and B, when relatively high and low concentrations of ATP were released. Because the decline of neither tension nor stiffness was exponential, each was characterized by the half-time required to reach relaxed levels. An estimate of the second-order rate constant for ATP-induced dissociation of rigor cross-bridges, which is based on the dependence of this half-time on the ATP concentration, is shown in Fig. 3. The half-times were measured from the laser pulse to when the tension reached the relaxed level. The ATP concentration was varied by keeping the concentration of caged ATP constant at 10 mM and varying the laser energy. The solid line drawn in Fig. 3 was fit to the data by the method of least squares. The slope of this relationship, 3.9×10^3 M^{-1} s^{-1}, approximates the second-order rate constant for ATP-induced rigor cross-bridge detachment. A similar value was obtained when the decline in stiffness was used as an estimate of the rate of cross-bridge detachment. Under these conditions, this rate was approximately three orders of magnitude slower than that reported from in vitro studies on bovine cardiac actomyosin: 1.4×10^6 M^{-1} s^{-1} (Taylor and Weeds, 1976).

A lower estimate for the rate of ATP-induced cross-bridge detachment would be expected if significant cross-bridge reattachment took place during relaxation from rigor. Accordingly, a better estimate for the rate of detachment could be made if cross-bridge reattachment were inhibited. One technique shown to reduce the amount of force generated by reattaching cross-bridges is the addition of millimolar concentrations of Pi to the caged ATP solutions (Hibberd et al., 1985). As shown in Fig. 4, the presence of 20 mM Pi during the release of ATP abbreviated, but did not eliminate the tension plateau in skinned cardiac muscle (compare trace *a* with trace *b*. Trace *c* will be described later). Pi also reduced both the transient change in the rate of stiffness decline and the increase in quadrature that normally occurred toward the end of the tension plateau. Pi had no effect on the initial rate of decline in stiffness during the tension plateau. This indicates that little cross-bridge reattachment occurred during this early period of the tension plateau and that Pi does not affect the rate of rigor cross-bridge detachment by ATP. Pi, however, was not used to inhibit reattachment during relaxation from rigor. At the time these experiments were initiated, the effects of Pi on stiffness were not known and its effects on tension were not well characterized. There was concern that Pi may directly affect the rate of detachment of rigor cross-bridges by ATP. Instead another method, described in Goldman et al. (1984a) was used. Their method used the rate of convergence of tension traces recorded after the release of ATP in a fiber held isometrically with traces

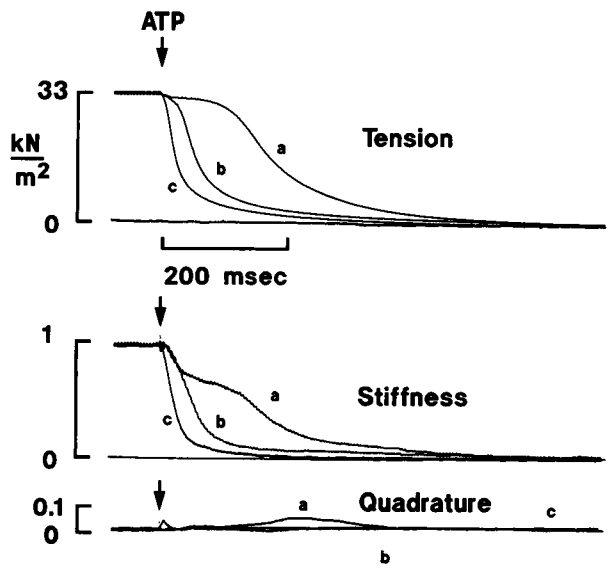

FIGURE 4 Effect of phosphate and apyrase on mechanical transients elicited by photolysis of caged ATP in the absence of calcium. At the arrow the fiber in rigor in the absence of calcium was relaxed by photolytic production of approximately 1 mM ATP (*a*), with 20 mM added, sodium phosphate (*b*) or after apyrase treatment (*c*). Final concentration of ATP = 1120 μM (*a*), 940 μM (*b*), 1050 μM (*c*). Fiber dimensions: 803 × 218 μm, sarcomere length: 2.20 μm.

recorded from the same fiber stretched just before ATP release to measure the initial cross-bridge detachment rate even in the presence of cross-bridge reattachment. This was based on the assumption that the amount of force produced by the reattaching cross-bridges was independent of the initial load during rigor.

Difference traces were generated from pairs of tension traces from the same trabeculae after the release of similar concentrations of ATP. The relaxation responses when the tissue was held isometrically (*i* in Fig. 2) was subtracted from the response when the tissue was stretched (~2% of its initial length), 1 s before the laser pulse (*s* in Fig. 2). Photolysis of caged ATP in the stretched trabeculae caused an immediate fall in tension and shortened the duration of the tension plateau. This initial decline in tension induced by stretching suggests that positive strain increased the apparent detachment rate of rigor cross-bridges. Similar results have been reported from studies on other muscle types (Goldman et al., 1984; Somlyo et al., 1988). The truncation of the tension plateau with stretch indicated that there was a limit to the amount of tension that attached cross-bridges could sustain. It was also consistent with the premise that the force produced by reattaching cross-bridges increases the duration of the tension plateau. By stretching the tissue, the load on the rigor cross-bridges was increased. This caused more rapid detachment upon the release of ATP, decreasing the number of cross-bridges attached to the thin filament. That, in turn, reduced the extent of thin filament suppression, which decreased the number of reattaching cross-bridges and thereby reduced the duration of the tension plateau. The rate of the loss of tension in stretched tissue slowed 50–100 ms after ATP release (see trace *s* in the upper panel in Fig. 2), indicating cross-bridge reattachment. Tension then increased again, falling to a level that was greater than the pre-stretched level of tension. The small amount of extra tension was probably due to extension of some noncontractile elastic elements that gave rise to the passive tension observed when cardiac tissue was lengthened beyond slack. The extra passive force prevented the tension records of stretched and isometric fibers from converging to the same level of force, even when fully relaxed. If the small extra tension attributed to passive elements (i.e., the difference between stretched and isometric fibers in relaxing solution) was subtracted from the relaxation time course of the stretched fiber, then the tension records of the stretched and isometric fibers converged before either was fully relaxed and remained identical throughout the remainder of relaxation. The difference traces were constructed without subtracting the contribution of the passive elements from the time course of the stretched trabecula, as illustrated by the difference traces ($s-i$) in Fig. 2. The abbreviation of the tension plateau in the stretched tissue tended to cause the tension to drop below what it was when held isometrically. The final plateau of the difference trace ($s-i$) corresponded to the convergence. The point at which the time course of the decline in tension in the isometric trial was identical to that in stretched trial.

The open square symbols in Fig. 3 show the relation between the initial cross-bridge detachment rate, estimated from the convergence of the tension traces, and the concentration of ATP released from the photolysis of caged ATP. A linear fit of this data is indicated by the dashed line and has a slope of $3.6 \times 10^3\ M^{-1}\ s^{-1}$. Given the scatter in the data it was not clear whether the y-intercepts of the lines shown in Fig. 3 were significantly different. More importantly, the lack of a significant difference in the slope of the relationship between estimates of the rate of detachment based on the half-time for the overall relaxation and those based on the convergence time indicates that in cardiac tissue the convergence of tension records occurs late in the relaxation time course. This is similar to insect flight muscle (Yamakawa and Goldman, 1991) but different from rabbit *psoas* fibers (Goldman et al., 1984a). Thus, under these conditions, convergence of the tension records is still complicated by the presence of reattaching cross-bridges and does not provide an accurate estimate of the initial cross-bridge detachment rate in skinned cardiac muscle.

These experiments revealed striking similarities between the characteristics of the mechanical responses of cardiac trabeculae in rigor to the photolytic release of ATP in the absence of calcium ions and those reported for rabbit *psoas* fibers but in the presence of MgADP. In both cases, there was an initial tension plateau or rise in tension after the release of ATP, dependence of the duration of the plateau on the concentration of MgATP, sensitivity of the tension plateau to Pi and increased load, and delay in the convergence of

tension records. These similarities prompted a series of experiments to test whether the presence of MgADP was responsible for the relatively slow and complex tension transients described above. There were at least three possibilities. First, any MgATP and/or MgADP contamination of the caged ATP preparation could be hydrolyzed by the tissue increasing the concentration of MgADP. Second, MgADP could remain bound to a significant number of cross-bridges during rigor. Finally, the observed mechanical responses to the release of ATP could simply reflect normal cross-bridge behavior for cardiac muscle.

MgATP and/or MgADP contamination of the caged ATP preparation was reduced to less than 2 μM, using a method described by Sleep and Burton (1990). On the day of the experiment, the caged-ATP solution was incubated with apyrase, an enzyme that has both ADP and ATPase activity. The apyrase was then removed from the solution by filtration (see Methods) before use. Lowering the concentration of uncaged nucleotide in the solution however had little effect on the shape and rate of the relaxation response. This suggested that MgATP and/or MgADP contamination of our caged-ATP preparation was not the cause of the slow and complex mechanical transients.

To estimate the amount of bound MgADP during rigor, skinned trabeculae were incubated for two minutes in an activating solution (pCa 4.5) containing [^{3}H]ATP and [^{14}C]-sucrose. The tissue was transferred to a rigor solution, incubated for a total of 15 min, and then extracted with perchloric acid. As shown in Table 1, the concentration of labeled MgADP present in the tissue after incubation in rigor solution was 28.2 ± 3.4 μM (mean ± SEM, n = 8). We have previously reported in Barsotti and Ferenczi (1988) that the concentration of myosin in this tissue was 80 or 160 μM S1. These results suggest that close to 18% of the cross-bridges in a trabeculae in rigor for 15 min have MgADP bound. To test whether this MgADP was bound to cross-bridges or at least to a site that readily exchanged with MgATP, the tissue was incubated in a relaxing solution containing 5 mM non-radiolabeled ATP for 2 min after incubation in rigor solution. The MgADP content of this tissue decreased to 5.7 ± 2.4 μM (mean ± SEM, n = 6), a value significantly lower than that measured in rigor (see Relax in Table 1). These results show that the MgADP still bound in the tissue in rigor was easily exchanged with MgATP and are consistent with the hypothesis that the MgADP retained during rigor was bound to the cross-bridges.

We tested the ability of apyrase to reduce the level of bound MgADP in skinned trabeculae by adding apyrase to the rigor solution and incubating the tissue for 15 min. The results are shown in Table 1. The amount of bound MgADP

TABLE 1 ADP Content of chemically skinned trabeculae

Rigor	28.2 ± 3.4 μM (n = 8)
Relax	5.7 ± 2.4 μM (n = 6)
Apyrase-treated	6.7 ± 1.1 μM (n = 12)

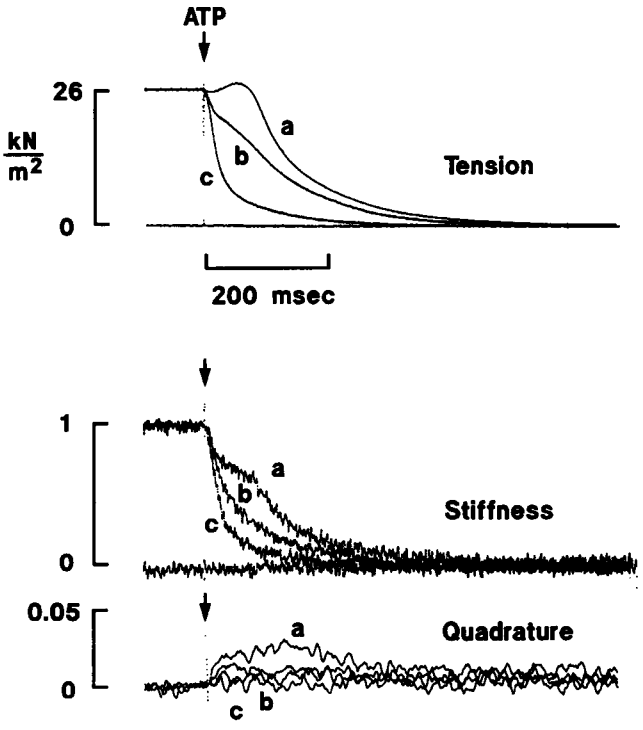

FIGURE 5 Effect of apyrase treatment on mechanical transients elicited by photolysis of caged ATP in the absence of calcium. Tension, stiffness, and quadrature after release of approximately 1 mM ATP (*arrow*) in skinned trabeculae in rigor in the absence of calcium and that have been treated with apyrase for (*a*) no treatment (*b*) 5 min, and (*c*) 15 min. Fiber dimensions: 1580 × 129 μm, sarcomere length: 2.17 μm.

remaining in the tissue decreased to 6.7 ± 1.1 μM (mean ± SEM, n = 12), an amount not significantly different from that measured in tissue in which the bound MgADP was chased with nonradiolabeled ATP (see Relax in Table 1). These results indicate that apyrase treatment was an effective means of reducing the concentration of MgADP present in skinned trabeculae during rigor.

To determine whether the presence of MgADP in skinned trabeculae in rigor was responsible for the mechanical transients observed during relaxation, we incubated the tissue in apyrase using the same conditions used to measure the tissue levels of MgADP. When fibers in rigor were treated with apyrase for 15 min, rigor tension decreased by approximately 5%. This decrease, however, was essentially the same as that observed when the fibers were kept in rigor solution without apyrase for 15 min. After apyrase treatment, the fibers spontaneously went into rigor 10 s after they were fully relaxed by photolysis of caged ATP. Presumably, the ATPase activity of the apyrase slowly depleted the ATP concentration within the tissue. Fig. 5 shows three relaxation responses of a single trabeculae after the release of similar ATP concentration: untreated (*a*); incubated for 5 min with apyrase in rigor solution (*b*); and then incubated for an additional 15 min (*c*). There was a progressive reduction in the duration of the plateau of tension after the release of ATP and an

increase in the rate of relaxation as incubation with the enzyme was increased. The transient changes in the rate of the decline in stiffness and the change in quadrature were significantly diminished. Similar results are illustrated by the records in Fig. 4; compare trace *a* (untreated) with trace *c* (apyrase-treated). These results together with those in Table 1, showing the presence of a significant concentration of MgADP in trabeculae during rigor, strongly suggested that the complex mechanical transients observed during relaxation from rigor were caused by the presence of MgADP, which was bound to some cross-bridges.

Apyrase-treated fibers fully relaxed after photolysis of caged ATP. Both tension and stiffness fell rapidly with little initial delay and with almost identical time courses, consisting of an early rapid phase followed by a slower phase. The time course of the decline in both tension and stiffness was fit well by an equation containing the sum of two exponential terms as shown in Fig. 6 and showed little evidence of cross-bridge rebinding. The delay in the decline in tension immediately after the laser pulse is probably the result of the rate of release of ATP from caged ATP. This brief delay required that the exponential fits to the mechanical responses be shifted approximately 5 ms after the laser pulse to exclude this region from the fit. Nevertheless, the early phase of the mechanical responses were well simulated by the reaction sequence shown in Scheme 1, which accommodates the rate of release of ATP from caged ATP.

$$\text{caged ATP} \xrightarrow{k_1} \text{ATP}$$

$$\text{ATP} + \text{AM} \xrightarrow{k_2} \text{AM.ATP} \xrightarrow{k_3} \text{M.ATP} + \text{A}$$

Scheme 1

Fig. 7 shows simulated tension records (*solid circles*) fit to original tension traces recorded after the release of 1.9 mM (upper left panel) and 1.1 mM ATP (upper right panel). The simulated traces were generated by computer and were based on the reaction sequence shown in Scheme 1 using the following rate constants: $k_1 = 100\ s^{-1}$ and $k_2 = 4.9$ or $5.3 \times 10^4\ M^{-1}\ s^{-1}$. Nominal values assigned for tension were AM = 1 and M.ATP = 0. To demonstrate the degree of freedom in fitting the rate of cross-bridge detachment, k_2, the lower panel in Fig. 7 illustrates the effect of varying this rate by a factor of 2 above (trace *c*) and below (trace *a*) the value that gave the best fit to the original tension record (trace *b*). The insets in Fig. 7 show the same traces on a slower time scale. These data indicate that apyrase treatment by a reduction in the number of cross-bridges with bound MgADP during rigor results in mechanical transients whose characteristics are much less complex than those of untreated fibers and that are consistent with those expected from the simple reaction mechanism shown in Scheme 1. Thus, apyrase treatment of tissue in rigor provided a means of directly measuring the rate of ATP-induced rigor cross-bridge detachment.

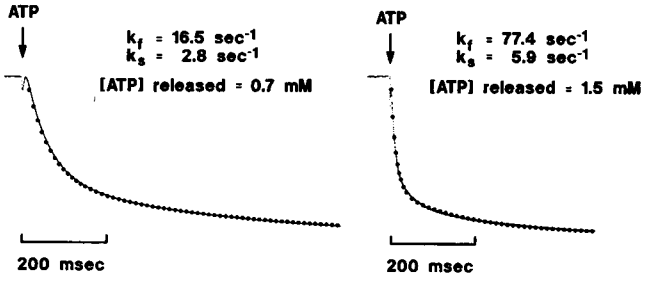

FIGURE 6 Fit of a double exponential to tension records of apyrase-treated trabeculae relaxed from rigor by photolysis of caged ATP at different laser energies. Tension transients after photolysis of caged ATP in apyrase treated skinned trabeculae were fit (●) to Eq. 1 (see Methods) using a nonlinear least-squares regression. Approximately 5 ms immediately after the laser pulse were excluded from the fit. Final concentration of ATP = 700 μM (*left panel*) and 1500 μM (*right panel*). Fiber dimensions: 940 × 213 μm, sarcomere length: 2.20 μm.

Fig. 8 shows the dependence of the apparent rate of cross-bridge detachment from rigor on the ATP concentration. In these experiments, the trabeculae were incubated with apyrase in rigor solution for 15 min before transfer into a solution containing caged ATP. The detachment rate was estimated by fitting the changes in tension (panel *A*) and stiffness (panel *B*) after the release of ATP with an equation containing the sum of two exponentials (Eq. 1). The solid and open square symbols in Fig. 8 represent the rate measured during the fast and slow phase of the relaxation transient. The rate of the rapid phase or k_f was 71 ± 10% of the total response (mean ± SEM, $n = 31$) and exhibited a strong dependence on the ATP concentration. The slope of the line fit by the method of least squares to k_f above 200 μM ATP was $3.9 \times 10^4\ M^{-1}\ s^{-1}$. The rate of the slow phase or k_s of relaxation from rigor exhibited no dependence on ATP. The observed dependence of k_f upon the ATP concentration was consistent with that expected for ATP mediated rigor cross-bridge detachment. The insensitivity of k_s to nucleotide concentration reflected the probability that this rate is determined by the viscoelastic properties of the tissue (Chiu et al., 1982; de Tombe and ter Keurs, 1992).

The apparent rate of the decline in tissue stiffness was also biphasic with a rapid early phase followed by a slower phase. The rate of both phases exhibited a dependence upon the ATP concentration similar to those of the tension. A plot of the rate of the decrease in tissue stiffness measured by exponential fitting versus ATP concentration is shown in Fig. 8, panel B. The slope of the relation was only 1.5 times that measured for tension. This indicates that there was little change in the amount of force per attachment during relaxation from rigor and suggests that no significant cross-bridge rebinding occurred during relaxation from rigor following the removal of bound MgADP by incubation with apyrase.

The apparent second-order rate of detachment, $3.9 \times 10^4\ M^{-1}\ s^{-1}$ was determined from the slope of the relation between the rate of the rapid phase (k_f) and the concentration of ATP. This rate was an order of magnitude faster than that measured in untreated tissue. Nevertheless, it was still significantly slower than the rate reported from in vitro studies of cardiac actomyosin-S1 (Taylor and Weeds, 1976). It still

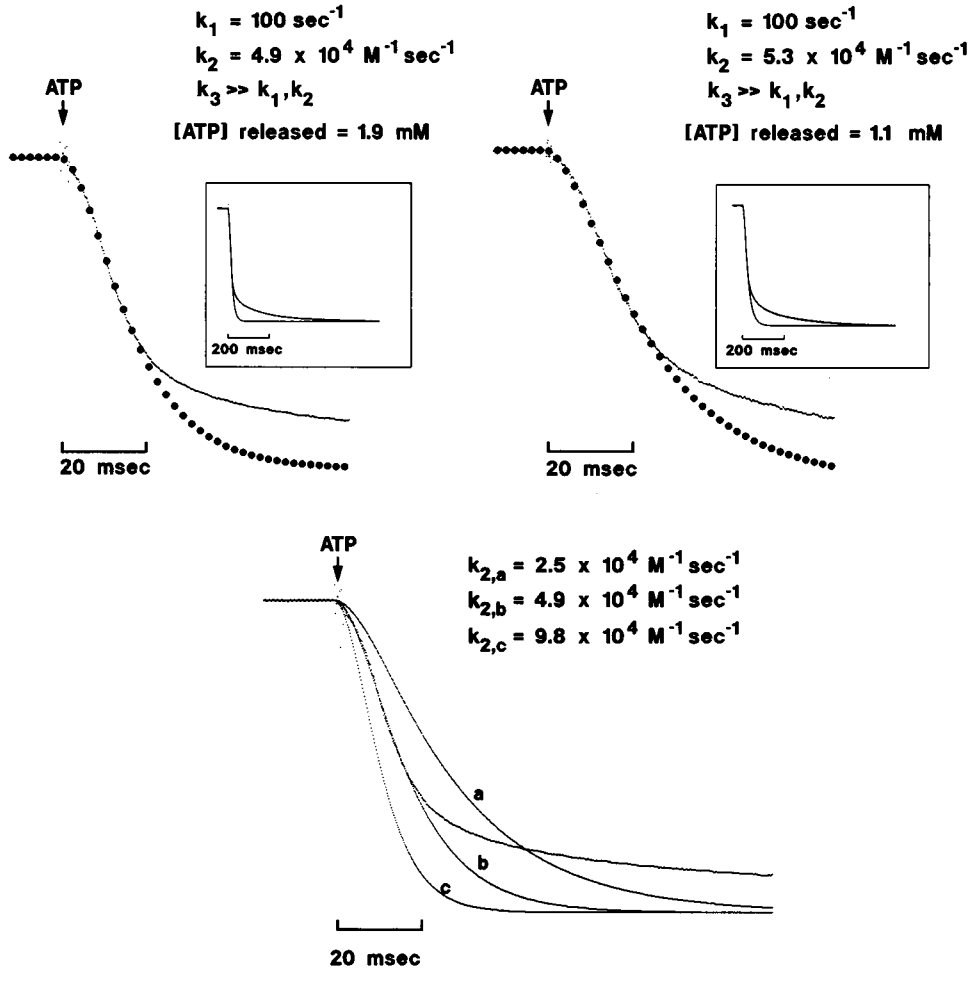

FIGURE 7 Fit of Scheme 1 to tension records of apyrase-treated trabeculae relaxed from rigor by photolysis of caged ATP at different laser energies. Scheme 1 was modeled (●) using the assumptions $k_3 \gg k_1, k_2$ and $k_1 = 100\ s^{-1}$, the concentration of ATP produced by photolysis (1.9 mM (panel *A*) and 1.1 mM (panel *B*)) as determined by procedures described in the text, and the value of k_2 that best fit the data as determined by eye. The insets show the same records with a longer time base. The lower panel illustrates the dependence of the fit shown in the upper left panel on the value assigned to k_2. Values of k_2 a factor of two below (*a*) and above (*c*) the best fit value (*b*) were chosen for this purpose. Fiber dimensions: 1300 × 188 μm, sarcomere length: 2.22 μm.

seemed possible, however, that despite the significant alteration of the rates and complexity of the mechanical transients after apyrase incubation, some degree of cross-bridge rebinding might still occur during relaxation and tend to slow the overall apparent rate of detachment. This was tested in two ways. First, difference traces were constructed in an attempt to subtract any of the tension caused by that generated by reattaching cross-bridges during relaxation from rigor. The protocol was similar to previous experiments with untreated tissue. Second, 20 mM BDM (2,3-butanedione monoxime) was added to the caged ATP solution before photolysis. BDM has been shown to inhibit tension production by slowing the apparent rate of formation of force producing cross-bridges (Lenart et al., 1989). In either case, the anticipated effect was an increase in the apparent rate of ATP-induced rigor cross-bridge detachment.

The half-time to the convergence of force was determined from difference traces for apyrase treated trabeculae, using isometric and pre-stretched tension transients obtained at approximately the same concentration of photolytically produced ATP. Compared to the difference records from untreated tissue, these traces have much shorter initial slow phase and a more rapid rate of decline (Data not shown). The slope of a line fitted to a plot of $1/t_{½}$ for the decline in the difference trace versus the ATP concentration was 3.1×10^4 $M^{-1}\ s^{-1}$, a value similar to that estimated by k_f, obtained from fitting the data with the sum of two exponentials. Similarly, the inclusion of 20 mM BDM in the caged ATP solution had little effect on this rate: $3 \times 10^4\ M^{-1}\ s^{-1}$. These results suggest that the relatively slow apparent second-order rate constant for ATP-induced cross-bridge detachment was not caused by cross-bridge rebinding and therefore represents the actual rate of this step in the cross-bridge cycle in cardiac muscle.

DISCUSSION

The kinetics of ATP-induced dissociation of rigor cross-bridges were studied to estimate the second-order rate constant controlling this process in skinned cardiac muscle. The measurements were based on the dependence of the observed rate of decline of tension or stiffness on the concentration of ATP produced by laser pulse photolysis of caged-ATP in the absence of calcium ions. In general, the relaxation response after the release of ATP was complex and exhibited numerous phases. This is uncharacteristic of the expected simple

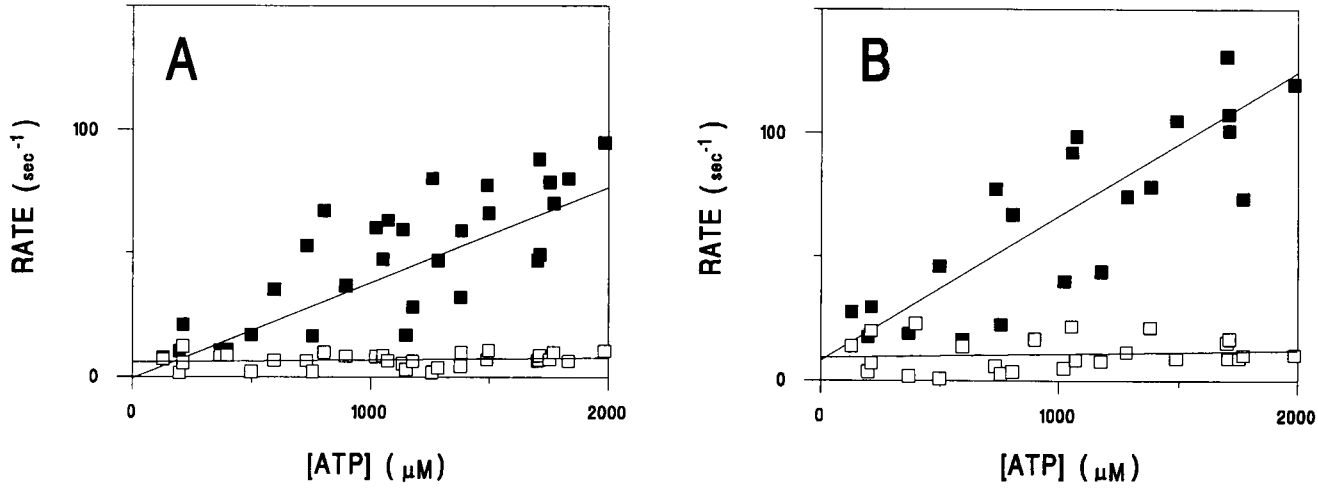

FIGURE 8 Rate of cross-bridge detachment as a function of ATP concentration determined from double exponential fits to the relaxation of apyrase treated skinned trabeculae. k_f (■) and k_s (□) were determined by fitting the equation $y = b_1 + c_1 \exp(-k_f t) + c_2 \exp(-k_s t)$ to tension (panel *A*) and stiffness (panel *B*) transients of skinned trabeculae treated with apyrase for 15 min and relaxed from rigor by photolytic production of ATP. Data are from 31 experimental trials on 11 different fibers. The lines are least-squares fit to the data and are described by the following equations: $y = 5.346 + 0.039x$, $r = 0.80$ and $y = 7.474 + 0.059x$, $r = 0.82$, for k_f for tension and stiffness. $y = 5.908 + 0.0008x$, $r = 0.17$, and $y = 9.21 + 0.0015x$, $r = 0.135$ for k_s for tension and stiffness.

two-step detachment process. Direct measure of the nucleotide content of tissue in rigor indicated the continued presence of MgADP, presumably bound to an estimated 18% of the cross-bridges. A reduction in this level of bound MgADP in tissue, by incubation in a solution containing apyrase dramatically altered the mechanical responses to photolytically released ATP. The time course of relaxation after apyrase incubation was consistent with a simple model of ATP release from caged ATP, followed by MgATP binding and cross-bridge detachment. The results implicate the presence of MgADP bound cross-bridges in rigor tissue as the cause of the transient changes in the rate of decline of both tension and stiffness observed during relaxation from rigor in skinned cardiac tissue in the absence of calcium ions.

Nucleotide content

Cross-bridges in muscle during rigor are thought to exist primarily in the ligand free state: AM. The data listed in Table 1, however, show that even after 15 min in rigor, levels of MgADP in skinned cardiac trabeculae were approximately 28 μM. This corresponded to about 18% of the cross-bridges in this tissue that may still have bound MgADP, assuming the concentration of myosin-S1 in this preparation was approximately 160 μM (Barsotti and Ferenczi, 1988). This MgADP was readily exchanged with nonradioactive ATP because 2 mins after the exchange of rigor solution with relaxing solution containing 5 mM nonradioactive MgATP, the concentration of labeled MgADP fell to 5.7 μM (see data listed as Relax in Table 1). The rapid release of radiolabeled MgADP was consistent with the hypothesis that nucleotide binding was on cross-bridges instead of a nonspecific, slowly exchanging site elsewhere in the tissue. This premise was supported by the results showing the significant alteration in the relaxation response of the tissue following apyrase incubation (see Fig. 5 and compare traces *a* and *c* in Fig. 4). Thus, the MgADP binding site was either the nucleotide binding site on the cross-bridge or some unidentified allosteric site, capable of affecting cross-bridge behavior.

The presence of MgADP in tissue in rigor was unexpected. In rigor, cross-bridges are thought to relieve the inhibition imposed by the thin filament regulatory system in the absence of calcium. They do this by binding with a high affinity to the actin filament, exposing actin binding sites for other cross-bridges not in the rigor state (Bremel et al., 1973). Thus the ATPase activity of these bound cross-bridges is potentiated by actin binding, resulting in a lower affinity for both Pi and MgADP. One explanation for the retention of MgADP within rigor tissue is that the MgADP released from one cross-bridge exchanged among adjacent cross-bridge binding sites. This would reduce the diffusion of MgADP out of the fiber, despite the large concentration gradient for MgADP favoring the diffusion of the molecule out of the tissue. The results from Table 1, showing that apyrase incubation decreased the MgADP content of rigor tissue, were consistent with the concept that MgADP was not irreversibly bound to a particular site, but exchanged either with the same site or among different sites.

Mechanics during relaxation from rigor

Typical mechanical responses of skinned cardiac muscle in rigor to the photolytic production of ATP from caged ATP in the absence of calcium ions are shown in Figs. 1, 2, trace *a* of Fig. 4, and Fig. 5. The level of tension occasionally dropped after the release of ATP but more often remained

constant or increased above the rigor level for 20–100 ms before falling to the relaxed level. Although a precise description of the cross-bridge states responsible for the tension transient is not yet established, the mechanical characteristics of the tissue during the latter phase of the tension plateau are consistent with the premise that this phase of the response is caused by cross-bridges that are reattaching and producing force (Goldman et al., 1984a). The in-phase component of stiffness decreased during the tension plateau, indicating an increase in the amount of force per attachment. The rate of decline in stiffness slowed toward the end of the plateau. Quadrature was normally positive, indicating actively cycling cross-bridges (Kawai and Brandt, 1980; Goldman et al., 1984a, b), and its peak occurred as the rate of the decline in stiffness slowed (see Figs. 1, 4, and 5). The duration of the tension plateau was sensitive to concentration of MgATP (see Fig. 2). As the concentration of ATP released after photolysis increased, the rate of ATP-induced cross-bridge dissociation also increased. This decreased the degree of suppression of the thin filament regulatory system caused by attached cross-bridges, reducing the number of reattaching cross-bridges and thereby shortening the plateau. The addition of 20 mM Pi to the caged ATP solution shortened the tension plateau and decreased the size of the transient change in the in-phase and quadrature components of stiffness. This should be expected if Pi inhibits tension production by reattaching cross-bridges via the redistribution of cross-bridge states from force producing states to detached states (Hibberd et al., 1985).

Although the tension and stiffness transients can be accounted for by the effects of reattaching cross-bridges, the lack of a decline in tension immediately after the release of ATP is more difficult to explain. A decrease would be expected as MgATP bound to rigor cross-bridges and caused detachment and a drop in tension. The fall in the in-phase component of stiffness indicates that cross-bridge detachment indeed takes place during the early phase of the plateau. Nevertheless, a decline in tension usually was not observed. A number of mechanisms could account for the lack of an accompanying drop in tension. The rate of MgADP dissociation from AM.ADP cross-bridges may be strain dependent as suggested by Dantzig et al. (1991). Such cross-bridges would be expected to release MgADP and eventually detach more rapidly than positively strained cross-bridges. This would cause a net increase in tension that could offset the decline in tension caused by the detachment of rigor cross-bridges. Alternatively, some cross-bridges with bound MgADP may be constrained in a high energy state and, upon the release of ATP, continue through their normal ATPase cycle, generating force before releasing MgADP and detaching. Both of these mechanisms, however, would result in a rapid decline in stiffness because any ligand-free or rigor cross-bridges should detach rapidly upon MgATP binding. As evidenced by the slow change in stiffness, it appears instead that cross-bridges in untreated tissue detach relatively slowly, compared to conditions when MgADP levels were reduced by apyrase (compare stiffness traces *a*, *b*, and *c* in Fig. 5). Additionally, although Pi significantly shortened the plateau, it had little effect on the early rate of the stiffness decrease (compare stiffness traces *a* and *b* in Fig. 4). These data suggest that the presence of AM.ADP cross-bridges causes both the tension plateau and the relatively slow initial rate of stiffness decline. Thus, some form of cooperation among cross-bridges might be present. In that case, MgADP binding to one head of the myosin molecule may slow the rate of ATP-induced detachment of the other head and/or promote force production by the other head while it is in the AM.ATP state.

The suggestion that MgADP bound cross-bridges are responsible for the tension plateau and the relatively slow cross-bridge detachment is supported by the results showing that a reduction in the MgADP content of cardiac trabeculae in rigor is accompanied by dramatic changes in the contractile behavior of the tissue during relaxation from rigor induced by the photolytic release of caged ATP. As illustrated in Figs. 4 and Fig. 5, the plateau of tension was abolished immediately after the laser pulse, as were the transient changes in the time course of the decline in stiffness (compare trace *a* and trace *c* in Figs. 4 and 5). The time course of relaxation in apyrase-treated tissue was biphasic, with an initial rapid phase followed by a slower phase. The responses were easily fit by a double exponential equation as shown in Fig. 6. In addition, the early phases of the response were well fit by a computer simulation of the reactions shown in Scheme 1. Thus, after MgADP depletion, the time course of relaxation from rigor is exponential. This is expected if ATP-induced cross-bridge detachment occurred by the reaction sequence illustrated in Scheme 1.

Additionally, the lack of any transient changes in the relaxation time course after the release of low concentrations of MgATP (~200 μM)—conditions that should promote rebinding—indicates that the cross-bridge state primarily responsible for relieving thin filament inhibition is the MgADP bound state. Thus, the results from the present experiments shift the responsibility for allowing cross-bridge rebinding from rigor or AM to ADP containing states: M.ADP or AM.ADP. These states, like rigor complexes, have been reported by Weber and co-workers (1972, 1973) to increase actin-activated ATPase activity of myosin-S1 in the absence of calcium. Other studies monitored the effects of various cross-bridge states on the conformation of troponin C (TnC) by replacing the endogenous form of TnC with dansylaziridine-labeled TnC. Those studies showed ligand bound cross-bridge states to have a larger effect in altering the conformation of TnC than rigor conformations (Güth and Potter, 1987; Morano and Rüegg, 1991). Either the process of cross-bridge binding to the thin filament directly promotes further cross-bridge binding or it promotes it indirectly by influencing the calcium affinity of TnC. In either case, however, if these mechanisms are fundamental in controlling the mechanical behavior of contracting muscle, they should be caused by cross-bridge states other than rigor. With concentrations of MgATP around 5 mM in most muscle cells, coupled with the rapid rate of ATP binding and cross-bridge

detachment, only a very small population of cross-bridges would exist in the rigor conformation during normal muscle contraction.

The complex time course of relaxation from rigor in skinned cardiac muscle was similar to that reported for every type of muscle studied by laser photolysis of caged ATP. The relaxation responses of various muscle types can be separated into two broad categories: fast muscle and slow muscle. The fast muscle types include rabbit *psoas* (Goldman et al., 1984a), insect flight muscle (Rapp et al., 1986; Yamakawa and Goldman, 1991), and fast fiber types from the outer region of the *iliofibularis* muscle of *Xenopus laevis* (Stienen and Ferenczi, 1991). These muscles show an initial decrease in tension after the release of ATP in the absence of calcium. But more importantly, they also show a relatively small transient rise in tension before declining to the relaxed level. Muscle fibers from slow muscle types—cardiac, rabbit soleus (Poole et al., 1988), slow fiber types from the *iliofibularis* (Stienen and Ferenczi, 1991), and smooth (Arner et al., 1987; Somlyo et al., 1989)—exhibit a relatively long tension plateau after ATP release. The complex time course for relaxation is thought to be caused by a population of rigor cross-bridges that in the absence of calcium allows cross-bridge reattachment and force production. Cross-bridge reattachment occurs presumably because rigor cross-bridges suppress the inhibition of the thin filament system normally present in the absence of calcium ions (Bremel and Weber, 1972). Although the overall rate of relaxation from rigor induced by the photolytic release of ATP varies approximately 100-fold from fast to slow muscle, the rate of ATP dissociation of actomyosin-S1—the cross-bridge transition presumably responsible for controlling the transients—shows only a 2–3 fold difference (Marston and Taylor, 1980). In turn, the rate of MgADP dissociation from actomyosin, one of the steps in the actomyosin ATPase cycle that exhibits the largest difference between fast and slow types of myosin, varies almost 40-fold (Siemankowski et al., 1985). Thus, it seems more likely that the differences in the time courses of relaxation from rigor between fast and slow muscle arise not from rigor cross-bridges, but by ligand bound cross-bridges. Slow muscle would tend to retain a relatively higher proportion of ligand bound cross-bridges in rigor. In turn, their presence would act to slow the rate of relaxation from rigor in at least two ways (Dantzig et al., 1991): MgADP must first dissociate from the cross-bridge (AM.ADP) before MgATP can bind and induce detachment; and by detaching slowly, MgADP-bound cross-bridges also permit more rigor cross-bridges to detach from the rigor conformation and then reattach in active force-producing conformations. Accordingly, the slower release of MgADP from cross-bridges in slow muscle would tend to exacerbate the tension transients. It is not clear whether the retention of a population of ligand bound cross-bridges is also responsible for the tension transient observed in fast skeletal muscle (Goldman et al., 1984a; Stienen and Ferenczi, 1991). Preliminary studies, however, using apyrase treatment of rabbit *psoas* fibers in rigor, have reported a reduction in the duration of the tension plateau and an increase in the rate of relaxation, similar to the effects shown in this study on cardiac muscle (Thirlwell et al., 1993).

Estimates of the second-order rate constant for ATP-induced detachment in untreated cardiac tissue based either on the overall half-time of the relaxation responses or on the rate of convergence of tension traces (Goldman et al., 1984a) (see Fig. 3) were three orders of magnitude slower than that reported from in vitro studies on cardiac actomyosin-S1 (Taylor and Weeds, 1976): 3.6×10^3 and 3.9×10^3 M^{-1} s^{-1} vs. 1.4×10^6 M^{-1} s^{-1}, respectively. This suggests that the presence of cross-bridges with bound MgADP during rigor acts to slow or delay the convergence, which is similar to the previous reports on the effects of MgADP on relaxation from rigor of skeletal muscle fibers (Dantzig et al., 1991). These effects could be explained if AM.ADP cross-bridges increase the tension produced upon reattachment of the adjacent head, thus delaying the convergence of the tension records.

As shown, pretreatment of the tissue during rigor with apyrase substantially reduced the complex mechanical transients during relaxation from rigor. The decay in rigor tension after the release of ATP in tissue treated with apyrase was consistent with that expected from the reaction scheme described earlier. The time course was biphasic: it consisted of an initial rapid phase, which represented 71% of the total decrease in the tension, followed by a slower phase. The rate of the decline in rigor tension during the slow phase, k_s, was not sensitive to the concentration of ATP and was probably determined by the viscoelastic properties of cardiac tissue (Chiu et al., 1982; de Tombe and ter Keurs, 1992) or to some amount of force in the AM.ATP cross-bridge state. The initial rapid phase, or k_f, was dependent on the ATP concentration (see *solid square symbols* in Fig. 8, panel A). The slope of the relation was 3.9×10^4 M^{-1} s^{-1}, a value one order of magnitude faster than in untreated tissue but still two orders of magnitude slower than that reported for bovine cardiac actomyosin-S1 (Taylor and Weeds, 1976). As shown, this relatively slow rate of ATP-induced detachment was not due to some small degree of cross-bridge rebinding that occurred in apyrase treated tissue—either from AM.ADP cross-bridges that remain or from rigor cross-bridges. We minimized cross-bridge reattachment in apyrase-treated tissue in two ways. First, we measured the rate of convergence of tension records and, in separate series of experiments, added 20 mM BDM to the caged ATP solution. BDM has been shown to inhibit tension production in skinned cardiac muscle (West and Stephenson, 1989). The mechanism is thought to operate via a reduction in the apparent rate constant for the formation of force generating cross-bridges (Lenart et al., 1989). Second, we used the rate of convergence of tension tracings as a measure of the detachment rate. The similarity in the estimates of the apparent second-order rate constant for ATP-induced rigor detachment determined by these two approaches suggests that little, if any, cross-bridge rebinding occurs during relaxation from rigor after the photolytic release of ATP in apyrase treated skinned cardiac muscle. Thus, we conclude that the assembly of the contractile proteins in the lattice present within cardiac cells

provides some form of steric constraints that limits either the accessibility of the ATP binding site on myosin or the rate of rigor cross-bridge detachment that follows ATP binding.

The authors gratefully acknowledge Professor Yale E. Goldman and Dr. Jody A. Dantzig for many thoughtful and stimulating discussions, Mr. Marcus Bell for expert electronics design and construction, Mr. James Smith for software support, Mr. Joseph Pili for the masterful construction of the mechanical apparatus, and Mr. Ray Iacobacci for spirited technical support. This work is supported by grant HL40953 from the National Institutes of Health.

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