# Activation of Skinned Trabeculae of the Guinea Pig Induced by Laser Photolysis of Caged ATP

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ABSTRACT The kinetics of force production in chemically skinned trabeculae from the guinea pig were studied by laser photolysis of caged ATP in the presence of $Ca^{2+}$. Preincubation of the tissue during rigor with the enzyme apyrase was used to reduce the population of MgADP-bound cross-bridges (Martin and Barsotti, 1994). In untreated tissue, tension remained constant or dipped slightly below the rigor level immediately after ATP release, before increasing to the maximum measured in pCa 4.5 and 5 mM MgATP. The in-phase component stiffness, which is a measure of cross-bridge attachment, exhibited a large decrease before increasing to 55% of that measured in rigor. Neither the rate of the decline nor of the rise in tension was sensitive to the concentration of photolytically released ATP. The rate of the decline in stiffness was found to be dependent on [ATP]: $1.8 \times 10^4\ M^{-1}/s^{-1}$, a value more than four times higher than that previously measured in similar experiments in the absence of $Ca^{2+}$. The rate of tension development averaged $14.9 \pm 2.5\ s^{-1}$. Preincubation with apyrase altered the mechanical characteristics of the early phase of the contraction. The rate and amplitude of the initial drop in both tension and stiffness after caged ATP photolysis increased and became dependent on [ATP]. The second-order rate constants measured for the initial drop in tension and stiffness were $8.4 \times 10^4\ M^{-1}\ s^{-1}$ and $1.5 \times 10^5\ M^{-1}\ s^{-1}$. These rates are more than two times faster than those previously measured in the absence of $Ca^{2+}$. The effects of apyrase incubation on the time course of tension and stiffness were consistent with the hypothesis that during rigor, skinned trabeculae retain a significant population of MgADP-bound cross-bridges. These in turn act to attenuate the initial drop in tension after caged ATP photolysis and slow the apparent rate of rigor cross-bridge detachment. The results also show that $Ca^{2+}$ increases the rate of cross-bridge detachment in both untreated and apyrase-treated tissue, but the effect is larger in untreated tissue. This suggests that in cardiac muscle $Ca^{2+}$ modulates the rate of cross-bridge detachment.

## INTRODUCTION

A detailed understanding of the energy transduction process in cardiac muscle is essential to explain the changes in mechanical performance that occur during both normal and pathophysiological states of the heart. To understand the transduction process we must characterize the steps in the cross-bridge cycle that control key points in the pathway, particularly those responsible for the rate of relaxation, force production, and maximum shortening velocity. This requires the direct study of cardiac muscle instead of relying on simply inferring such information from studies on skeletal muscle, because it is likely that differences exist between these two types of muscle. To date, reports from a number of studies are available describing the characteristics of some of these steps in fast skeletal muscle fibers (for review see Millar and Homsher, 1990). Far fewer, however, are available concerning cardiac muscle.

This study extended the previous work on the kinetics of relaxation from rigor of skinned cardiac muscle, induced by laser photolysis of caged ATP in the absence of $Ca^{2+}$ (Martin and Barsotti, 1994). In the prior experiments, the time course of tension exhibited a plateau or a transient rise immediately after ATP release in the absence of $Ca^{2+}$ before declining to the relaxed level. The in-phase component of stiffness declined during this tension plateau, but the decrease in both tension and stiffness was more than two orders of magnitude slower than that reported for the dissociation of cardiac actomyosin (Taylor and Weeds, 1976; Marston and Taylor, 1980). Likewise, the time course of the changes in tension and stiffness were inconsistent with a relatively simple process of ATP-cross-bridge binding followed by detachment. The tension transient during relaxation from rigor, however, was characteristic of all muscle types yet studied using this technique. In general, tension transients after ATP release in the absence of $Ca^{2+}$ are most pronounced in slow skeletal muscle (Poole et al., 1988; Steinen and Ferenczi, 1991) and in cardiac. Direct chemical measurement of nucleotide content of skinned cardiac tissue in rigor showed that after 15 min, nearly 20% of the cross-bridges retain bound MgADP. Reduction in this population of ligand-bound cross-bridges by preincubation of the tissue during rigor with apyrase, an enzyme with ADPase and ATPase activity, was associated with elimination of the plateau, and an increase in the rate of decline in both tension and stiffness. The results suggested that MgADP-bound cross-bridges were responsible for relieving the inhibition normally imposed by the thin-filament regulatory system, thereby allowing transient cross-bridge binding and force production. The higher affinity of slow muscle myosin for MgADP and thus the relatively higher retention of this ligand during rigor could explain the larger and longer-lasting tension transient normally observed in slow muscle, including cardiac, when compared with fast muscle such as rabbit psoas (Goldman et al., 1984a), and the

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outer fibers of the iliofibularis muscle of *Xenopus laevis* (Stienen and Ferenczi, 1991).

The goal of this study was to answer two questions: 1) what effect does MgADP, normally present in skinned cardiac muscle in rigor, have on the time course of tension and the changes in stiffness during activation from rigor; and 2) does $Ca^{2+}$ affect the rate of rigor cross-bridge detachment induced by the photolysis of caged ATP? The ATP binding and cross-bridge detachment steps in the cycle are probably too rapid to regulate significantly the rate of relaxation, force production, or maximum shortening velocity. Nevertheless, any change in the rate of these steps with $Ca^{2+}$ would indicate that the control of cardiac muscle contraction by $Ca^{2+}$ and the thin-filament regulatory system is too sophisticated to be explained solely by a steric blocking model in which the regulatory system controls only the number of available binding sites on the thin filament.

## MATERIALS AND METHODS

### Tissue preparation and solutions

Chemically skinned cardiac trabeculae <250 $\mu$m in diameter were prepared from male Dunkin-Hartley guinea pigs (350–450 g) as described in Smith and Barsotti (1993).

Unless otherwise indicated all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity and grade available. All experimental solutions were prepared based on calculations using a computer program that employed the association constants for the various constituents from the literature. Unless otherwise stated, all solutions contained 100 mM TES(*N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid) buffered at pH 7.1 and 21°C, 5 mM MgATP, 1 mM free $Mg^{2+}$, and the ionic strength was adjusted to 200 mM using 1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (Aldrich Chemical Co., Milwaukee, WI). Relaxing and activating solutions contained 30 mM $Ca^{2+}$-EGTA (pCa 4.5) and EGTA (pCa >8). Tissue treatment with apyrase was carried out as described in Martin and Barsotti (1994). 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 preactivating solution was used to lower the EGTA concentration to 0.1 mM. The concentration of caged ATP used in the photolysis experiments was 10 mM with 20 mM glutathione added to bind 2-nitroacetophenone that is released upon photolysis of the caged compound.

Apyrase was used to eliminate low levels of contaminating MgADP and MgATP from caged ATP solutions. On the day of the experiment, the caged ATP solutions were incubated with 17.6 U/ml for 1 h, similar to the method described in Sleep and Burton (1990). Before using the solution, the apyrase was removed by centrifugal filtration through an Ultra Free-MC, 10-kDa NMWL Millipore filter (Bedford, MA) (see Martin and Barsotti, 1994).

### Apparatus

T-shaped aluminum foil clips were crimped around the ends of the skinned trabeculae and were mounted in the apparatus described in Martin and Barsotti (1994). The tissue was stretched 1.13 times above slack, resulting in an average sarcomere length of 2.16 ± 0.02 mm (mean ± SE, $n$ = 32). The transducer was a semiconductor element of a silicon strain gauge (Akers 801, SensoNor, Norway). The resonance frequency with connections was 4 kHz. Small (<0.5%) sinusoidal length changes of 1 kHz 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 piezoelectric device. The lock-in amplifier performed real-time demodulation of the oscillation in the tension signal into components in-phase and 90° out-of-phase with the imposed length oscillation. A PC-based program that emulates a lock-in amplifier was used to demodulate the tension recordings in some experiments instead of the lock-in amplifier. The in-phase component of the tension oscillation is an estimate of the number of attached cross-bridges. A positive change in the 90° out-of-phase component or quadrature is primarily due to changes in the viscoelastic properties of the fiber and is thought to indicate the presence of actively cycling cross-bridges (Kawai and Brandt, 1980; Goldman et al., 1984a, b).

### Synthesis and 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.

Caged ATP was photolyzed by a 50-ns pulse of 347 nm light produced using a frequency-doubled Q-switched ruby laser (Laser Applications, Winter Park, FL). The beam was focused on the tissue by a cylindrical lens with a portion of the beam masked by an adjustable slit placed between the lens and the specimen trough. This prevented the beam from striking the hooks extending from the transducer and piezoelectric device. A single laser pulse of 100 mJ typically photolyzed 10% of 10 mM caged ATP. The rate of release of ATP was assumed to be 100 $s^{-1}$, as reported in Goldman et al. (1984a). 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 high-performance liquid chromatography using a paired ion method on a C18 column as described in Martin and Barsotti (1994).

### Data analysis

Tension records of trabeculae not treated with apyrase were fit to the sum of two exponential terms. These fits were started at the onset of a net increase in the level of tension. Tension records from trabeculae treated with apyrase were fit to the simple kinetic scheme described in the Results section using the Levenberg-Marquardt routine of nonlinear least squares (Press et al., 1988). Where used, regression lines were calculated by the method of least squares. Data are expressed as means ± standard error of the mean (SE).

## RESULTS

Fig. 1 *A* illustrates a typical response of skinned trabeculae to the photolytic release of ATP from caged ATP in the presence of calcium ions (pCa 4.5). The level of tension immediately after the laser pulse usually dipped slightly (top trace, Fig. 1 *A*), but in some tissue remained relatively constant (not shown) before rising to the level measured in pCa 4.5. The in-phase component of stiffness (middle trace), a measure of the cross-bridge attachment, declined immediately after the pulse and then rose slightly to 55 ± 6% ($n$ = 23) of that measured in rigor. The minimum in the decline in the in-phase component of stiffness occurred during the early rising phase of tension, indicating that net cross-bridge detachment continued during this phase. The smaller decrease in tension relative to that of stiffness immediately after the laser pulse suggests that net cross-bridge detachment occurs during this phase of the response and results in an increase in the amount of force per attachment. Quadrature, the 90° out-of-phase stiffness, (bottom trace) increased 10 ms after the laser pulse. This positive-phase lead of tension over length has been shown to be caused by the presence of

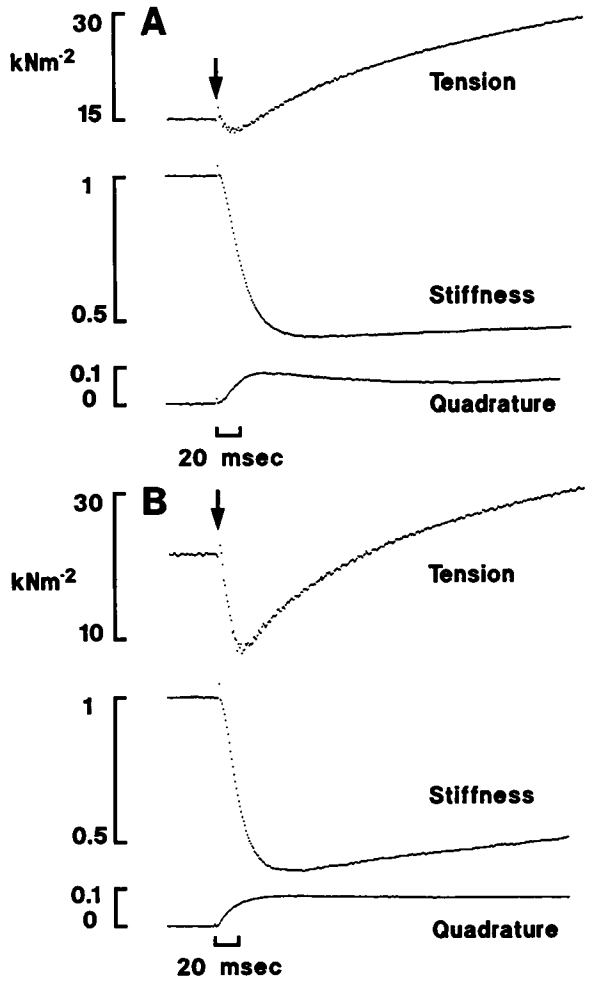


FIGURE 1 Examples of mechanical transients induced by photolysis of caged ATP in skinned trabeculae in the presence of calcium (pCa 4.5). Laser was triggered at the arrow releasing ~1 mM ATP. Each panel shows tension, stiffness, and quadrature from the same trabecula untreated (*A*) and incubated with apyrase (*B*). Final concentrations of ATP shown in (*A*) and (*B*) were 1204 μM and 927 μM, respectively. Fiber dimensions: 800 μm × 192 μm (length × diameter), sarcomere length: 2.18 μm.

actively cycling cross-bridges (Kawai and Brandt, 1980; Goldman et al., 1984a, b). The increase in quadrature normally was maintained throughout the contraction. We previously reported that in the absence of $Ca^{2+}$, the increase in the quadrature signal was transient (Martin and Barsotti, 1994).

The amplitude of the initial decline in tension varied from one trabecula to another and was not dependent upon the concentration of ATP produced. The rate of force development after the initial dip or delay was also not dependent on the concentration of ATP. This rate was measured by fitting the tension responses to a single exponential expression beginning from the onset of the rise in tension. The rate of tension development averaged 14.9 ± 2.5 $s^{-1}$ ($n$ = 23). The time course of the change in the in-phase component of stiffness was also fit to a single exponential expression to estimate the rate of cross-bridge detachment. The rate of decline in stiffness was sensitive to the ATP concentration. The slope of a plot of the rate of change in stiffness as a function of the concentration of ATP photolytically produced provides an estimate of the second-order rate constant for rigor cross-bridge detachment in the presence of $Ca^{2+}$, $1.8 \times 10^4$ $M^{-1}$ $s^{-1}$. (The line is described by $y = 21.4 + 1.82 \times 10^4 x$, $r^2 = 0.45$, 24 determinations on 11 trabeculae; data not shown). This rate is more than four times that reported previously from identical experiments in the absence of $Ca^{2+}$ (Martin and Barsotti, 1994), in which the rate of decline in stiffness was estimated from the half-time of the response. These results suggest that either calcium ions directly affected the rate of rigor cross-bridge detachment in cardiac muscle or they affected the rate by promoting MgADP release from the cross-bridges, which in turn increased the apparent rate of detachment.

The size of the drop in tension immediately after the laser pulse was smaller than expected. The detachment of rigor cross-bridges caused by ATP binding should result in a pronounced fall in tension from the rigor level, as was found in similar studies by Goldman et al. (1984b) on skinned rabbit psoas fibers. We previously reported, however, that during rigor skinned trabeculae retain ~20% of the cross-bridges with MgADP-bound (Martin and Barsotti, 1994). Dantzig et al. (1991) reported that the presence of MgADP attenuated the amplitude of the transient fall in rigor tension in psoas fibers after the photolytic release of ATP in the presence of $Ca^{2+}$. We tested whether a reduction in the amount of MgADP in trabeculae during rigor would alter the time course of the mechanical responses to ATP release in the presence of $Ca^{2+}$ by treating the tissue with the enzyme apyrase (Martin and Barsotti, 1994).

Fig. 1 shows the time course of tension (top trace), in-phase stiffness (middle trace) and quadrature (bottom trace) after the photolytic production of ATP in the presence of calcium ions in a trabecula untreated (*A*) and another pre-incubated with apyrase (*B*). Treatment with apyrase significantly increased the size of the initial tension decline, presumably by reducing MgADP (Martin and Barsotti, 1994). The rate of the decline in the in-phase component of stiffness increased after apyrase treatment. The ATP dependence of the rate in the decline in stiffness was approximately eight times that measured in trabeculae before apyrase incubation: $1.5 \times 10^5$ versus $1.8 \times 10^4$ $M^{-1}$ $s^{-1}$. The time course of the change in stiffness exhibited a sharper transition from the falling phase to the rising phase in apyrase tissue. The level of stiffness when peak force was reached, however, was not significantly different. Apyrase-treated tissue would go into rigor 4–5 s after the laser pulse. This resulted in a further slow but relatively small increase in tension while the in-phase stiffness would reach the rigor level. Neither the delay before the increase in quadrature nor the rate of the change in quadrature was significantly altered by apyrase incubation.

The dependence of the mechanical transients on ATP was examined by altering the concentration of ATP released upon photolysis from 300 mM to 2 mM in presence of $Ca^{2+}$ (pCa 4.5). This was done by holding the concentration of caged ATP at 10 mM and varying the laser energy. Figs. 2 and 3

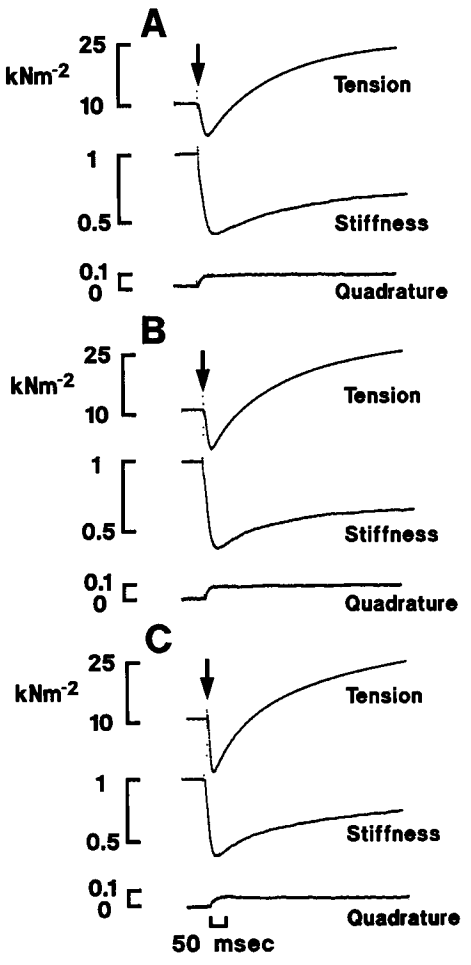


FIGURE 2 The effect of altering the ATP concentration in the presence of $Ca^{2+}$ on the time course of tension development, the change in the in-phase (stiffness) and quadrature. The arrows indicate when the laser was triggered. The final ATP concentrations were 332, 976, and 1801 $\mu$M shown in (*A*), (*B*), and (*C*), respectively. Fiber dimensions: 930 $\mu$M × 184 $\mu$m (length × diameter), sarcomere length: 2.20 $\mu$m.

show the mechanical responses of two trabeculae, treated with apyrase, to the photolytic release of different concentrations of ATP in the presence of $Ca^{2+}$. As the concentration of ATP released increased, the amplitude of the initial decline in tension increased, and the minimum tension occurred earlier. The amplitude of drop in the in-phase component of stiffness was not as sensitive to the ATP concentration as that of tension, whereas the dependence of the rate of the decline in stiffness on ATP concentration was similar to that of tension (see Fig. 2).

The mechanical transients are consistent with a simple model where the initial decline in tension is caused by the detachment of rigor cross-bridges followed by a transition to an active, force-producing conformation. The sequence is illustrated by Scheme 1 below. In this simplified model, the detached states produce no force and the active state represents all the hypothesized cross-bridge states that produce force.

$$\text{Rigor} \xrightarrow{k_d} \text{Detached} \xrightarrow{k_r} \text{Active} \qquad \text{Scheme 1}$$

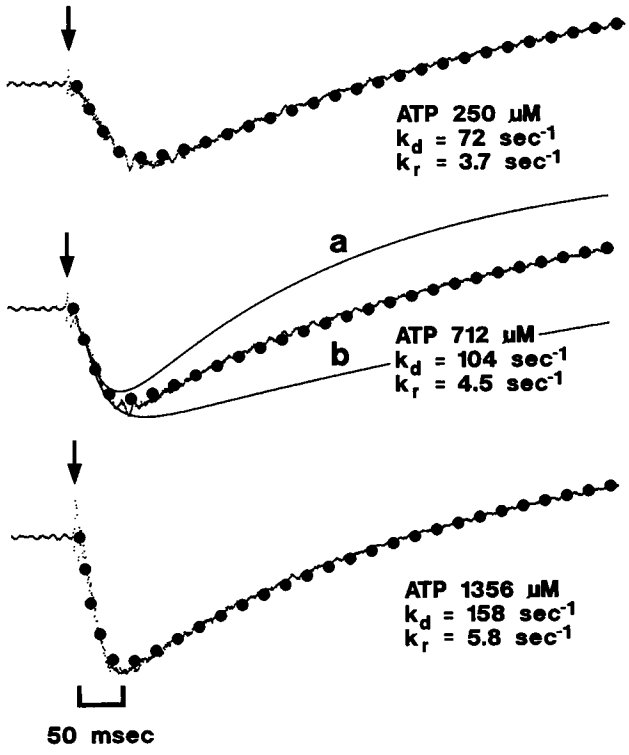


FIGURE 3 Computer-generated fits of Eq. 1 to tension records from an apyrase-treated trabecula activated from rigor by the photolytic release of different concentrations of ATP in the presence of $Ca^{2+}$. The large dots represents the fit to the tension records. The values for $k_d$ and $k_r$ from each fit are shown below each trace along with the concentration of ATP produced upon photolysis. The traces labeled *a* and *b* in the middle panel illustrate the dependence of the fit on an assigned value of $k_r$: 9 $s^{-1}$ (*a*) and 2.2 $s^{-1}$ (*b*). Fiber dimensions: 520 $\mu$m × 70 $\mu$m (length × diameter), sarcomere length: 2.22 $\mu$m.

By assuming that cross-bridge detachment and force production are first-order processes, the tension expected after the release of ATP is given by the following relation:

$$\text{Relative Force} = f e^{-k_d t} + 1 - \left(\frac{k_r}{k_r - k_d}\right) e^{-k_d t} - \left(\frac{k_d}{k_d - k_r}\right) e^{-k_r t} \qquad (1)$$

The value $f$ represents the fraction of active to rigor force and is measured from the strip chart recordings of tension. $k_d$ and $k_r$ represent the rate of cross-bridge detachment and force production. Distinct values for $k_d$ and $k_r$ were obtained by fitting the above expression to the tension recordings. This is demonstrated in Fig. 3, which shows tension recordings from a single trabecula after the release of 0.25, 0.71, and 1.36 mM ATP. The dots superimposed on the records represent the fit to the tension data, using the above expression. A delay of a few ms in the decline in tension immediately after the laser pulse was expected. This was caused by the release of ATP from caged ATP at 100 $s^{-1}$. The delay was usually lost, however, in the mechanical noise caused by the laser pulse. Although the $k_d$ varied more than twofold in the records shown in Fig. 3, the tension records nevertheless were fit well by Eq. 1. The uniqueness of the determination of $k_r$ is illustrated by traces *a* and *b* superimposed on

the middle record in Fig. 3. $k_r$ is set a factor of 2 above (trace *a*, 9 $s^{-1}$) and below (trace *b*, 2.2 $s^{-1}$) the rate determined by the fitting routine (4.5 $s^{-1}$).

The rate of the initial decline in tension, $k_d$, increased as the concentration of photolytically produced ATP increased. A plot of $k_d$ versus ATP released is shown in Fig. 4. A line fit to the data by the method of least squares has a slope of $8.4 \times 10^4$ $M^{-1}$ $s^{-1}$ and is a measure of the second-order rate constant for ATP-induced detachment of rigor cross-bridges in the presence of $Ca^{2+}$. A similar rate of $1.5 \times 10^5$ $M^{-1}$ $s^{-1}$ was measured for the ATP dependence of the rate of the decline in stiffness. These values are more than twice the respective rate previously reported in the absence of calcium ions (Martin and Barsotti, 1994). Thus, in apyrase-treated skinned cardiac muscle, in which the number of ligand-bound cross-bridges is reduced, calcium ions affect the kinetics of rigor cross-bridge detachment. The magnitude of this effect of calcium ions on rigor cross-bridge detachment in apyrase-incubated tissue was smaller than the fourfold effect of $Ca^{2+}$ on detachment measured in untreated tissue.

As illustrated by the data in Fig. 3, the rate of force development, $k_r$, tended to increase with increasing concentrations of ATP released, but the dependence was small. No significant relation between $k_r$ and the ATP concentration was found if the data from all the trabeculae were plotted as individual points (see Fig. 4, □). If, instead, the $k_r$ data from each trabecula were normalized to the maximum rate measured for that trabecula after the release of ~2 mM ATP, then the trend is discernible. This is shown in Fig. 5, in which the data from only five trabeculae are shown for clarity. The rate of tension development that was estimated by averaging the data from all trabeculae incubated with apyrase and after the release of up to 2 mM ATP was 9.3 ± 1.4 $s^{-1}$ ($n$ = 15).

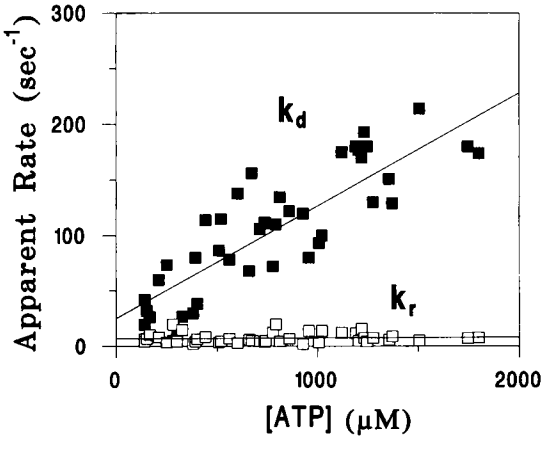


FIGURE 4 Dependence of cross-bridge detachment and force development on the ATP concentration. The detachment rate, $k_d$, and force development, $k_r$, were estimated by fitting Eq. 1 to the tension records. The solid lines are linear least-squares fit to the data (43 determinations from 16 trabeculae) and are described by the following equations: $y = 40.06 + 0.00844x$, $r^2 = 0.58$; and $y = 8.80 + 0.00041x$, $r^2 = 0.001$, for $k_d$ and $k_r$, respectively.

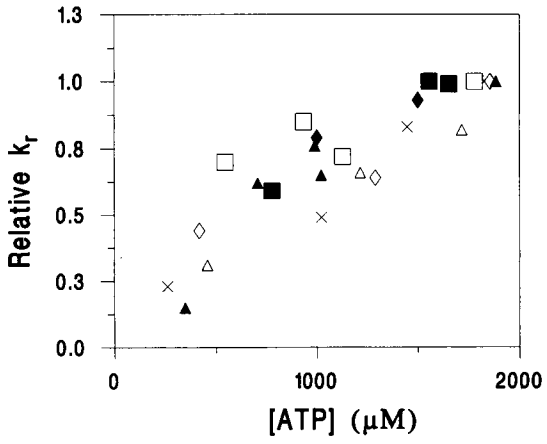


FIGURE 5 The dependence of $k_r$ on the ATP concentration. Some of the data from Fig. 4 normalized to the maximum value of $k_r$ for the individual trabecula. Each type of symbol represents a single trabecula. Data from only five trabeculae are plotted.

## DISCUSSION

The results show that in skinned cardiac muscle, 1) the time course of tension development and the change in the in-phase component of stiffness induced by laser photolysis of caged ATP in the presence of $Ca^{2+}$ were affected markedly by pre-incubation with the enzyme apyrase; and 2) $Ca^{2+}$ affected the kinetics of rigor cross-bridge detachment in both untreated and apyrase-treated tissue.

### Effects of incubation with apyrase on mechanical transients

The time course of tension development of skinned trabeculae did not exhibit an initial drop from the rigor level before rising to its maximal level (see Fig. 1, *A*) as would be anticipated if the cross-bridge reaction pathway followed that shown in Scheme 1. Accordingly, the rate and amplitude of the decline in both tension and the in-phase component of stiffness should be dependent on the ATP concentration as rigor cross-bridges bind ATP and detach before entering force-producing states. Only the rate of decline in the in-phase component of stiffness was found to be dependent upon the ATP concentration.

The lack of immediate drop in tension after ATP release was consistent with earlier studies of skinned cardiac muscle. Those studies showed that in the absence of $Ca^{2+}$ (pCa > 8.0) rigor tension remained either constant or frequently increased after caged ATP photolysis before declining to the relaxed level (Barsotti and Ferenczi, 1988). In a more recent study, it was shown that the in-phase component of stiffness declined continually throughout the relaxation response, albeit at different rates. The drop in stiffness observed during the tension plateau indicated that although tension remained constant at the rigor level or occasionally increased, net cross-bridge detachment occurred during this phase of the relaxation response from rigor (Martin and Barsotti, 1994).

Thus, in the present experiments, if $Ca^{2+}$ did not significantly alter the rates of the steps in the cross-bridge cycle controlling relaxation from rigor, it would be expected that little or no change in tension would be observed directly after ATP release. In turn, a large decrease in stiffness would be expected before both tension and stiffness reached levels normally observed in 5 mM MgATP and pCa 4.5.

It was previously reported that during rigor ~20% of the cross-bridges in skinned cardiac muscle were bound with MgADP (Martin and Barsotti, 1994). Incubation with apyrase reduced this population of AM.ADP or M.ADP cross-bridges to near undetectable levels. This reduction was associated with the elimination of the plateau in tension and a 10-fold increase in the rate of relaxation from rigor, induced by the photolysis of caged ATP in the absence of $Ca^{2+}$. That study concluded that in cardiac muscle, ligand-bound cross-bridge states were responsible for maintaining force near the rigor level and for slowing the rate of relaxation from rigor after ATP release. In the present study, incubation of the tissue with apyrase resulted in comparable changes in the time course of tension development and the change in stiffness. Both the amplitude of the initial drop in tension from the rigor level immediately after caged ATP photolysis, and the rate of decrease in tension and stiffness before the onset of tension production increased. These results indicate that in the presence of $Ca^{2+}$, ligand-bound cross-bridges affect the time course of tension development and the change in stiffness after the photolytic release of ATP.

Other observations were consistent with the existence of a population of the ligand-bound cross-bridges. First, the drop in stiffness after the laser pulse was smaller in untreated tissue. Second, the apex of the stiffness minimum was broader in untreated tissue (see Fig. 1). The release of MgADP from cross-bridges is thought to be strain dependent (Dantzig et al., 1991) and, therefore, during an isometric contraction the AM.ADP state is expected to be relatively long lived when compared with the rigor state, AM. The presence of AM.ADP cross-bridges and transition of these cross-bridges to the rigor state could account for both observations. The presence of MgADP cross-bridges limits the size of the stiffness drop after ATP by reducing the cross-bridge population available to bind ATP during rigor, whereas the slow transition of cross-bridges from the AM.ADP state to AM would act to broaden the stiffness minimum.

After incubation in apyrase, the changes in both tension and stiffness more closely followed those predicted by the reaction sequence shown in Scheme 1: 1) tension decreased immediately upon ATP release; 2) the amplitude of this drop in tension was dependent on the concentration of ATP; 3) the tension minimum occurred earlier; and 4) the rates of the decline in both tension and stiffness were sensitive to the ATP concentration.

## Influence of $Ca^{2+}$ on rigor cross-bridge detachment

The second goal of this study was to determine whether $Ca^{2+}$ affected the second-order rate constant for ATP-induced dissociation of rigor cross-bridges. In untreated tissue, it was not possible to estimate the detachment rate based on tension because little fall in tension occurred after ATP release. On the other hand, the time course of the change in tissue stiffness could be fit to a single-order exponential expression and with a rate that was dependent on the ATP concentration. The apparent second-order rate constant of this process was $1.8 \times 10^4\ M^{-1}\ s^{-1}$, approximately five times that measured in the absence of $Ca^{2+}$, $3.9 \times 10^3\ M^{-1}\ s^{-1}$ (Martin and Barsotti, 1994). These results show that $Ca^{2+}$ enhances the apparent rate of rigor cross-bridge detachment. One explanation for this effect is that $Ca^{2+}$ increases the rate of the transition of cross-bridges from the AM.ADP state to AM. Indeed, we previously reported that in rigor, the presence of MgADP-bound cross-bridges acts to slow the decline in tension and stiffness after the release of ATP in the absence of $Ca^{2+}$ (Martin and Barsotti, 1994). An increase in the rate of ADP dissociation by $Ca^{2+}$ would account for the observed increase in the rate of decline in stiffness because fewer MgADP-bound cross-bridges would be present to slow detachment. In apyrase-treated trabeculae, in which the population of MgADP-bound cross-bridges is reduced, $Ca^{2+}$ also increased the apparent second-order rate constant for rigor cross-bridge detachment but to a smaller extent, approximately two times. Thus, $Ca^{2+}$ may increase both MgADP release and the rate of rigor cross-bridge dissociation.

The precise mechanism for observed $Ca^{2+}$-induced increase in the rate of detachment in apyrase-treated trabeculae is not clear. The most interesting possibility is that $Ca^{2+}$ directly affects rigor cross-bridge detachment. This may be caused by either an increase in the on rate of ATP or an increase in the rate of the conformational change that is induced by ATP binding and that leads to detachment. Thus $Ca^{2+}$, either via the thin-filament regulatory system or by direct binding to myosin (Metzger and Moss, 1992) may promote a conformation of myosin in which ATP may have easier access to its binding site. Alternatively, $Ca^{2+}$ may speed the structural changes required for cross-bridge detachment that follow ATP binding. In similar studies of rabbit psoas fibers (Goldman et al., 1984b) and insect flight muscles (Yamakawa and Goldman, 1991), $Ca^{2+}$ was reported to have no effect on the rate of rigor cross-bridge detachment induced by laser photolysis of caged ATP. We have no explanation for the apparent difference between cardiac muscle and these other muscle types except to suggest that it reflects differences in myosin (Swynghedauw, 1986) or troponin-tropomyosin isoforms (Ohtsuki et al., 1986).

An alternative explanation that cannot be ruled out is that $Ca^{2+}$ does not enhance the detachment rate but instead reduces the tissue compliance, which in turn results in the observed increase in the rate of decline in both tension and stiffness. When untreated tissue was transferred from a rigor solution containing 30 mM EGTA to one containing $Ca^{2+}$ (pCa 4.5) rigor tension and stiffness increased to approximately the same extent (~20%, data not shown). A $Ca^{2+}$-dependent decrease in compliance should cause a proportionally greater increase in stiffness than tension. In addition,

there was no significant increase in tension or stiffness upon the addition of $Ca^{2+}$ in apyrase-treated tissue (results not shown). This suggests that the increase in tension and stiffness observed with the addition of $Ca^{2+}$ in untreated tissue results from additional cross-bridge attachment possibly due to the continued presence of MgADP-bound cross-bridges during rigor that remain detached or weakly attached in the absence of $Ca^{2+}$, not simply a change in compliance.

As illustrated in Fig. 1, net cross-bridge detachment as demonstrated by the drop in the in-phase component of stiffness occurs immediately after ATP release in untreated tissue with little change in tension. A decrease in stiffness without a concomitant change in tension has been reported from similar studies on other slow muscle types, vertebrate smooth muscle (Somlyo et al., 1988) and molluscan smooth muscle (Abe et al., 1989). In previous studies on fast muscle, rabbit psoas (Goldman et al., 1984b), both tension and stiffness decreased below the rigor level immediately after ATP release. The inclusion of MgADP in the caged ATP solution with $Ca^{2+}$ was reported to attenuate the early decline in tension in rabbit psoas fibers (Dantzig et al., 1991). The resulting time courses of tension and stiffness were similar to those observed in slow muscle. We have previously suggested that the larger tension transients observed in slow muscle during relaxation from rigor by laser photolysis of caged ATP in the absence of $Ca^{2+}$ were due to the relatively higher affinity of slow myosin for MgADP and the resulting higher retention of MgADP in slow muscle fibers during rigor (Martin and Barsotti, 1994).

Our results in cardiac muscle along with those of Dantzig et al. (1991) from skeletal muscle show that ligand-bound cross-bridges act to maintain tension at the rigor level immediately after ATP release in the presence and absence of $Ca^{2+}$. Although the precise mechanism is not known, Dantzig et al. (1991) modeled the interactions of cross-bridges with MgATP and MgADP and proposed two ways that MgADP may act to reduce the magnitude of the tension dip. According to one model, in the presence of MgADP, the rapid detachment of negatively strained AM.ADP cross-bridges may limit the size of the tension drop. Alternatively, the interactions of MgADP-bound cross-bridges may function to allow other attached cross-bridge states (AM.ATP and AM.ADP.Pi), modeled to bear significant force, to be in rapid equilibrium with detached cross-bridge states (M.ATP and M.ADP.Pi). In the first model, tension would be maintained by the detachment of negatively strained cross-bridges, whereas stiffness, an index of the number of attached cross-bridges, would fall rapidly. In the second case, the fall in both tension and stiffness would be expected to be slowed when AM.ADP cross-bridges are present. The latter model is consistent with the results from this study and from our earlier report showing that ADP-bound cross-bridges slow the apparent rate of rigor cross-bridge detachment induced by the photolytic production of ATP in the absence of $Ca^{2+}$ (Martin and Barsotti, 1994). The results in the present study show that although $Ca^{2+}$ increased the rate of ATP-induced rigor cross-bridge detachment in both apyrase-treated and untreated tissue, the apparent second-order rate constant was still significantly slower in untreated tissue: $1.5 \times 10^5$ $M^{-1}$ $s^{-1}$ versus $1.8 \times 10^4$ $M^{-1}$ $s^{-1}$.

As expected, the number of attached cross-bridges dropped upon the detachment of rigor cross-bridges. This decline continued even during the early rise in tension before increasing to ~55% of that measured during rigor. The continued fall in stiffness during the early rise in tension indicated that the force per attachment begins to increase during this phase, a property consistent with the formation of active force producing cross-bridge states. The lower stiffness during active contraction compared with during rigor indicated that fewer cross-bridges were attached. Although a model of the reaction scheme described above would show that nearly all of the cross-bridges are attached during an isometric contraction, the number of attached cross-bridges can be reduced by allowing detachment during the cross-bridge cycle as has been postulated by Huxley (1957) and more recently by Lombardi et al. (1992) and Piazzesi et al. (1992).

X-ray diffraction studies of intact and skinned papillary muscle have suggested that 80% of the myosin heads transfer to actin during maximal $Ca^{2+}$ activation (Matsubara et al., 1989). The apparent discrepancy with our results (55%) can be resolved, however, if not all the myosin heads detected by x-ray diffraction in the vicinity of the actin filament are bound to the thin filament. Alternatively, with a 1-kHz sinusoidal oscillation, one might only detect cross-bridges that are strongly bound to the thin filament, whereas the equatorial intensity distribution of x-rays scattered by skinned papillary muscle would report both strongly and weakly bound cross-bridges. A direct study in cardiac muscle of the relation between stiffness and the intensity of the equatorial reflections during rigor and full activation is required to fully account for this apparent discrepancy.

The rate of tension development from the minimum was ~9 and 15 $s^{-1}$, respectively, for treated and untreated tissue. Force production is thought to result from the isomerization of a low-force-producing AM.ADP.Pi state to a high-force-producing state (Dantzig et al., 1992). Because the trabeculae were activated by the photorelease of ATP in the presence of $Ca^{2+}$, the rate tension development should approximate the rate of this isomerization step, assuming that the rates of any intervening steps are more rapid. Accordingly, the apparent slowing of the rate of tension development with apyrase incubation and presumably a reduction in the number of MgADP-bound cross-bridges was unexpected because the rate of this isomerization should not be altered. In rabbit psoas fibers MgADP concentrations <100 $\mu$M had no effect on the rate of tension development when activated by laser photolysis of caged ATP, whereas higher concentrations slowed tension development (Dantzig et al., 1992). The mechanism by which MgADP bound cross-bridges increases the rate of tension development in cardiac muscle is not known but, like the maintenance of rigor tension observed after ATP release in untreated tissue discussed earlier, AM.ADP cross-bridges may also increase the rate of this force producing isomerization in other cross-bridges.

Another unexpected result was the small dependence of the rate of force development, $k_r$, on the concentration of ATP released in trabeculae incubated with apyrase: as the ATP concentration increased, $k_r$ increased (see Figs. 3 and 5). This dependence is in the opposite direction from that expected if changes in tissue end-compliance were responsible for the effect. These results suggest that rigor cross-bridges may limit the rate of force development. Although the dependence of $k_r$ on the ATP concentration is slight, this observation coupled with the apparent slowing of the rate of force development with apyrase incubation suggests that cross-bridges are not independent but that their mechanical behavior is affected by the state of other cross-bridges. This effect presumably can be mediated by cross-bridges directly or through the thin-filament regulatory system. In either case, whether incubated with apyrase or not, the rate of force development in cardiac muscle activated by photolysis of caged ATP was much slower than that reported for rabbit psoas fibers, 87 $s^{-1}$ (Goldman et al., 1984b), but faster than the rates reported for thiophosphorylated tonic, 0.1 $s^{-1}$ and phasic, 0.4 $s^{-1}$, smooth muscle (Horiuti et al., 1989). In all of these studies, the major $Ca^{2+}$ regulatory system was activated before the release of ATP. In the studies of striated muscle, experiments were carried out in pCa 4.5, whereas in the studies of smooth muscle, myosin was thiophosphorylated before ATP release. Thus, the major determinant for the rate of tension development in different muscle types is not the rate of the various steps in their respective activation pathways, but an intrinsic property of the actin-myosin interaction. This premise is further supported by the work of Marston and Taylor (1980) who studied the transient kinetics of actomyosin isolated from various muscle types. They found that the rate of formation of a complex between actin and M.ADP.Pi, a biochemical correlate of cross-bridge binding and perhaps force production, paralleled the steady-state rate of ATP hydrolysis of the muscle type.

It is also of interest to compare the rate of the rise in tension in cardiac muscle after activation by laser photolysis of caged ATP with $k_{tr}$, the rate of tension redevelopment after a period of rapid shortening and an immediate restretch. $k_{tr}$ is thought to be a measure of the rate of force development as cross-bridges change from weakly to strongly bound states, (Brenner, 1988). If the steps in the cross-bridge cycle that occur after ATP binding and that lead to force production are relatively rapid, then the rate of tension development after ATP release should be similar to $k_{tr}$. Hancock et al. (1993) reported a value of 50 $s^{-1}$ for $k_{tr}$ in intact ferret papillary muscle at 27°C, following a rapid step release in length. This estimate is approximately five times faster than $k_r$ found in this study. This difference suggests that $k_{tr}$ is a measure of a cross-bridge transition distinct from that limiting force development after ATP release. It is probable, however, that this apparent difference between $k_{tr}$ and $k_r$ is due to the differences in the animal species and temperature in the two studies, rather than representing estimates of distinct cross-bridge processes (skinning alone does not affect cross-bridge kinetics of cardiac tissue (Saeki et al., 1991)). In a preliminary study of skinned rat trabeculae, Hancock et al. (1994) found values of 7 and 16 $s^{-1}$ for $k_{tr}$ at 15°C after a release to 60% of the maximum force and restretch and a 4% step decrease in segment length, respectively, values similar to the rate of force development found in this study. More direct comparisons of $k_{tr}$ and $k_r$ on the same preparation and under identical conditions must await future experiments.

In conclusion, MgADP retained by skinned cardiac muscle during rigor markedly affected the time course of tension production and the change in tissue stiffness induced by the photolysis of caged ATP in the presence of $Ca^{2+}$. Incubation with apyrase before photolysis resulted in 1) a time course of both tension development and a change in stiffness consistent with that expected from a reaction scheme of ATP binding to rigor cross-bridges, followed by detachment and tension production; and 2) an increase in the rate of rigor cross-bridge detachment. $Ca^{2+}$ was found to increase the second-order rate constant for ATP-induced rigor cross-bridge detachment in both apyrase-treated and untreated tissue, but the effect was larger in untreated tissue. The rate of cross-bridge detachment in the intact cardiac cell with an intracellular ATP concentration of 5 mM would be nearly 500 $s^{-1}$, a rate much too rapid to limit relaxation, force development, or shortening velocity. Nevertheless, the effects of $Ca^{2+}$ on cross-bridge detachment suggest that in cardiac muscle, regulation of contraction is not mediated solely by the control of available cross-bridge binding sites on the thin filament. Other potential regulatory mechanisms include direct $Ca^{2+}$ binding to cross-bridges, thick-filament cooperativity whereby cross-bridges directly affect other cross-bridges or indirectly, being mediated via the thin-filament regulatory system.

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