

# Cross-Bridge Kinetics, Cooperativity, and Negatively Strained Cross-Bridges in Vertebrate Smooth Muscle

## *A Laser-Flash Photolysis Study*

AVRIL V. SOMLYO, YALE E. GOLDMAN, TARO FUJIMORI,  
MEREDITH BOND, DAVID R. TRENTAM, and ANDREW P. SOMLYO

From the Pennsylvania Muscle Institute and the Departments of Physiology and Pathology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104, and the National Institute for Medical Research, London, NW7 1AA, England

**ABSTRACT** The effects of laser-flash photolytic release of ATP from caged ATP [ $P^3$ -1(2-nitrophenyl)ethyladenosine-5'-triphosphate] on stiffness and tension transients were studied in permeabilized guinea pig portal vein smooth muscle. During rigor, induced by removing ATP from the relaxed or contracting muscles, stiffness was greater than in relaxed muscle, and electron microscopy showed cross-bridges attached to actin filaments at an  $\sim 45^\circ$  angle. In the absence of  $Ca^{2+}$ , liberation of ATP (0.1–1 mM) into muscles in rigor caused relaxation, with kinetics indicating cooperative reattachment of some cross-bridges. Inorganic phosphate ( $P_i$ ; 20 mM) accelerated relaxation. A rapid phase of force development, accompanied by a decline in stiffness and unaffected by 20 mM  $P_i$ , was observed upon liberation of ATP in muscles that were released by 0.5–1.0% just before the laser pulse. This force increment observed upon detachment suggests that the cross-bridges can bear a negative tension. The second-order rate constant for detachment of rigor cross-bridges by ATP, in the absence of  $Ca^{2+}$ , was estimated to be  $0.1\text{--}2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ , which indicates that this reaction is too fast to limit the rate of ATP hydrolysis during physiological contractions. In the presence of  $Ca^{2+}$ , force development occurred at a rate ( $0.4 \text{ s}^{-1}$ ) similar to that of intact, electrically stimulated tissue. The rate of force development was an order of magnitude faster in muscles that had been thiophosphorylated with ATP $\gamma$ S before the photochemical liberation of ATP, which indicates that under physiological conditions, in non-thiophosphorylated muscles, light-chain phosphorylation, rather than intrinsic properties of the actomyosin cross-bridges, limits the rate of force development. The release of micromolar ATP or CTP from caged ATP or caged CTP caused force development of up to 40% of maximal active tension in the absence of  $Ca^{2+}$ , consistent

Address reprint requests to Dr. Avril V. Somlyo, Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, B42 Anatomy-Chemistry Bldg., Philadelphia, PA 19104.

with cooperative attachment of cross-bridges. Cooperative reattachment of dephosphorylated cross-bridges may contribute to force maintenance at low energy cost and low cross-bridge cycling rates in smooth muscle.

#### INTRODUCTION

The presence of myosin filaments and cross-bridges in smooth muscle (A. P. Somlyo et al., 1973; Ashton et al., 1975; A. V. Somlyo et al., 1981) is consistent with the sliding filament mechanism of contraction mediated by cyclic attachment of myosin cross-bridges to actin filaments, which has been characterized in striated muscle (A. F. Huxley and Niedergerke, 1954; H. E. Huxley and Hanson, 1954; Hanson and Huxley, 1955; A. F. Huxley, 1957). The length-tension, force-velocity (reviewed in Murphy, 1980), and length-energy consumption (Hellstrand and Paul, 1982) curves of smooth muscle are qualitatively similar to those in skeletal muscle, where these functions have been related to the number of attached cross-bridges. Mechanical transients observed in single smooth muscle cells (Warshaw and Fay, 1983) and the kinetics of the actomyosin ATPase in solution (Marston and Taylor, 1980; Rosenfeld and Taylor, 1984) also resemble the corresponding data, ascribed to cyclic cross-bridge attachment and detachment, in skeletal muscle (A. F. Huxley and Simmons, 1971; Lymn and Taylor, 1971; Eisenberg and Hill, 1985). In fact, as noted by A. F. Huxley (1957), the first clear statement that muscle contraction is due to relative sliding of molecules rather than folding was made by Bozler (1936) in his studies of stress relaxation in smooth muscle.

Regulation of contraction, however, is substantially different in the two muscle types. Normal smooth muscle contraction is initiated when the regulatory light chains of myosin are phosphorylated by an enzyme, myosin light-chain kinase (MLCK). The latter is activated when calmodulin combines with  $\text{Ca}^{2+}$  and forms a  $\text{Ca}_4^{2+}$ -calmodulin-MLCK complex (for review, see Kamm and Stull, 1985; Hartshorne, 1987). In contrast, in vertebrate striated muscles, the thin-filament regulatory proteins troponin and tropomyosin suppress the cross-bridge cycle until  $\text{Ca}^{2+}$  binding to troponin removes the suppression (Ebashi and Endo, 1968; for review, see El-Saleh et al., 1986).

We examined in the present study the kinetics of cross-bridge attachment and detachment and the rate of force development in chemically skinned smooth muscle strips, to compare them with the kinetics of isolated smooth muscle actomyosin and to determine which steps in the regulatory pathway or in the cross-bridge cycle control the rate of contraction. In order to circumvent the kinetic limitation of diffusion into the smooth muscle strips, the light-chain kinase reaction and the cross-bridge cycle were initiated by a photochemical method. Caged ATP (Kaplan et al., 1978), a biologically inert precursor, can be photolyzed using near-ultraviolet radiation to form ATP within the muscle preparation. The tension and stiffness transients following liberation of ATP relate to chemical and mechanical reactions of the contractile apparatus on the millisecond time scale. This method has been used to characterize the cross-bridge cycle in striated muscle (Goldman et al., 1984*a, b*; Hibberd and Trentham, 1986; Goldman, 1987*a*).

Photolysis of caged ATP in skinned smooth muscle strips initiated mechanical transients that could be related to the MLCK reaction and to the cross-bridge cycle. Surprisingly, we have also found evidence of cooperative attachment of cross-bridges in the absence of  $\text{Ca}^{2+}$ . Some of our observations also indicate that, as suggested by A. F. Huxley (1957) for striated muscle, cross-bridges in smooth muscle can be negatively strained and the detachment of negatively strained cross-bridges results in positive force development. Preliminary reports of some of these results have been presented at the EMBO Symposium on Smooth Muscle in Maria Alm (A. P. Somlyo et al., 1986) and at the Satellite Symposium on Smooth Muscle of the International Physiological Congress (A. V. Somlyo et al., 1987).

## METHODS

### *Smooth Muscle Strip Preparation*

The portal anterior mesenteric vein was removed from ~400-g male guinea pigs, which were killed by rapid cervical dislocation and exsanguination. The adventitia was carefully removed from the 60- $\mu\text{m}$  thick vessel wall and longitudinal strips 0.1–0.25 mm wide and 2–3 mm long were cut parallel to the muscle bundles, avoiding the transverse bands of connective tissue, which are easily visualized with dark-field illumination in the light microscope. Fresh razor knives were used to cut the strips. The cell membranes were permeabilized by freeze-glycerination (Peterson, 1982), either with or without prior exposure to 50  $\mu\text{g}/\text{ml}$  saponin in relaxing solution for 10 min. The permeabilizing solution was a 1:1 mixture of glycerol and relaxing solution containing 1  $\mu\text{M}$  leupeptin and 40 mM reduced glutathione as a thiol protecting agent. Strips were stored at  $-18^\circ\text{C}$  until used, usually within 24 h.

T-shaped aluminum foil clips made by a photolithographic process (Goldman and Simmons, 1984) were folded around each end of the strip under silicone oil at  $10\text{--}12^\circ\text{C}$ . The clips were applied so as to orient the endothelial layer, which was less transparent than the muscle cells, away from the photolysis laser beam. In most experiments, the laser energy measured with a disk calorimeter ranged from 90 to 140 mJ. The fall-off in laser intensity across the whole strip of tissue, as well as across the longitudinal muscle bundle of interest, was estimated by positioning a photomultiplier tube against the outer wall and a sheet of tissue against the inner wall of a cuvette. 56% transmission at 336–344-nm light from a mercury arc lamp was measured through the tissue, indicating significant light scattering. However, the transmission would be considerably higher in the cells of interest, since the longitudinal smooth muscle bundles occupy the outer ~50% of the tissue cross-section of the guinea pig portal vein and are oriented to face the laser. The remaining thickness of the strip consists of connective tissue and endothelial cells. On the basis of measurements made on smooth muscle preparations of varying thicknesses, we estimate ~70–80% intensity at the back edge of the muscle bundle. The tissue at the endothelial side of the preparations would receive less photolysis energy than that facing the laser, but 70–80% intensity at the back edge of a strip would be a lower limit, because some of the ultraviolet light scattered in the strip would still cause photolysis without being transmitted to the photomultiplier.

### *Composition of Solutions*

The solutions used for the experiments are shown in Table I. The concentrations of the ingredients were calculated by a computer program, using published affinity constants

(Godt and Lindley, 1982) for the binding equilibria of the components. HDTA (1,6-diaminohexane-*N,N,N',N'*-tetraacetic acid) was purchased from Fluka, A. G., Buchs, Switzerland, and ATP $\gamma$ S from Boehringer Mannheim Biochemicals, Indianapolis, IN. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

### Apparatus and Techniques

The laser-flash photolysis technique and the configuration of the optical components used with the frequency-doubled ruby laser, as well as the muscle trough arrangement and solution exchanger, have been described in detail (Goldman et al., 1984a). One end of the fiber is attached to a force transducer (AE801, Akers, Horten, Norway) and the other

TABLE I  
Solutions

	ATP	PIPES	EGTA	Mg	CaEGTA	Ca <sup>2+</sup>	EDTA	P <sub>i</sub>	HDTA	CP*	Gluta-thione	Caged ATP or CTP
Relax	2.0	30	20	5.7						21.4	40	
Activate	2.0	30	0.1	4.6	19.9	0.03				21.8	40	
Thio <sup>‡</sup>		30	0.1	3.6	19.9	0.03			22.1		40	
Rigor + Ca		30	0.1	1.8	19.9	0.03			25.5		40	
Rigor, 0 Ca <sup>§</sup>		30	20	2.9					25.1		40	
Rigor, 0 Ca, 0 Mg		30	20				10		8.1		40	
Rigor + Ca + CP		30	0.1	2.7	19.9	0.03			5.2	20	40	
Rigor, 0 Ca + CP		30	20	3.8					4.8	20	40	
Photolysis + Ca		30	0.1	2.5	19.9	0.03			3.7		40	10
Photolysis, 0 Ca		30	20	3.7					3.4		40	10
Photolysis + Ca + CP		30	0.1	3.1	19.9	0.03				10.5	20	10
Photolysis, 0 Ca + CP		30	20	4.2						10.1	20	10
Photolysis, 0 Ca + P <sub>i</sub>		30	20	4.1				10	4.1		20	10
Photolysis, 0 Ca + P <sub>i</sub>		30	20	4.6				20	1.3		10	10
Photolysis, 0 Ca + CP + P <sub>i</sub>		25	14.9	4.4				10		10.1	20	10

All concentrations are in millimolar; pH 7.1; ionic strength, 0.20 M. Solutions were made up at room temperature (20–22°C). The photolysis solutions were calculated for an ionic strength of 0.20 M and 1.5 mM Mg<sup>2+</sup> after photolysis. All solutions contained protease inhibitors, 1  $\mu$ M leupeptin, 1 mM phenylmethylsulphonyl fluoride and mitochondrial inhibitors, 1 mM KCN, plus 1  $\mu$ g/ml oligomycin or 1  $\mu$ M FCCP [carbonyl cyanide 4-(trifluoroethoxy phenylhydrazone)]. Free Ca<sup>2+</sup> (10<sup>-7</sup> M) was present in all solutions without added Ca. 5  $\mu$ M calmodulin was present in Ca<sup>2+</sup>-containing solutions.

\* All solutions with creatine phosphate (CP) had 50 U/ml of creatine kinase.

<sup>‡</sup> Thiophosphorylating solution; with 2 mM ATP- $\gamma$ -S, and diadenosine pentaphosphate added to inhibit myokinase.

<sup>§</sup> Hexokinase (20 U/ml) and glucose (5 mM) were added when necessary to deplete tissue ATP.

to a piezoelectric element (P-173, Physik Instrumente, GmbH, Waldbronn, Federal Republic of Germany) applying a 500-Hz, 1- $\mu$ m sinusoidal length oscillation for measurement of fiber stiffness. The length oscillation caused a small amplitude of sinusoidal tension to be superimposed on the steady tension. The component of this sinusoidal tension in phase with the length change was demodulated by a lock-in amplifier (393, Ithaco, Inc., Ithaca, NY), and its amplitude was recorded as the stiffness signal (Goldman et al., 1984a). To determine the effects of cross-bridge strain on tension transients, length changes of 0.5–1% of total muscle length were made <30 s before photolysis, using a manual micrometer drive that held the tension transducer.

### *Rigor Protocol*

Low-tension rigor was induced by transferring the muscle from a relaxing solution containing 2 mM ATP to a rigor solution without ATP or  $\text{Ca}^{2+}$ . This resulted in a significant increase in stiffness and either no change or a small rise in tension.

A high-tension rigor state, also described by Arner and Rüegg (1984), was produced by transferring the muscle to rigor solutions following maximal activation with  $\text{Ca}^{2+}$  and ATP (Fig. 3A). Maximal forces developed by the permeabilized strips in activating solution were ~80% of the maximal force developed by intact strips. After development of maximal steady active tension, the strip was transferred to  $\text{Ca}^{2+}$  rigor solution for 3 min, and then to the  $\text{Ca}^{2+}$ -free rigor solution. The rigor force obtained with this protocol is ~50% of the maximal active force. The muscles were fully in rigor, as indicated by the lack of tension redevelopment following length releases in the rigor solutions. The addition of  $\text{Ca}^{2+}$  to fibers in low-tension rigor or its removal during high-tension rigor had no effect on rigor stiffness.

Fibers were transferred to rigor solution containing 10 mM caged ATP, and at least 2 min was allowed for the caged ATP to diffuse into the muscle before the laser was triggered to liberate ATP. In control experiments, the laser pulse itself, in the absence of caged nucleotide, had no effect on muscle force or stiffness, except for a small instantaneous drop of force in some of the recordings, as previously reported for skeletal muscle (Goldman et al., 1984a).

### *Caged Nucleotides*

$\text{P}^3$ -1(2-nitrophenyl)ethyladenosine-5'-triphosphate (caged ATP) and  $\text{P}^3$ -1(2-nitrophenyl)ethylcytidine-5'-triphosphate (caged CTP) were synthesized by treating the parent nucleotide with 1(2-nitrophenyl)diazoethane in rapidly stirred  $\text{CHCl}_3/\text{H}_2\text{O}$  for 18 h at 21°C and pH 4–5. The diazoethane was prepared by  $\text{MnO}_2$  oxidation of the hydrazone of 2-nitroacetophenone.

The concentrations of ATP and CTP liberated by laser photolysis were measured by anion-exchange high-pressure liquid chromatography (HPLC) of samples taken from the photolysis trough after each experimental trial. In some experiments, the ATP or CTP concentration was varied by altering the intensity of the laser pulse by interposing a varying number of glass microscope slides between the laser and the muscle strip.

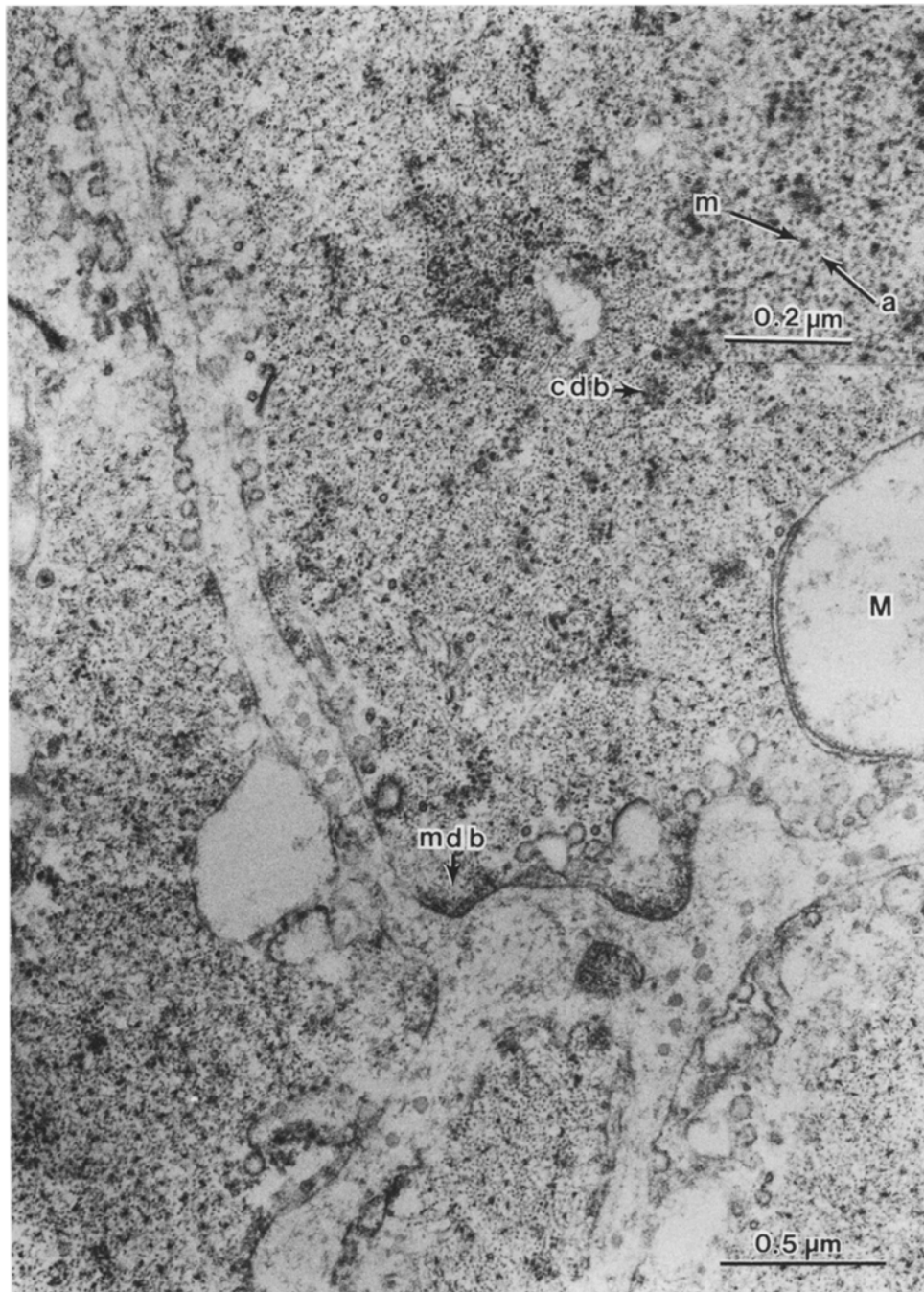
### *Electron Microscopy*

Muscle strips prepared as described above were fixed in 2% glutaraldehyde followed by 0.2% tannic acid, osmium, and uranyl acetate en bloc, and dehydrated and embedded in Spurr's resin. Some muscles were fixed after photolysis.

## RESULTS

### *Morphology of Permeabilized Smooth Muscle Cells*

The general appearance of the smooth muscle cells and the myofilament density were similar across the entire ~30–40- $\mu\text{m}$ -thick media of the portal vein. The use of tannic acid before osmication resulted in a marked increase in contrast in the freeze-glycerinated cells compared with intact cells, owing to better penetration of the tannic acid. In muscles fixed in relaxing solution either before or after photolysis of caged ATP, the incidence and arrangement of myofilaments and of cytoplasmic and plasmalemmal dense bodies (Fig. 1) were similar to those



**FIGURE 1.** Transversely sectioned smooth muscle cells from the guinea pig portal vein permeabilized by freeze-glycerination, showing regular arrays of myofilaments (enlarged inset in upper right-hand corner), as well as cytoplasmic and surface membrane-associated dense bodies in all of the cells. The mitochondria are swollen because of the presence of mitochondrial blockers necessary for induction of the rigor state. The muscle was incubated in relaxing solution (Table I) before fixation in glutaraldehyde. *M*, mitochondria; *m*, myosin; *a*, actin; *cdb*, cytoplasmic dense body; *mdb*, membrane-associated dense body.

observed in intact cells. The mitochondria were swollen, owing to the presence of mitochondrial blockers. There was no evidence of structural changes after repeated photolysis of caged ATP. However, in several muscles that were thiophosphorylated with ATP $\gamma$ S and had then undergone a rapid large contraction induced by photolysis of caged ATP, the regular myofilament arrangement was disrupted, with clustering of the filaments toward the center of the cell. In such fibers, the areas beneath the plasma membrane were devoid of filaments. These muscles gave only very small force responses if, after the rapid contraction induced by photolysis of caged ATP, the ATP was removed and then reapplied. This loss of contractility was consistent with the disorganization of the contractile apparatus. Consequently, after thiophosphorylation, muscles were used only for a single photolysis trial.

Longitudinal views of muscles fixed in the rigor state displayed regular arrays of cross-bridges attached to actin at an  $\sim 45^\circ$  angle (Fig. 2). Occasionally, regions of alternating actin and myosin filaments were found where clearly visible chevron patterns of cross-bridges were evident (arrowheads, Fig. 2).

#### *Relaxation from Rigor*

Muscles were put into high-tension rigor (see Methods) and then relaxed by release of 0.5–1 mM ATP liberated from caged ATP in the absence of Ca $^{2+}$  (Figs. 3A and 4A). After photolysis of caged ATP, tension decreased in several phases: (a) a rapid (50–100 ms) drop to  $\sim 80\%$  of the rigor tension, in muscles stretched by 0.5–1% before photolysis (a smaller drop occurred in nonstretched muscles), (b) a plateau lasting  $\sim 400$  ms, and (c) a subsequent fall to the relaxed baseline level. Stiffness decreased continuously during the relaxation. These events are reminiscent of transients ascribed to detachment, transient cooperative reattachment, and final detachment processes in vertebrate skeletal muscle (Goldman et al., 1984a). However, in a smooth muscle that is myosin-regulated, transient reattachment of cross-bridges in the absence of Ca $^{2+}$  was not expected. Therefore, we performed several experiments to investigate the underlying mechanisms.

#### *Mechanism of Tension Plateau*

We considered the possibility that the plateau during photolysis-induced relaxation was due to myosin light chains being phosphorylated via Ca $^{2+}$ -independent kinases, with photogenerated ATP as the substrate. This possibility was investigated using a new nucleotide analogue, caged CTP. CTP is a substrate of the actomyosin ATPase, but is not used as a phosphate donor by the myosin light-chain kinase (Cassidy and Kerrick, 1982). Caged CTP (10 mM) was diffused into fibers that were in high-tension rigor (Fig. 3B). Liberation of CTP in the absence of Ca $^{2+}$  caused relaxation, with a plateau of tension before the final relaxation, as with ATP (Fig. 4B). Although the relaxation after release of CTP from caged CTP was not as rapid or as large in amplitude as that caused by equimolar ATP, the two nucleotides could be compared by decreasing the laser energy for photolysis of caged ATP, until approximately the same rate of relaxation was achieved. In that case, the shapes of the tension and stiffness transients evoked

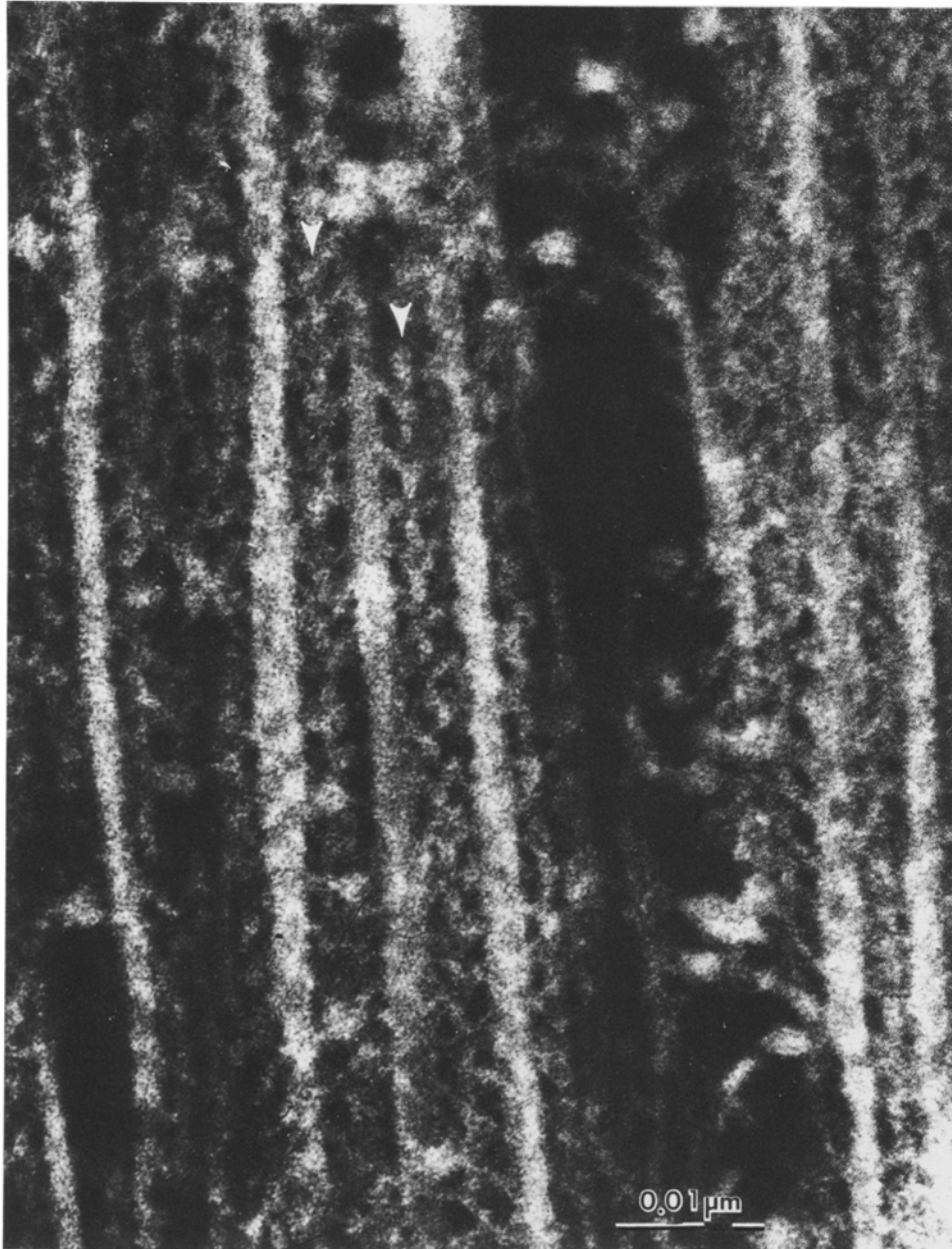


FIGURE 2. Longitudinal view of a permeabilized guinea pig portal vein smooth muscle cell in the rigor state. Alternating myosin and actin filaments with typical rigor chevron patterns of cross-bridges, with cross-bridges making an  $\sim 45^\circ$  angle of attachment with actin, are indicated (arrowheads).



by the two nucleotides were indistinguishable (Fig. 4, A and B). These results provide evidence that the plateau is not due to rapid phosphorylation of myosin light chains during the relaxation process. Phosphorylation of myosin light chain

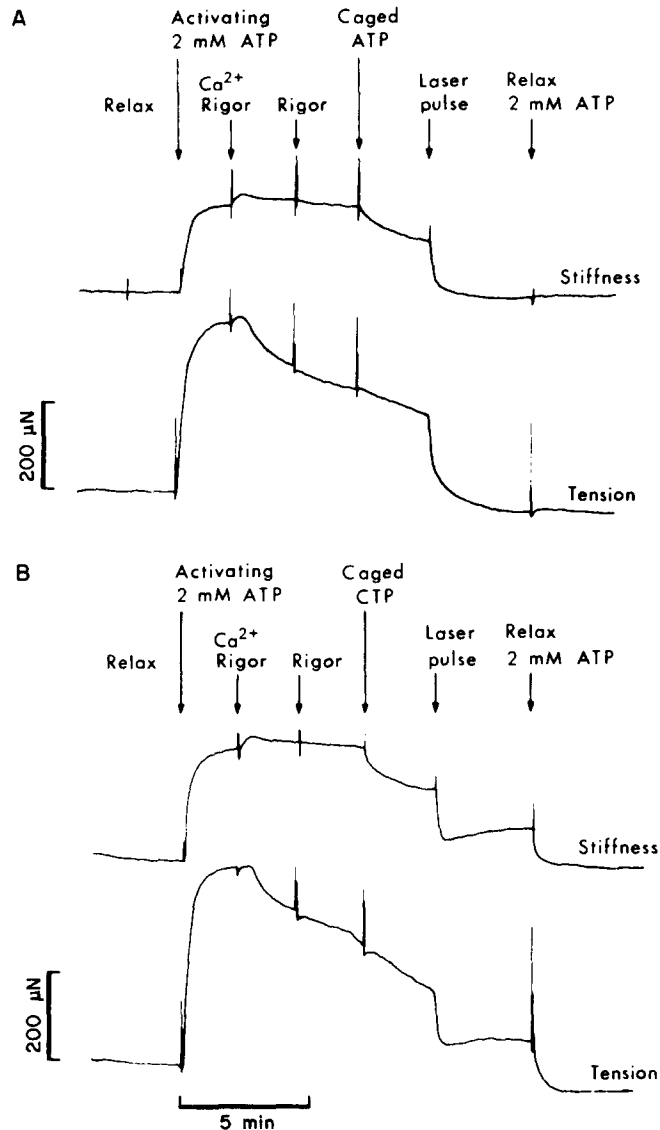


FIGURE 3. Force and in-phase stiffness records illustrating the experimental protocol used for recording the detachment of rigor cross-bridges by photolysis of caged ATP (A) or caged CTP (B). A high-tension rigor state is achieved by transferring the muscle from a relaxing solution to an activating solution containing  $\text{Ca}^{2+}$  and ATP, followed by removal of the ATP and ATP-regenerating system and subsequent removal of  $\text{Ca}^{2+}$  before adding 10 mM caged ATP. A 50-ns laser pulse photolyzed caged ATP or caged CTP, releasing from 0.5 to 1 mM nucleotide into the myofilament lattice.

during rigor (determined by Dr. T. Butler in five muscle strips subjected to the same series of solution exchanges as in the photolysis experiments shown in Fig. 3A, up to the point of adding caged ATP) was <11%; this is similar to the 12% baseline value measured in paired muscles incubated in relaxing solution, and is probably due to charge modification of unphosphorylated myosin (Driska et al., 1981; Haerberle et al., 1984).

Force development at low ATP concentrations in the absence of  $\text{Ca}^{2+}$  in skeletal muscle is thought to be caused by cooperative switching on of the

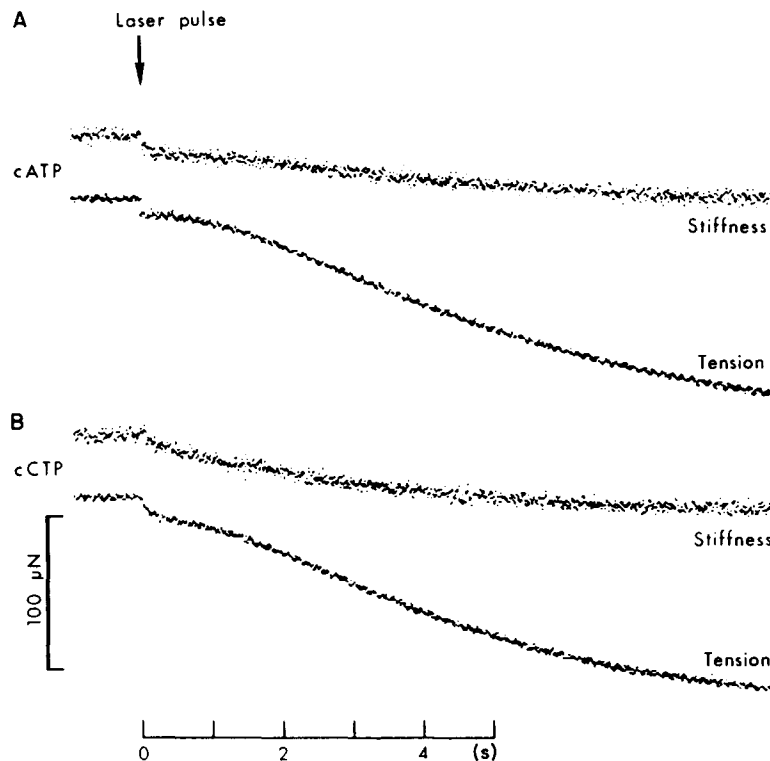
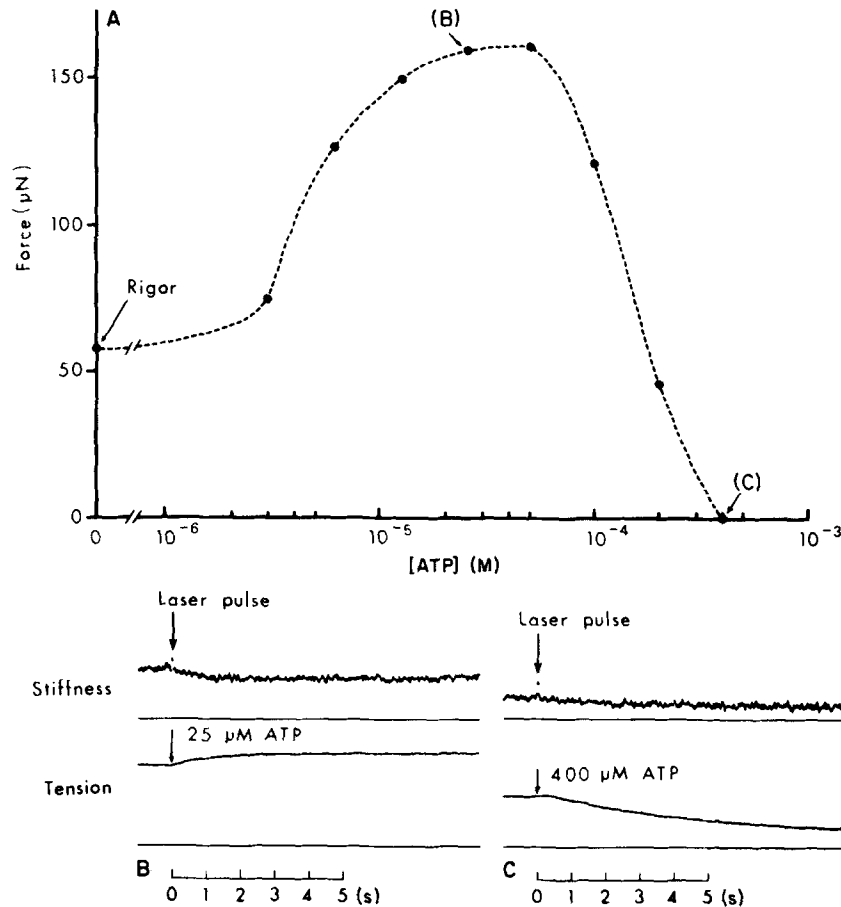


FIGURE 4. A pair of tension and stiffness transients from the same muscle, illustrating the similar shape of the transients with photolysis of either caged ATP (cATP) or caged CTP (cCTP). The protocol is given in Fig. 3.

regulated thin filaments by nucleotide-free (rigor) cross-bridges. The plateau or transient force development after photolysis of caged ATP in striated muscle has been ascribed to this type of cooperativity on the basis of transient reattachment of cross-bridges following detachment by ATP. Therefore, to determine whether cooperativity that could account for the plateau of tension during the relaxation induced by caged ATP photolysis also occurs in smooth muscles, we tested whether, in the absence of  $\text{Ca}^{2+}$ , low ATP concentrations also cause tension development. We did not observe force development when diffusing micromolar ( $\geq 6 \mu\text{M}$ ) ATP into the fibers (data not shown), but we found that force was produced by photolyzing caged ATP.

Small but rapid increments in the ATP concentration within the myofilament lattice of rigor muscles were achieved by varying the laser energy for photolysis of caged ATP. The release of 3–50  $\mu\text{M}$  ATP in the absence of  $\text{Ca}^{2+}$  ( $n = 12$



**FIGURE 5.** Tension vs.  $[\text{ATP}]$  relationship for a single muscle strip, showing force development in the absence of  $\text{Ca}^{2+}$ . The muscle had been carried through the low-tension rigor series of solutions with an additional  $\text{Mg}^{2+}$ -free, 10 mM EDTA step in order to deplete ADP from the myofibrillar space. The  $\text{Ca}^{2+}$ -free rigor solution containing caged ATP did not include an ATP-regenerating system. Sequential increments of ATP in the myofilament space were obtained by varying the energy of the laser pulse. Stiffness and tension traces for points *B* and *C* are shown beneath in panels *B* and *C*. At concentrations of  $<50 \mu\text{M}$  ATP, the muscles contracted; at higher concentrations, the overall effect was a fall in tension. In-phase stiffness fell with all concentrations of ATP tested. The horizontal lines beneath the stiffness and tension transients in *B* and *C* represent the relaxed baseline levels.

observations in six strips) induced contraction of the muscle strip, whereas the higher ATP concentrations caused a fall in steady force (Fig. 5). Fig. 5, *B* and *C*, shows the stiffness and tension traces following the release of, respectively, 25 and 400  $\mu\text{M}$  ATP, by photolysis of caged ATP. In the absence of an ATP-

regenerating system,  $\sim 25 \mu\text{M}$  ATP produced the largest and the most rapid increase in force, which amounted to 20–40% of maximal active tension measured at the end of the experiment. The stiffness trace declined at the lowest ATP concentration tested ( $6 \mu\text{M}$ ) and declined further as the ATP concentration increased up to full relaxation.

In the presence of a creatine phosphate/creatine kinase ATP-regenerating system, the ATP concentration at which tension decreased was shifted to lower values ( $10 \mu\text{M}$ ). No net increase of tension was seen at any concentration of ATP released, presumably because residual ADP acted as a source of ATP and prevented the internal ATP concentration from falling below the levels required to demonstrate cooperativity.

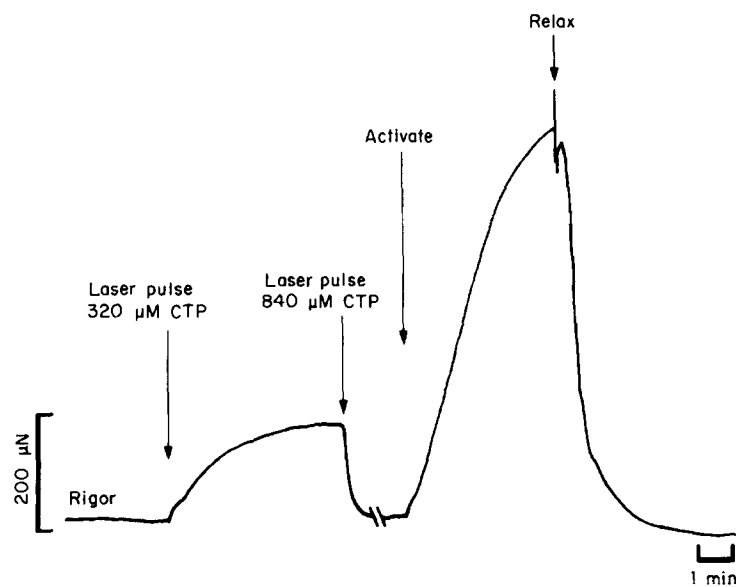


FIGURE 6. Force transients initiated from low-tension rigor in the absence of  $\text{Ca}^{2+}$  by photolysis of caged CTP. The release of  $320 \mu\text{M}$  CTP resulted in a contraction that was 25% of the maximal force produced by transferring the muscle into activating solution (Table I). Release of  $840 \mu\text{M}$  CTP induced a relaxation. In-phase stiffness fell after both of the laser pulses (not shown).

The above experiments were repeated with caged CTP to rule out the possibility that the force production after release of low concentrations of ATP in the absence of Ca involved phosphorylation of the myosin light chains by a  $\text{Ca}^{2+}$ -insensitive kinase (Walsh et al., 1982). Cumulative increments in the CTP concentration within the myofilament lattice were achieved by varying the laser energy for photolysis of caged CTP. A biphasic curve similar to that shown in Fig. 5 was observed, but the curve was shifted to the right of that for ATP. Fig. 6 shows tension records during the release of  $320$  and  $840 \mu\text{M}$  CTP by photolysis of caged CTP starting from low-tension rigor. The magnitude of the force developed in response to  $320 \mu\text{M}$  CTP was  $\sim 25\%$  of the maximal force achieved by an activating solution (Table I). These experiments strongly suggest that the plateau phase of relaxation is due to cooperative attachment of cross-bridges (see Discussion).

*Initial Rapid Tension Changes*

Prestretching the smooth muscle strips by 0.5–1% of their length a few seconds before the laser pulse markedly enhanced the amplitude of the initial fast relaxation, which was complete in ~100 ms (Fig. 7, lower trace). The fast phase accounted for ~20% of the total fall in force to the relaxed baseline and for ~60% of the drop in stiffness after a 1% prestretch. The rate of the initial fast phase of relaxation also increased with increasing concentrations of ATP (see below). These results suggest that the initial rapid phase of relaxation represents the detachment of rigor cross-bridges by ATP.

When muscles, either in high- or low-tension rigor, were prereleased by 0.5–1% of muscle length before photolysis, the release of 0.35–1 mM ATP caused a rapid increase in force within the first 100 ms (Fig. 8), followed by a slow decline

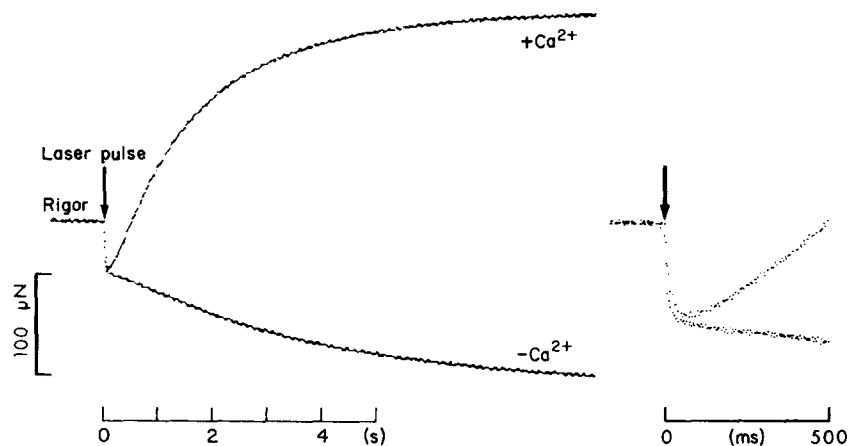


FIGURE 7. Two superimposed tension transients after liberation of ATP by photolysis of caged ATP in the presence (+Ca<sup>2+</sup>) and absence (-Ca<sup>2+</sup>) of Ca<sup>2+</sup>. The muscle was stretched by 1% of its length before liberation of ATP. Both transients show a similar initial rapid fall in force caused by the detachment of rigor cross-bridges by the released ATP. In the presence of Ca<sup>2+</sup>, the muscle is capable of generating sufficient force so that the two curves diverge in <50 ms (inset). In the absence of Ca<sup>2+</sup>, after the rapid detachment, the transient has a complex shape with a plateau phase of ~400 ms, followed by a slow fall to the relaxed baseline.

to the relaxed baseline. The rapid component of tension production was much faster than the tension development in the absence of Ca<sup>2+</sup>, ascribed in the previous section to the cooperative reattachment and cycling of cross-bridges (compare Fig. 5B with Fig. 8). The rapid component of tension production in prereleased muscles was also faster than the subsequent active tension generation that occurred when Ca<sup>2+</sup> was present (Fig. 9).

A decrease in stiffness was coincident with the rapid rise in force, which indicates the detachment of cross-bridges. The stiffness recordings included considerable series compliance at the ends of the preparations and also presumably between smooth muscle cells. Therefore, quantitative conclusions should not be drawn from the amplitude of the stiffness transients. However, nonlinearity of the series compliance has the effect of causing the apparent stiffness to increase when tension increases. This occurred, for instance, when small stretches

were applied to muscles in rigor (not shown). The decrease in stiffness during the tension increase in Fig. 8 (see also Fig. 4B) is thus not due to series compliance, but is evidence of cross-bridge detachment during the first 100 ms after the laser pulse.

These results suggest that the initial detachment of cross-bridges caused positive force development, which implies that in pre-released muscles a significant proportion of the cross-bridges are negatively strained.

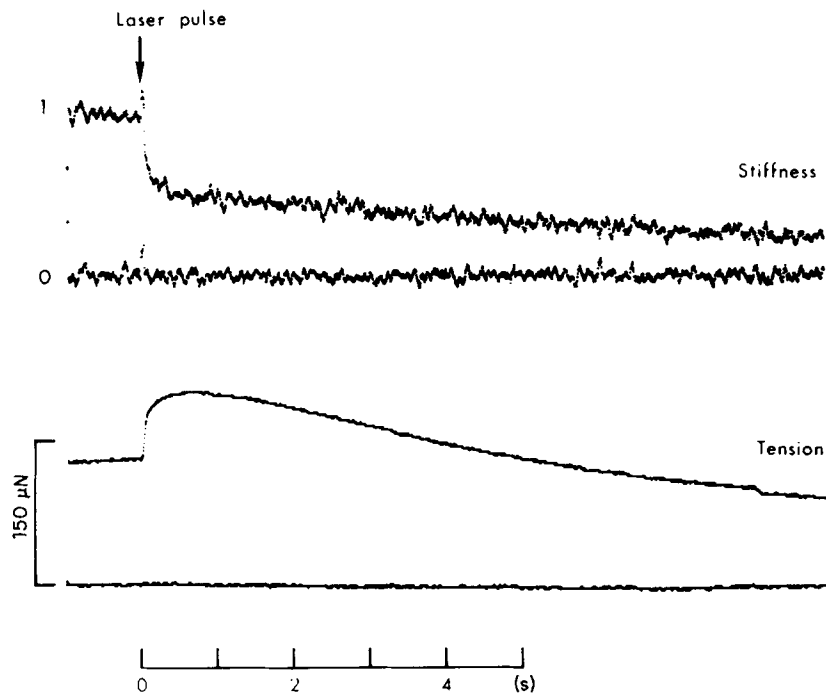


FIGURE 8. Tension and in-phase stiffness transients recorded from a muscle strip in the high-tension rigor state and released by 0.5% muscle length before the release of 350  $\mu\text{M}$  ATP by photolysis of caged ATP (arrow), in the absence of  $\text{Ca}^{2+}$ . Despite the absence of  $\text{Ca}^{2+}$ , there was a rapid initial increase in tension, with a coincident fall in in-phase stiffness, followed by a slow fall in tension to the relaxed baseline.

#### *Rate of ATP-induced Cross-Bridge Detachment*

In order to compare the kinetics of ATP-induced cross-bridge detachment with the rate of actomyosin dissociation in solution, the kinetics of relaxation from rigor were measured over a range of ATP concentrations. The muscles were put into the low-tension rigor state and prestretched before the laser pulse to enhance the amplitude of the fast component of relaxation. After photolysis, the muscle length was returned to the original value before further rigor-photolysis trials at altered concentrations of liberated ATP. At most, four photolysis trials were applied to an individual muscle strip.

When very low concentrations of ATP (i.e.,  $<100 \mu\text{M}$ ) were released in the absence of creatine phosphate and creatine kinase, tension and stiffness declined but then slowly increased again. This may have been due to cross-bridges returning to the rigor state after hydrolysis of these low concentrations of ATP, but the increased force could also represent active tension generation caused by the cooperative reattachment (see above) of the small population of cross-bridges that were detached by these low concentrations of ATP.

Even at the highest concentrations of ATP released ( $800 \mu\text{M}$ ), the rapid component of tension decrease did not reach the relaxed baseline. This incomplete rapid relaxation may have been due to force generation by cross-bridges that reattached cooperatively, as discussed above, or to a population of cross-bridges that detaches more slowly than those represented in the initial tension

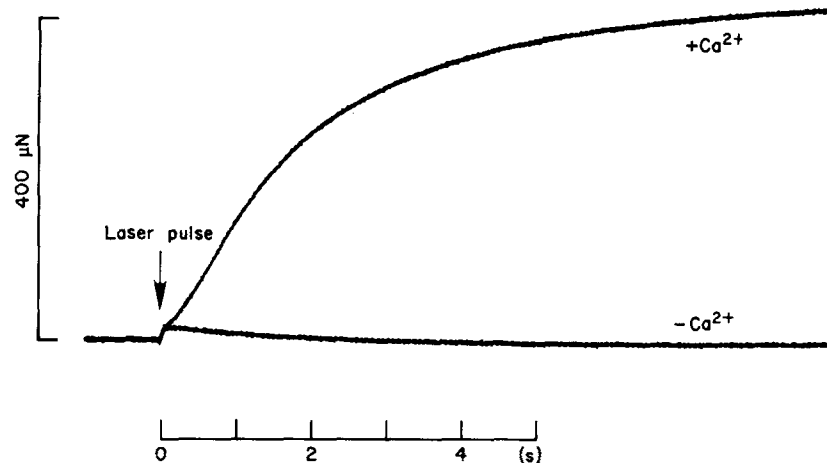


FIGURE 9. Two superimposed tension transients obtained from the same muscle in the presence and absence of  $\text{Ca}^{2+}$  and released by 0.5% before photolysis of caged ATP (arrow). In-phase stiffness fell both in the presence and absence of  $\text{Ca}^{2+}$  after photolysis (not shown). The initial rapid increase in force is independent of  $\text{Ca}^{2+}$  and represents the detachment of negatively strained cross-bridges. The baseline force in relaxing solution was at the same level for both traces as that shown before the laser pulse.

decay. Because a contribution from more slowly detaching cross-bridges could not be ruled out, two methods of analysis were used to obtain upper and lower estimates of the cross-bridge detachment rate (Fig. 10A, inset). The upper estimate was made by directly fitting an exponential function to the fast phase of the tension decline. The apparent rate constants for detachment ( $1/\tau_1$ ) obtained from this exponential fitting procedure are plotted as circles in Fig. 10B. These data would represent the more rapidly detaching population of cross-bridges, if there were a range of detachment rates. The detachment rates of the other cross-bridges are indeterminate.

A lower limit for the detachment rate was obtained by fitting a straight line to the initial segment (50–120 ms) of the tension decline and extrapolating that

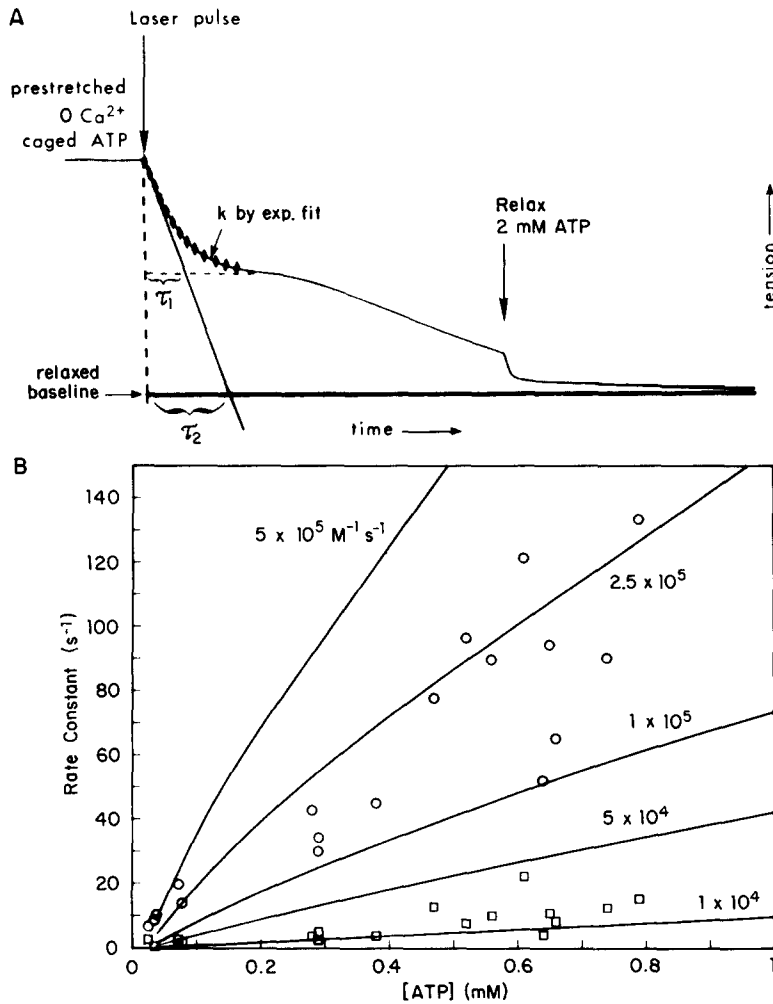


FIGURE 10. (A) Schematic illustration of a relaxation transient, such as shown in Fig. 7, indicating the two approaches used for estimating values for the rate of ATP-induced cross-bridge detachment. The upper estimate was made by direct fitting of an exponential function to the fast phase of tension decline (diamonds). The rate constant data are plotted as circles in B. A lower limit for the detachment rate was obtained by fitting a straight line to the initial segment (50–120 ms) of the tension decline and extrapolating that line to the intersection with the relaxed baseline at time  $\tau_2$ . If tension ( $T$ ) of detaching cross-bridges before significant reattachment is given by  $T = T_0 \exp(-t/\tau_2)$ , where  $T_0$  is the starting rigor tension and  $t$  is time, then the line fitted to the initial slope is given by  $Y = T_0 - T_0 t/\tau_2$ , which intercepts 0 at  $t = \tau_2$ . The apparent detachment rates, given by the reciprocal of  $\tau_2$ , are plotted as squares in B. The apparent rate of cross-bridge detachment from rigor was measured as a function of ATP concentration using the two approaches described above and in the text. The lines represent the expected relation between the rate constant and the ATP concentration determined from a computer simulation as described in the text. The data cluster between the lines plotted for values of the second-order rate constant for detachment by ATP of  $k_d = 1 \times 10^4$ – $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ .



line to the intersection with the relaxed baseline at time  $\tau_2$ . The apparent detachment rates, given by the reciprocal of  $\tau_2$  and plotted as squares in Fig. 10, would correspond to the cross-bridge detachment rate if the plateau phase were due entirely to reattachments. The abscissa in Fig. 10B is the liberated ATP concentration measured by HPLC of the contents of the muscle trough after photolysis. The rates of detachment did not saturate with ATP concentrations up to 1 mM.

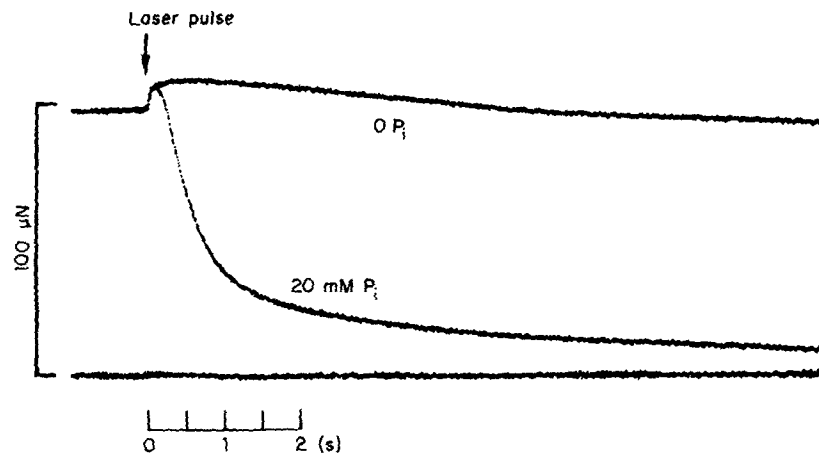
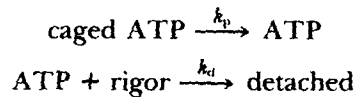


FIGURE 11. Two superimposed tension transients recorded from the same muscle strip after photolysis of caged ATP in the presence and absence of  $P_i$ . The muscle, initially in a high-tension rigor state without  $Ca^{2+}$ , was released by 0.5% before the laser pulse. In the absence of  $P_i$ , 0.6 mM ATP was liberated upon photolysis, and in the presence of 20 mM  $P_i$ , 0.7 mM ATP was liberated. Note that the initial rapid tension rise is independent of the  $P_i$  concentration, and may represent the detachment of negatively strained cross-bridges. A coincident fall of in-phase stiffness (not shown) occurred with the initial rise in force.  $P_i$  markedly accelerated the subsequent rate of relaxation.

The continuous lines plotted in Fig. 10B are the rate constants (determined by a computer simulation) expected on the basis of the following reactions:



The rate constant ( $k_p$ ) of the release of ATP from caged ATP by the laser photolysis is  $118 \text{ s}^{-1}$  (Goldman et al., 1984a). The concentration of myosin heads within the smooth muscle cells was assumed to be  $75 \mu\text{M}$  (Cohen and Murphy, 1978). The data are clustered between the lines plotted for values of the second-order rate constant for detachment by ATP of  $k_d = 1.0 \times 10^4$ – $2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ . The final slow phase of relaxation was not appreciably dependent on the ATP concentration. The average rate was  $0.2 \pm 0.03 \text{ s}^{-1}$  (SD;  $n = 10$ ).

The effect of  $Ca^{2+}$  on the detachment rate was determined (Fig. 7) in three smooth muscle strips prestretched before photolysis of caged ATP. The detachment rates, obtained by fitting an exponential function, were not significantly different in the presence of  $Ca^{2+}$  from those in the absence of  $Ca^{2+}$  and fell

within the range of the circles shown in Fig. 10. These data show no effect of  $\text{Ca}^{2+}$  on the rate of detachment of rigor cross-bridges by ATP.

In two experiments using caged CTP, the presence of  $\text{Ca}^{2+}$  also did not appear to affect the detachment rates. The rates with CTP were slower than those with ATP, which is consistent with CTP being a less effective substrate. Liberation of CTP in the presence of  $\text{Ca}^{2+}$  was not followed by a positive force development, such as that evoked by ATP, consistent with the inability of CTP to act as a substrate for myosin light-chain kinase.

#### *Effects of Inorganic Phosphate*

Inorganic phosphate ( $\text{P}_i$ ) had no detectable effect on rigor tension, but inclusion of  $\text{P}_i$  in the medium markedly accelerated relaxation from rigor initiated by photolysis of caged ATP. Fig. 11 shows transients recorded from a preparation in the presence of 20 mM  $\text{P}_i$  and in the absence of  $\text{P}_i$ . The muscle was prereleased

TABLE II  
*Rate of Force Development by Smooth Muscle Activated from Rigor*

Conditions	$k^*$ $s^{-1}$	$n$
I. Skinned muscles		
(A) Non-phosphorylated before activation		
(1) Low-tension rigor		
(a) Isometric	0.3±0.12	29
(b) Stretched	0.5±0.13	6
(2) High-tension rigor, isometric	0.5±0.05	7
(3) Low-tension rigor plus MLCK, isometric	0.4±0.04	6
(B) Thiophosphorylated before activation, isometric		
(1) + $\text{Ca}^{2+}$	3.9±1.50	11
(2) - $\text{Ca}^{2+}$	3.7±0.90	5
II. Intact, non-skinned muscles		
(A) Electrical stimulation		
	0.4±0.09	4
(B) High-potassium contracture		
	0.3±0.09	10

\* Values are given ± SD.

by 0.5% shortly before each laser pulse and ~700  $\mu\text{M}$  ATP was released in each trial. The rate and amplitude of the initial tension rise were not affected by the presence of  $\text{P}_i$ , but at 100 ms after the laser pulse, the traces separated and the muscle then relaxed more rapidly in the presence of  $\text{P}_i$ .

#### *Activation from Rigor: the Effect of Thiophosphorylation*

When  $\text{Ca}^{2+}$  was present during photolysis experiments, tension rapidly decreased (pre-stretched muscles) or increased (pre-released muscles) during the initial cross-bridge detachment, and then increased more slowly to an active contraction level (Figs. 7 and 9, upper traces). Comparison of the transients in pre-stretched (Fig. 7) and pre-released (Fig. 9) muscles shows that the tension traces were not altered by the presence of ~30  $\mu\text{M}$   $\text{Ca}^{2+}$  for the first 50–100 ms after the laser pulse. Thereafter, in the presence of  $\text{Ca}^{2+}$ , tension increased. The rate of force development after the rapid tension change was not significantly different

whether the muscles were activated from the low- or high-tension rigor state or prestretched before photolysis (Table II).

The rate of force development from rigor after release of 0.5–1 mM ATP from caged ATP (Fig. 12*B*, from low-tension rigor) was similar to that observed in intact preparations stimulated electrically with extracellular electrodes (Fig. 12*C*, Table II) or by high potassium (Table II). A major difference between the intact and permeabilized muscles was the long latency observed after electrical

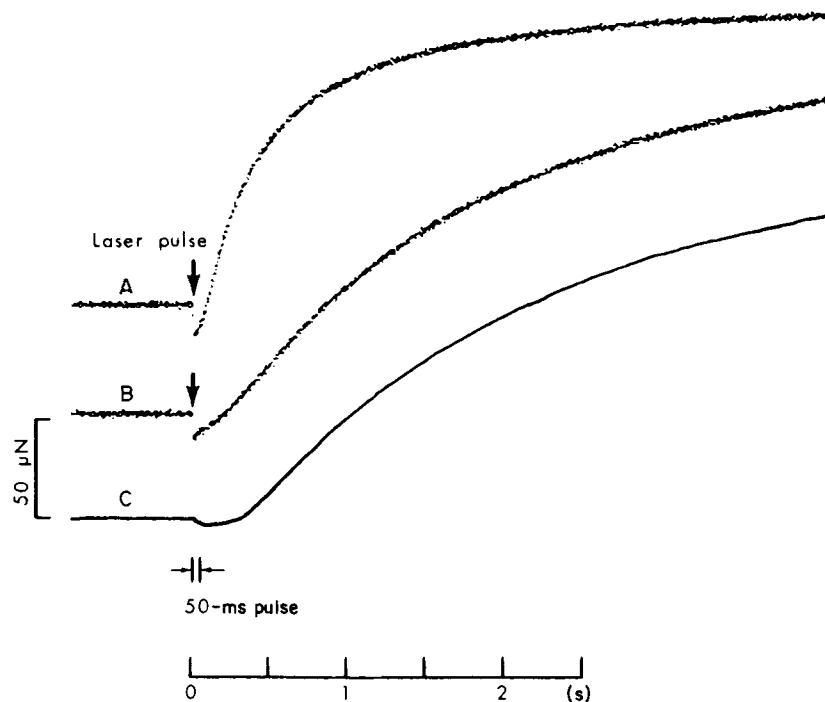


FIGURE 12. Rates of force development of guinea pig portal vein are shown for the following conditions: (A) the muscle strip has been permeabilized and the light chains have been thiophosphorylated by incubation with ATP $\gamma$ S, Ca $^{2+}$ , and calmodulin before photolysis of caged ATP (arrow) in the presence of Ca $^{2+}$ ; (B) permeabilized muscle strip, not thiophosphorylated before photolysis of caged ATP (arrow); the protocols for A and B are shown in Fig. 13; (C) a non-permeabilized intact muscle strip that was electrically stimulated by a 50-ms DC pulse. Note the similar rates of force development for B and C, the 10-fold-faster rate for A, and the long delay before the onset of tension development in C.

stimulation ( $280 \pm 60$  ms, SD,  $n = 4$ ) and during high-K $^{+}$  depolarization ( $570 \pm 180$  ms, SD,  $n = 8$ ). The latency between ATP release and the onset of contraction in permeabilized muscle preparations was either absent or much shorter (Fig. 12).

The possible involvement of light-chain phosphorylation in controlling the onset of these contractions was investigated by thiophosphorylating the 20-kD myosin light chains before the photolysis of caged ATP. The protocol is illus-

trated in Fig. 13*B*; thiophosphorylation of light chains was induced by incubating muscle strips, initially in the low-tension rigor state, in 2 mM ATP $\gamma$ S in the presence of Ca<sup>2+</sup> and calmodulin. Although ATP $\gamma$ S is a slowly hydrolyzed analogue of ATP (Goody and Hofmann, 1980), a small and variable amount of force was developed on exposure to ATP $\gamma$ S, possibly because of contamination by ADP. Tension decayed slowly upon re-exposure to the Ca<sup>2+</sup> rigor solution. The tension development initiated by the release of 0.5–1 mM ATP from caged

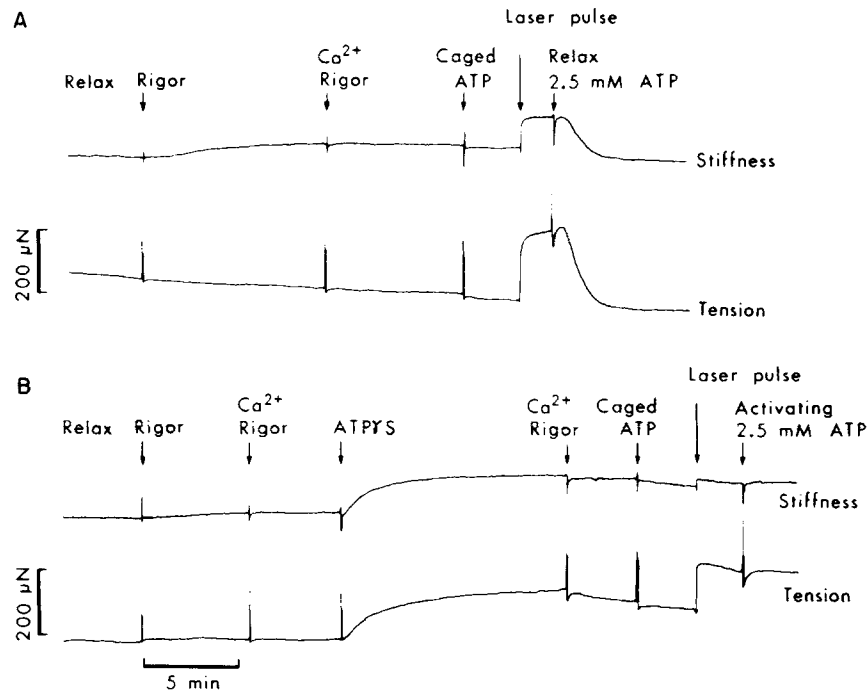


FIGURE 13. Tension and stiffness traces showing the experimental protocol used for measuring the rates of force development upon release of ATP by photolysis of caged ATP, in the presence of Ca<sup>2+</sup>. The muscle was in the rigor state with non-phosphorylated light chains (A) or with the light chains thiophosphorylated with ATP $\gamma$ S (B). In both instances, the muscles were transferred through the rigor solutions as indicated and described in Table I, in order to induce the low-tension rigor state. The thiophosphorylated muscle was incubated for 10 min in a Ca<sup>2+</sup> rigor solution containing 2 mM ATP $\gamma$ S and 5  $\mu$ M calmodulin, followed by a washout of the ATP $\gamma$ S with Ca<sup>2+</sup> rigor solution.

ATP was approximately an order of magnitude faster after exposure to ATP $\gamma$ S (Fig. 12*A*) than in muscles without thiophosphorylated light chains (Fig. 12*B*, Table II). The rates of force development by muscles having pre-thiophosphorylated light chains were also not significantly affected by the presence or absence of Ca<sup>2+</sup> (Table II).

In the non-thiophosphorylated muscles, force development did not seem to be limited by the amount of light-chain kinase present, as the rate did not increase when skinned fibers were incubated for 13–17 min before photolysis, at saturat-

ing ATP concentrations, with 20  $\mu\text{g/ml}$  myosin light-chain kinase (kindly provided by Dr. D. J. Hartshorne, University of Arizona, Tucson, AZ).

#### DISCUSSION

Our results provide substantial further evidence indicating that cyclic attachment and detachment of cross-bridges occurs in smooth muscle, paralleling the cyclic association and dissociation reactions of actomyosin ATPase in solution (Marston and Taylor, 1980), and that the cross-bridge cycle represents the molecular mechanism of contraction. A rigor state was induced when ATP was removed from skinned smooth muscle, as indicated by increased mechanical stiffness and electron-microscopic evidence of cross-bridges between thick and thin filaments. The release of ATP by photolysis of caged ATP caused cross-bridge detachment, indicated by a decrease in rigor tension and stiffness and followed, in the presence of  $\text{Ca}^{2+}$ , by force development. We also found evidence of cooperative cross-bridge behavior, which may contribute to tonic force maintenance at low levels of light-chain phosphorylation.

The existence of rigor in smooth muscle has previously been inferred (A. P. Somlyo and Somlyo, 1968) from the relaxant effect of ATP on glycerinated vascular smooth muscle (Schirmer, 1965) and from the mechanical properties of substrate-depleted smooth muscles (Lowy and Mulvany, 1973; Bose and Bose, 1975). We have established that a rigor state can be reproducibly induced in skinned smooth muscle (present study; Arner and Rüegg, 1985), and our present results suggest that in smooth, as in skeletal, muscle, the binding of ATP to a nucleotide-free (rigor) cross-bridge causes detachment that is too rapid to be rate-limiting of the overall ATPase cycle.

#### *Cross-Bridge Detachment Rate*

In the absence of  $\text{Ca}^{2+}$ , the relaxation of stiffness and tension following laser-induced liberation of ATP from caged ATP consisted of a rapid, approximately exponential phase and a more complicated plateau or slow phase (Figs. 4, 7, and 11). The amplitude and direction of the initial phase of the tension traces were modified when the stress on the cross-bridges was altered by stretching or releasing the muscle immediately before photolysis. The exponential rate of the initial tension change was correlated with the amount of ATP liberated (Fig. 10). The apparent second-order rate constant for ATP binding and cross-bridge detachment calculated from our measurements was  $0.1\text{--}2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ , and can be compared with the corresponding rate,  $2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ , of dissociation of gizzard actomyosin subfragment-1 (S-1) in solution (Marston and Taylor, 1980). In skeletal muscle fibers, the ATP-induced dissociation rate is about an order of magnitude faster in solution than in muscle fibers (Goldman et al., 1984a). Thus, assembly of the proteins into the filament lattice apparently slows the rate of cross-bridge detachment.

The effective rate of detachment from the actomyosin state during a normal contraction can be estimated by considering that the MgATP concentration within smooth muscle cells is 3–5 mM (Butler and Davies, 1980). The calculated rates of detachment are then between  $30 \text{ s}^{-1}$  ( $10^4 \text{ M}^{-1}\text{s}^{-1} \times 3 \text{ mM}$ ) and  $1,250 \text{ s}^{-1}$  ( $2.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \times 5 \text{ mM}$ ). These values are greater than the rate of ATP

hydrolysis,  $1-2 \text{ s}^{-1}$  per myosin head (Sellers, 1985; Hartshorne 1987), which indicates that detachment of actomyosin cross-bridges is not the rate-limiting step for the cross-bridge cycle or ATPase activity.

The rate of detachment, in our experiments, was not directly affected by  $\text{Ca}^{2+}$ . However, the rate constants discussed above reflect the dissociation of nucleotide-free, dephosphorylated myosin heads from actin, while in intact muscle the dissociation rate may be controlled by the rate of ADP release from the cross-bridge (Siemankowski et al., 1985). The effects of bound nucleotide and phosphorylation of the myosin light chain on cross-bridge detachment, and the possible modulation of these effects by  $\text{Ca}^{2+}$ , remain to be determined.

#### *Force Owing to Detachment of Negatively Strained Cross-Bridges*

An unexpected feature of the transient response to the release of ATP was the rapid, initial phase of force development by the smooth muscles that were mechanically released before photolysis (Figs. 8, 9, and 11). The high rate, ATP and strain dependence, lack of sensitivity to  $\text{Ca}^{2+}$  and  $\text{P}_i$ , and, most importantly, the accompanying decrease in stiffness suggest that this transient represents net force development owing to detachment of cross-bridges that were under negative strain in the rigor state. If there is variation in the relative position of thick and thin filaments in the axial and radial directions, then the rigor cross-bridges will exist in a range of distortions with respect to the neutral, zero-force position. Some of the cross-bridges might attach with little or no force. A prerelease that decreases the net force in the muscle will then push such low-force rigor cross-bridges into the region of strain corresponding to negative force. Detachment of that population of cross-bridges will lead to a rapid tension increase and a stiffness decrease as observed (Figs. 8, 9, and 11). An important feature of A. F. Huxley's (1957) cross-bridge model was that attached cross-bridges could be displaced by filament sliding past the zero-force position into the region of negative force. Evidence for cross-bridges bearing negative force was obtained in skeletal muscle fibers (Ford et al., 1977; Goldman, 1987*b*; Goldman et al., 1988). The present results support their existence in smooth muscle.

#### *Cross-Bridge Attachment in the Absence of $\text{Ca}^{2+}$ : Cooperativity*

The relaxation induced by photolysis of caged ATP included a slow phase or plateau of  $\sim 400$  ms. A similar slow phase occurs in striated muscle, albeit on a faster time scale, and has been ascribed to the rapid reattachment of detached cross-bridges (Goldman et al., 1984*a*). Such reattachment, in the absence of  $\text{Ca}^{2+}$ , is thought to be due to cooperative behavior either between myosin heads or among groups of cross-bridges linked through the thin-filament (actin-tropomyosin) system (Weber and Murray, 1973). The latter type of cross-bridge cooperativity is manifested in the "bell-shaped" curve of the actomyosin ATPase (Bremel et al., 1972) or tension response (Reuben et al., 1971; Fabiato and Fabiato, 1975; Kawai and Brandt, 1976; Arata et al., 1977; Goldman et al., 1984*a*) as a function of ATP concentration in the absence of  $\text{Ca}^{2+}$ .

Micromolar ATP, released with laser-flash photolysis in the absence of  $\text{Ca}^{2+}$ , also induced steady state force in the present experiments, while high ATP



$P_i$  accelerates ATP-induced relaxation of skinned smooth muscle from rigor (Arner et al., 1986; present study). The time resolution of the present study permitted us to establish that  $P_i$  accelerated only the slow phase, and did not affect the initial, rapid phase of relaxation. This is consistent with our conclusion that the rapid phase of relaxation is due to detachment of rigor cross-bridges (steps 1 and 2) that would be unaffected by  $P_i$  (see scheme above). Some relationship between the plateau of the transients described in this article and the high-force, low-ATPase state of tonically contracted smooth muscle is suggested by the fact that relaxation from both conditions is facilitated by  $P_i$  (Schneider et al., 1981; Gagelmann and Güth, 1987; present study). It is interesting to consider the possibility that similar reattachment of unphosphorylated cross-bridges, facilitated in intact muscles by cooperative action of attached, phosphorylated cross-bridges, could cause the tonic maintenance of tension associated with minimal ATPase activity ("catch-like state" or "latch") (A. V. Somlyo and Somlyo, 1967; Siegman et al., 1976; Driska et al., 1981).

#### *Cross-Bridge Attachment and the Rate of Force Development*

The rate of force development attainable by smooth muscle that was prethiophosphorylated, and thus not rate-limited by light-chain kinase/phosphatase kinetics, was an order of magnitude faster ( $\sim 4 \text{ s}^{-1}$ ) than under conditions rate-limited by light-chain phosphorylation. The similar rates ( $\sim 0.4 \text{ s}^{-1}$ ) of force development in non-thiophosphorylated skinned preparations and in electrically stimulated, intact smooth muscle suggest that light-chain phosphorylation also determines the rate of contraction in intact muscle. This conclusion also follows from the close temporal relationship between light-chain phosphorylation and increased stiffness in intact smooth muscle (Kamm and Stull, 1986) and from the role of myosin light-chain phosphorylation as the primary mechanism of activation in smooth muscle (for review, Hartshorne, 1987). The rate of force development ( $4 \text{ s}^{-1}$ ) limited by actomyosin (in thiophosphorylated muscle) was similar to the maximal rate of ATPase activity of isolated actomyosin ( $1\text{--}2 \text{ s}^{-1}$ ; Sellers, 1985; Hartshorne, 1987). A possible relationship between the respective rates was previously observed in striated muscle (Brenner and Eisenberg, 1986).

The onset of force in electrically stimulated, intact muscle showed a lag phase or latency (Fig. 12,  $\sim 250 \text{ ms}$ ; see also Fay, 1977; Kamm and Stull, 1986) much greater than observed with skinned muscle preparations (Figs. 7, 9, and 12). This lag suggests that additional steps preceding the light-chain kinase reaction (e.g.,  $\text{Ca}^{2+}$  release,  $\text{Ca}^{2+}$  binding to calmodulin, and/or  $\text{Ca}^{2+}$ -calmodulin binding to MLCK) are kinetically important during activation of intact smooth muscle.

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