The ADP Release Step of the Smooth Muscle Cross-Bridge Cycle Is Not Directly Associated with Force Generation

Jody A. Dantzig,* Robert J. Barsotti,# Scott Manz,* H. Lee Sweeney,* and Yale E. Goldman*

*Department of Physiology and Pennsylvania Muscle Institute, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6083 and #Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 USA

ABSTRACT When smooth muscle myosin subfragment 1 (S1) is bound to actin filaments in vitro, the light chain domain tilts upon release of MgADP, producing a ~3.5-nm axial motion of the head-rod junction (Whittaker et al., 1995. *Nature.* 378:748–751). If this motion contributes significantly to the power stroke, rigor tension of smooth muscle should decrease substantially in response to cross-bridge binding of MgADP. To test this prediction, we monitored mechanical properties of permeabilized strips of chicken gizzard muscle in rigor and in the presence of MgADP. For comparison, we also tested psoas and soleus muscle fibers. Any residual bound ADP was minimized by incubation in Mg^{2+}-free rigor solution containing 15 mM EDTA. The addition of 2 mM MgADP, while keeping ionic strength and free Mg^{2+} concentration constant, resulted in a slight increase in rigor tension in both gizzard and soleus muscles, but a decrease in psoas muscle. In-phase stiffness monitored during small (<0.1%) 500-Hz sinusoidal length oscillations decreased in all three muscle types when MgADP was added. The changes in force and stiffness with the addition of MgADP were similar at ionic strengths from 50 to 200 mM and were reversible. The results with gizzard muscle were similar after thiophosphorylation of the regulatory light chain of myosin. These results suggest that the axial motion of smooth muscle S1 bound to actin, upon dissociation of MgADP, is not associated with force generation. The difference between the present mechanical data and previous structural studies of smooth S1 may be explained if geometrical constraints of the intact contractile filament array alter the motions of the myosin heads.

INTRODUCTION

During muscle contraction, mechanical work is generated by transduction of the chemical energy liberated from hydrolysis of ATP to ADP and inorganic phosphate (P_i) (reviewed in Huxley, 1980; Cooke, 1997; Goldman, 1998). This energy transduction is accomplished by a cyclic interaction between the globular head (subfragment 1, S1) of myosin projecting from the backbone of the thick filaments and actin in the thin filaments. Relaxed muscle contains predominantly detached or weakly attached heads with tightly bound products from ATP hydrolysis (M·ADP·P_i and AM·ADP·P_i; Scheme 1). Upon activation, S1 attaches to the actin filament and then tilts to produce a sliding force on the filaments. Force generation seems to be coupled with release of P_i from the active site (Scheme 1, steps 5 and 6), which causes local rearrangements of the protein structure near the phosphate-binding site. These small structural changes in the catalytic domain are amplified into 5–20 nm of motion at the head-rod junction by the light chain region, which probably serves as a lever arm (Rayment et al., 1993; Uyeda et al., 1996). If the mechanical load on the muscle is moderate, then tilting of the light chain region accompanies filament sliding (step 7), followed by ADP release (step 8) and then rapid ATP binding (step 1), which induces cross-bridge detachment (step 2). Hydrolysis of ATP by S1 reforms M·ADP·P_i and AM·ADP·P_i (step 3).

Evidence that the power stroke is linked most closely with an isomerization of AMADP·P_i (step 5), release of P_i from the active site (step 6), and an isomerization of AM·ADP (step 7) comes from biochemical studies with the purified proteins and from physiological studies on skeletal muscle fibers. In vitro, about half of the total free energy available from ATPase hydrolysis is liberated on P_i release from acto-S1 (White and Taylor, 1976). Active force production by permeabilized (skinned) muscle fibers (Cooke and Pate, 1985) is decreased in the presence of P_i. The rate constant for contraction after laser photolysis of caged ATP is increased with the concentration of P_i (Hibberd et al., 1985). These results imply that the force generating transition is linked to P_i release. Oxygen exchange between bound P_i and the solvent indicates that P_i release (step 6) is reversible during active contraction in muscle fibers, which is expected only if this step is coupled to production of the force-generating intermediate (Webb et al., 1986). Experiments with caged P_i (Dantzig et al., 1992) and frequency-dependent stiffness (Kawai and Halvorson, 1991) are most compatible with an isomerization of a low-force AM·ADP·P_i state to a force-producing AM′·ADP·P_i state followed by rapid P_i release.

The release of ADP from actomyosin (step 8) has not generally been thought to be coupled to a major portion of the power stroke. Exchange between radiolabeled ATP and P_i during ATP hydrolysis is independent of ADP concentration, suggesting that AM′·ADP in Scheme 1 is a higher energy state than AM·ADP and that the chemomechanical coupling is completed in step 7 (Sleep and Hutton, 1980).

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Address reprint requests to Dr. Yale E. Goldman, Pennsylvania Muscle Institute, School of Medicine, University of Pennsylvania, D700 Richards Building, Philadelphia, PA 19104-6083. Tel.: 215-898-4017; Fax: 215-898-2653; E-mail: goldmany@mail.med.upenn.edu.

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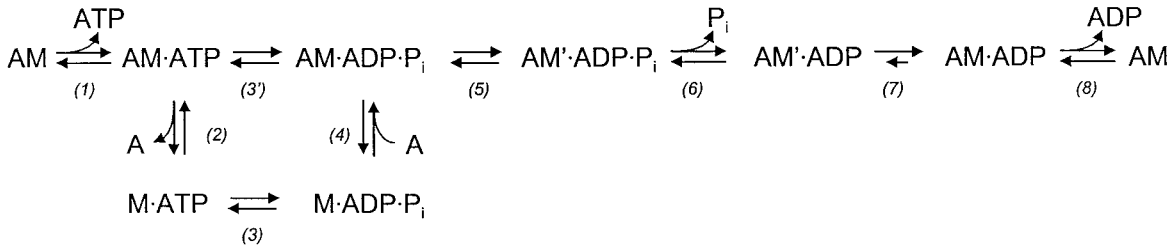

Scheme 1

ADP release from AM·ADP is readily reversible, even in the absence of mechanical strain (e.g., with isolated proteins in solution; White and Taylor, 1976; White, 1977; Kodama, 1985; Greene and Eisenberg, 1980; Geeves, 1989), and the dissociation constant of actomyosin for MgADP is in the same range as the free MgADP concentration in intact skeletal muscle fibers (Marston, 1973; Schoenberg and Eisenberg, 1987; Cooke and Pate, 1985; Biosca et al., 1988; Rosenfeld and Taylor, 1987a,b). Thus little, if any, energy can be liberated on release of ADP (Marston and Tregear, 1972; Dantzig et al., 1991; Gollub et al., 1996). Binding of MgADP to the cross-bridges in skinned skeletal muscle preparations in rigor (the absence of ATP) causes only very small changes in force (Marston et al., 1979; Schoenberg and Eisenberg, 1987; Dantzig et al., 1991) and structural signals, such as the orientation of fluorescent (Allen et al., 1996) or spin label (Fajer et al., 1990) probes placed on the myosin regulatory light chain, fiber birefringence (Obiorah and Irving, 1989), and x-ray diffraction spectra (Takemori et al., 1995), all indicating only small movements of the head when MgADP binds to AM.

Thus it was surprising when Whittaker et al. (1995) reported cryoelectron microscopic evidence that binding and release of MgADP to smooth muscle S1 decorating actin filaments tilt the light chain domain of the molecule axially by ~23°, resulting in a 3.5-nm movement at the head-rod junction. If the power stroke of smooth muscle S1 is comparable to that of skeletal myosin (5–10 nm), tilting associated with MgADP release would contribute a significant component of the power stroke. According to Whittaker et al. (1995), this change represents an extra movement of smooth S1 beyond the rigor conformation of skeletal S1. In similar experiments with a nonmuscle myosin I from intestinal brush border, Jontes et al. (1995) and Jontes and Milligan (1997) obtained an even greater tilt on binding MgADP. The difference between effects of MgADP on the myosin isoforms was further investigated using paramagnetic probes bound to the regulatory light chain of both smooth and skeletal S1 diffused into a skeletal muscle preparation stretched beyond filament overlap (Gollub et al., 1996). The exogenous S1 decorates the actin filaments. With the addition of MgADP, the probes bound to smooth S1 tilted by 20°, whereas probes bound to skeletal myosin hardly changed. Thus tilting of the light chain domain induced by MgADP release occurs in a subset of myosins.

The function of the extra tilt in smooth and nonmuscle myosin I heads is not clear. The main goal of the present studies was to determine whether this movement contributes to filament sliding during smooth muscle contraction. If the structural change detected when MgADP binds to smooth muscle S1 decorating actin filaments takes place within the intact filament lattice of smooth muscle cells, then addition of MgADP to a skinned smooth muscle in rigor should result in a substantial reduction of tension. We tested this prediction in skinned strips of chicken gizzard cells. For comparison, we also tested the effect of MgADP on rigor tension in slow (soleus) and fast (psoas) rabbit skeletal muscle. The addition of MgADP to the gizzard muscle during rigor did not cause the anticipated decrease in rigor tension, but rather, tension increased slightly. This observation implies that the change in orientation detected in previous structural studies on smooth muscle S1 is not closely linked to a force-producing component of the power stroke. Possible causes and functions of this motion are discussed. Some of these results have been presented in abstract form (Dantzig et al., 1997).

MATERIALS AND METHODS

Muscle preparations

Strips of triton-skinned chicken gizzard fibers were prepared as described by Barsotti et al. (1987). Briefly, fresh gizzards from female chickens were obtained from Tony and Sam's Live Poultry (Philadelphia, PA) and packed on ice. Within 1 h, the surface fascia on the lateral sides of the gizzard were removed carefully with a pair of fine forceps. With a scalpel, circumferential 5-mm-deep cuts were made ~3 mm apart. Thin sheets of tissue, ~1 mm thick, were gently removed from the gizzard and transferred into a sylgard-coated dish containing skinning solution (Table 1). The sheets were pinned at a slightly taught length and incubated for at least 3 h at 4°C. The solution was exchanged for a storage solution (Table 1), and the preparation was left overnight (~14 h) at 4°C. The next morning, the preparation was rinsed with fresh storage solution and then stored at −20°C for up to 1 week.

On the day of an experiment, small strips of tissue, ~3–5 mm long and 100–400 μm in diameter were isolated from a strip. Each end of the strip was trimmed, wrapped in T-shaped aluminum clips (Goldman and Simmons, 1984), and transferred via a fine glass rod to a 60-μl solution trough in the apparatus. The T-clips were placed over sharpened steel hooks to suspend the preparation between a force transducer (Goldman et al., 1984) and a servo-controlled moving coil motor (step response of 180 μs; Lombardi and Piazzesi, 1990). The muscle strip was initially stretched by a few percent over slack length to produce a small amount of passive force.

Individual 1–2-mm-wide strips of rabbit psoas and soleus muscle fibers were dissected from male New Zealand white rabbits (greater than 8 pounds, but not fatty), tied with silk suture to wooden rods, skinned, and stored as described by Goldman et al. (1984). Protease inhibitors, leupeptin (Roche Molecular Biochemicals, Indianapolis, IN; 0.01 mg/ml), phenyl-

TABLE 1 Solutions used

Solution	TES	IMID	CaEGTA or EDTA	EGTA	HDTA	$MgCl_2$	Mg^{2+}	Nucleotide	Mg-nucleotide	CP	GLH	Γ
Relaxing												
5.0 REL	100	—	—	25	—	7.7	1	5.2	5	19	10	200
0.1 REL	100	—	—	30	—	2.7	1	0.12	0.1	21.6	10	200
Preactuvating												
5.0 PreACT	100	—	—	0.1	24.9	6.9	1	5.2	5	19.5	10	200
ATPγS PreACT	100	—	—	0.1	52	2.4	1	1.1	1	—	10	200
Activating												
5.0 ACT	100	—	25	—	—	6.8	1	5.2	5	19.5	10	200
ATPγS ACT	100	—	20	—	32	2.2	1	1.1	1	—	10	200
Rigor												
RIG (R-200-1)	100	—	—	53	—	3	1	—	—	—	10	200
R-14-2	—	10	—	1	—	2	2	—	—	—	1	14
R-50-2	—	10	—	1	11	2.3	2	—	—	—	1	50
R-50-4	—	10	—	1	9	4.4	4	—	—	—	1	50
R-75-1	100	—	—	23	—	1.7	1	—	—	—	1	75
EDTA-RIG (R-200-0)	100	—	15	—	27	—	—	—	—	—	10	200
ADP-rigor												
ADP-RIG (D-200-1)	100	—	—	20	26	4	1	4.8	2	—	10	200
D-50-4	—	10	—	1	6	12	4	10	8	—	1	50
D-75-1	100	—	—	1.9	—	8.6	1	16	7.5	—	1	75
Gizzard skinning solution*	100	—	—	30	—	7.2	1.0	5.2	5.0	—	2	200
Gizzard storage solution#	100	—	—	30	—	7.2	1.0	5.2	5.0	—	2	200

Values listed are in mM. All solutions are titrated to pH 7.1 at 20°C. The rigor and ADP solutions all contained 2 mM hexokinase, 200 mM glucose, and 250 μM AP_5A. Nucleotide is ATP for REL, PreACT, ACT, and skinning solutions, ATPγS and ADP are in their respective solutions. ATPγS solutions contained 1–5 mM calmodulin to activate the myosin light chain kinase activity. ACT or activating solutions contained CaEGTA, and EDTA-RIG contained EDTA.

*The gizzard skinning solution contained 44.6 mM Na-propionate as the major anion and 1% Triton X-100 to disrupt the membrane.

#The gizzard storage solution contained 49.8 mM K-propionate as the major anion and 50% glycerol.

methylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO; 0.2 mM), and trypsin inhibitor (Sigma Type IIS, 0.5 mg/ml) were added to the storage solution. On the day of an experiment, single muscle fibers were dissected from a strip under silicone oil (viscosity 10 cs; Dow Corning), which was cooled to 12°C. T-clips were folded around the ends of a 3-mm-long segment (usually 75–100 μm in diameter), and then the fiber was transferred and suspended in the same apparatus.

Purification of ADP

ADP was purified (>99.8%) by DEAE column chromatography as described by Dantzig et al. (1991). The purified triethylammonium salt of ADP was dissolved in deionized water, adjusted to pH 7.0, partitioned into single-use aliquots, rapidly frozen with liquid nitrogen, and stored at −80°C. Aliquots were used within 4 months. Storage of ADP at −20°C did not maintain purity sufficient for these experiments.

Solutions

A computer program, originally developed by J. Thorson, D. S. C. White, and Y. E. Goldman and subsequently upgraded to a PC Windows application by A. Fielding and R. J. Barsotti (personal communication), was used to design the solutions listed in Table 1. The program solves the multiple metal-ligand binding equilibria of the constituents based on stability constants obtained from published data and/or tabulated in Martell and Smith (1974).

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. and were of the highest purity available. 1,6-Diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (HDTA) was purchased from Fluka (Ronkokoma, NY), and ATPγS was purchased from CalBiochem (San Diego, CA). The pH of all solutions was adjusted to 7.1 at 20°C. All rigor solutions with and without added ADP contained 5–10 units/ml hexokinase (Type F-300), 200 mM glucose, and 250 μM diadenosine pentaphosphate (AP_5A) to minimize contaminant ATP.

Solution exchanges

The bathing solution was exchanged either by transferring the tissue to another trough containing the new solution (Goldman et al., 1984; Dantzig et al., 1998) or by manually flowing the desired solution into one end of the trough with a pipette, while applying suction at the other end to evacuate the excess solution and maintain a stable meniscus (Dantzig et al., 1991). The latter method minimized mechanical artifacts caused by passage through a solution/air interface during the transfer of solutions and was usually used when the tissue was in rigor.

Protocol

For strips of smooth muscle, rigor was induced either by exchanging the relaxing solution with several rinses of a nucleotide-free rigor solution and then stretching the muscle to produce tension, or by first activating the relaxed strip and then transferring to the rigor solution. Steady activation was achieved by transferring the muscle strip to a solution containing 1–5 μM calmodulin and pCa 4.5. Alternatively, the muscles were thiophosphorylated in a solution containing ATPγS (Table 1, ATPγS-ACT), 30 μM free Ca^{2+}, and 5 μM calmodulin. When the regulatory light chain was thiophosphorylated, the strip was activated by incubating in relaxing solution (5.0 REL, pCa 8) for a Ca^{2+}-free contraction. The solution was then exchanged for rigor solution without added Ca^{2+} or nucleotides (RIG, Table 1). A >2 min wash of the strip with EDTA rigor solution was used to reduce MgADP bound to the muscle preparations. The strip was then returned to the standard rigor solution. Tension and the in-phase component of the 500-Hz stiffness were monitored throughout the experiment.

To induce rigor in skeletal muscle experiments, the fiber was rinsed in a low (0.1 mM) MgATP solution (0.1 REL, Table 1) twice for 2 min each time and then transferred to the rigor solution.

RESULTS

Glycerol-extracted strips of chicken gizzard muscle or single glycerol-extracted fibers from rabbit psoas or soleus muscle were put into rigor as described in Materials and Methods. The effects on rigor force and stiffness of adding 2 mM MgADP to the medium were tested in each muscle type (Fig. 1). In these experiments an enzymatic ATP-depleting system (glucose, hexokinase, and AP_5A) was included in all rigor and ADP-containing solutions. The ionic strength (200 mM) and free Mg^{2+} (1 mM) concentration were kept equal to their values in the ADP-free rigor solution.

In the smooth (Fig. 1 *A*) and soleus (Fig. 1 *B*) muscle preparations, addition of 2 mM MgADP to the rigor solution (ADP-RIG) caused a relatively small but consistent increase in tension, with a concomitant decrease of the in-phase component of 500-Hz sinusoidal stiffness. In psoas fibers (Fig. 1 *C*), both rigor tension and in-phase stiffness decreased when MgADP was added to the rigor solution, as reported before (Dantzig et al., 1991). Average magnitudes of the tension and stiffness in MgADP relative to the rigor values are shown in Fig. 2 *A* and Table 2. The direction and magnitudes of these MgADP-induced mechanical changes were similar in solutions with ionic strengths of 50–200 mM and free Mg^{2+} concentrations of 1–4 mM (Table 2), provided the rigor solutions with and without MgADP were matched in ionic strength and [Mg^{2+}] (matched solutions).

In psoas and soleus fibers, the mechanical effects of adding MgADP were reversed by several washes of the nucleotide free rigor solution containing the ATP-depleting system. Rigor tension and stiffness slowly returned to nearly their original rigor levels (Fig. 1, *B* and *C*). In smooth muscle, the effects of MgADP were partially reversible.

One possible explanation for the small effect of MgADP on rigor tension is the presence of a substantial population of cross-bridges with MgADP bound in rigor. This is especially likely in smooth muscle, which has a higher myosin affinity for MgADP than striated muscle. To reduce the residual MgADP binding in rigor, the smooth muscle strips were incubated in an EDTA-rigor solution (Table 1, EDTA-RIG) for 2 min or more to chelate the Mg^{2+} and reduce the actomyosin affinity for ADP. The strips were then returned to the standard rigor solution (RIG, Table 1). The data in Figs. 1 and 2 were obtained using this protocol. Without the preincubation in EDTA-RIG solution, addition of MgADP to the rigor fiber induced a small (4–10%) decrease in the stiffness, while tension remained nearly constant.

The effectiveness of EDTA in the removal of MgADP was tested by measuring the rate of relaxation from the rigor state. Because MgADP and MgATP compete for the same site on myosin, the presence of bound MgADP would be expected to slow relaxation from rigor when ATP is added; conversely, reducing the amount of bound ADP would speed up relaxation. After exposure to MgADP, ATP-in-

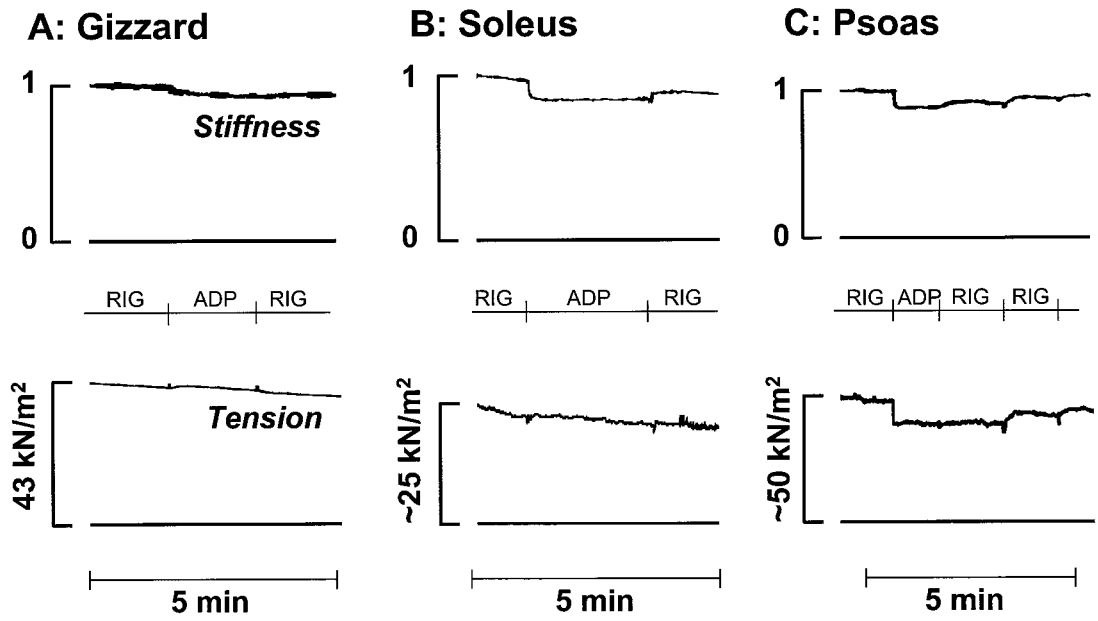

FIGURE 1 Tension and stiffness recordings from a skinned strip of chicken gizzard muscle (*A*) and single skinned fibers of rabbit soleus (*B*) and psoas (*C*) muscle. The upper and lower traces in each panel are recordings of the in-phase, 500-Hz sinusoidal stiffness (relative to stiffness in RIG) and rigor force, respectively. The flat lines below each recording represent the baseline levels (measured in 5.0 REL for smooth muscle and 0.1 REL for skeletal muscle) for stiffness and tension. Tick marks on the central bar indicate solution exchanges alternating between rigor (RIG) and ADP-RIG (ADP; see Table 1). All solutions were 200 mM ionic strength and contained 1 mM free Mg^{2+}, an ATP depletion cocktail of glucose, hexokinase, and AP_5A (described in Materials and Methods). Chicken gizzard strip dimensions: length = 3.55 mm, cross-sectional area = 25,768 μm^2. The fiber diameters used to normalize the tension measurements for both the soleus (5730 μm^2) and psoas (6000 μm^2) fibers represent average values for these fiber types. The lengths of these fibers were ~3 mm.

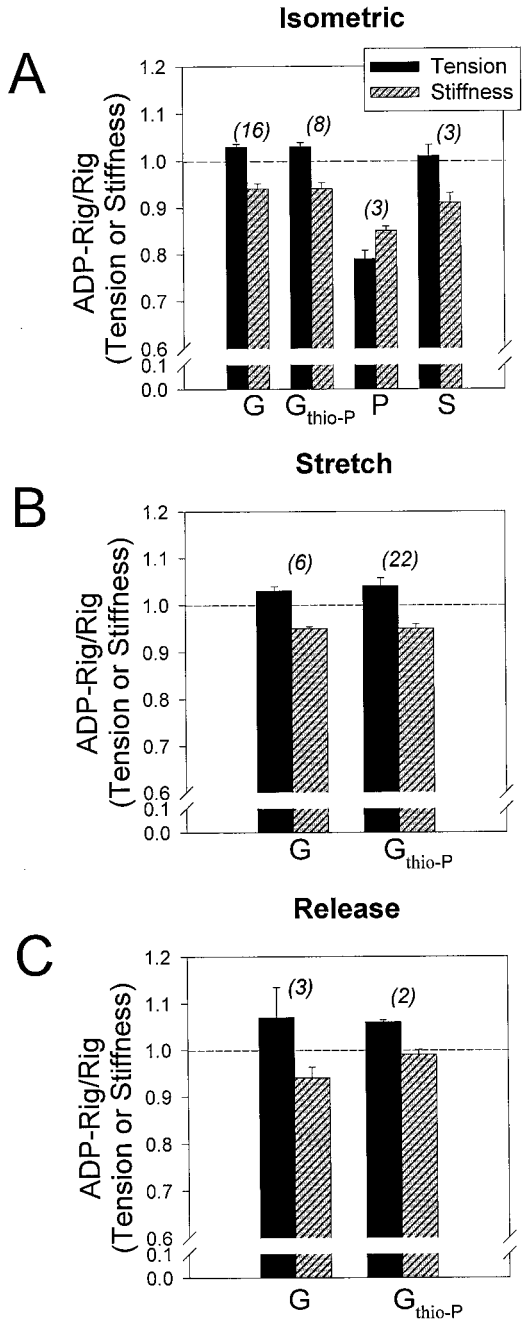

FIGURE 2 Tabulated ratios of tension and stiffness (ADP-RIG/RIG) from skinned strips of gizzard muscle without (G) and with thiophosphorylation (G_{thio-P}) and single skinned fibers of psoas (P) and soleus muscle (S). The values are means ± SEM of tension (■) and stiffness (▨) relative to their values in rigor (RIG) after flow through of MgADP. The numbers of fibers are indicated above each set. In *A*, the preparations were held at constant length throughout the protocol. In *B* and *C*, rigor tension of gizzard strips was approximately doubled or halved, before the introduction of MgADP, by applying several stretches or releases to the preparation (see Fig. 4). These values are also listed in Table 2.

TABLE 2 Average magnitudes of the tension and stiffness in MgADP relative to the rigor values

	Tension ratio	Stiffness ratio
Isometric D-200-1:R-200-1		
Gizzard	1.03 ± 0.03 (16)	0.94 ± 0.04 (16)
$Gizzard_{thio-P}$	1.03 ± 0.02 (8)	0.94 ± 0.04 (8)
Psoas	0.79 ± 0.03 (3)	0.85 ± 0.02 (3)
Soleus	1.01 ± 0.04 (3)	0.91 ± 0.04 (3)
Stretch D-200-1:R-200-1		
Gizzard	1.03 ± 0.02 (6)	0.95 ± 0.01 (5)
$Gizzard_{thio-P}$	1.04 ± 0.03 (3)	0.95 ± 0.02 (3)
Psoas	0.82 ± 0.04 (2)	0.89 ± 0.01 (2)
Release D-200-1:R-200-1		
Gizzard	1.07 ± 0.11 (3)	0.94 ± 0.04 (3)
$Gizzard_{thio-P}$	1.06 ± 0.01 (2)	0.99 ± 0.01 (2)
Psoas	0.68 (1)	0.87 (1)
D-50-2:R-50-2		
Gizzard	1.02 ± 0.02 (4)	0.98 ± 0.02 (4)
$Gizzard_{thio-P}$	1.06 ± 0.00 (2)	0.96 ± 0.06 (2)
Psoas	0.78 ± 0.08 (2)	0.87 ± 0.01 (2)
D-75-1:R-75-1		
Gizzard	1.03 (1)	0.53 (1)
Soleus	1.03 (1)	0.67 (1)
D-50-4:R-14-2		
Gizzard	0.80 ± 0.10 (13)	0.80 ± 0.09 (13)
$Gizzard_{thio-P}$	0.77 ± 0.07 (6)	0.84 ± 0.06 (6)
Psoas	0.67 ± 0.09 (3)	0.80 ± 0.04 (3)
Soleus	0.86 ± 0.04 (4)	0.78 ± 0.11 (4)

All values are mean ± standard deviation for *n* fibers (in parentheses). Note that error bars in Fig. 2 are the standard errors of the mean. The subscript thio-P indicates that the regulatory light chains of the gizzard muscle were thiophosphorylated.

duced relaxation of gizzard strips was much faster ($t_{1/2}$ = 18 ± 6 s, mean ± SEM, n = 8) after a brief wash in EDTA-RIG solution (Fig. 3 *A*) than when the EDTA wash was eliminated ($t_{1/2}$ = 99 ± 35 s, n = 8; Fig. 3 *B*). This difference is mostly caused by an increased amplitude of an initial component of relaxation within the first 20 s. These results indicate that MgADP was retained within the strip in the rigor solution, but the amount was reduced by exposure to the EDTA-RIG solution.

A number of other factors were explored. These included the phosphorylation state of myosin, mechanical strain, free Mg^{2+} concentration, ionic strength, and the series compliance of gizzard skinned muscle.

Phosphorylation

One of the important mechanisms of smooth muscle regulation is phosphorylation of the regulatory light chain (RLC). Because isolated S1 is constitutively switched on whether phosphorylated or dephosphorylated as in the in vitro studies, we tested whether the effects of MgADP on gizzard muscle mechanics in rigor are modulated by RLC phosphorylation. The RLCs were thiophosphorylated by incubating the muscles in an activating solution containing ATPγS (Table 1, ATPγS-ACT), ~30 μM free Ca^{2+}, and 5 μM calmodulin. Approximately 60–90% of the myosin regulatory light chains in permeabilized strips of chicken gizzard muscle are thiophosphorylated under these conditions (Kenney et al., 1990).

Thiophosphorylation of the smooth muscle RLC did not appreciably alter the direction or magnitude of the changes

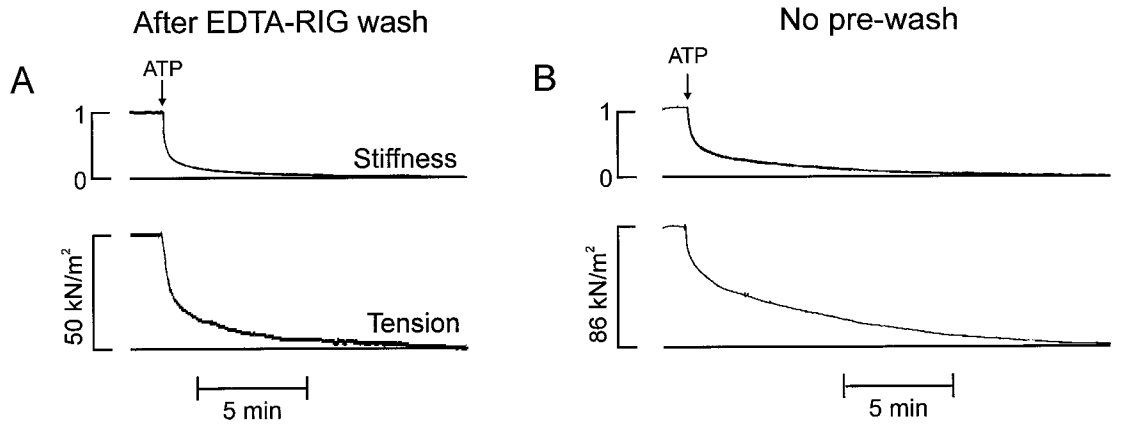

FIGURE 3 Stiffness and tension recordings from skinned strips of chicken gizzard muscle in rigor upon the addition of MgATP (5.0 REL). In *A* the strip was briefly incubated in EDTA-RIG to reduce the amount of MgADP-bound cross-bridges, returned to the standard rigor solution (RIG), and then relaxed. The preincubation with EDTA-RIG was eliminated for the transients shown in *B*. This EDTA-RIG wash markedly reduced the half-time of relaxation. Note that the amplitude of a fast component of relaxation is reduced in *B*. Strip dimensions were (*A*) cross-sectional area = 9035 μm^2, length = 3.2 mm; (*B*) cross-sectional area = 15,486 μm^2, length = 2.76 mm.

in either rigor force or stiffness associated with the addition of MgADP (Table 2 and Fig. 2, *bars labeled* $G_{thio\text{-}P}$). These changes in force and stiffness with the addition of MgADP were similar at ionic strengths from 50 to 200 mM or free [Mg^{2+}] from 1 to 4 mM, whether the RLC was phosphorylated or not.

Mechanical strain

Mechanical strain imposed on the cross-bridge by the filament lattice of the muscle cell might affect motions of the myosin head resulting from nucleotide binding. To test whether effects of MgADP on rigor mechanics in smooth muscle are strain-dependent, muscle strips were stretched or released by small amounts before the rigor solution was exchanged for the ADP-rigor solution (Fig. 4). Because tension recovered toward the isometric level after these length changes, additional small stretches or releases were applied to the strip until tension was approximately double or half of the isometric rigor tension.

When rigor tension was approximately doubled by the applied stretch, addition of MgADP caused tension to increase slightly (3–4%; Fig. 4 and Table 2) and stiffness decreased by ~5%. These changes are virtually identical to the isometric condition and took place whether the RLC was unphosphorylated (Fig. 4 *A*) or thiophosphorylated (Fig. 4 *B*). When the isometric tension was approximately halved by applying a small release to the gizzard strip, MgADP caused tension to increase by 6–10% and stiffness to decrease by ~6%, values that are slightly higher than the isometric ones (Table 2 and right side of Fig. 4).

In none of these cases was rigor tension observed to decrease as a result of changes in MgADP concentration, as would be expected if the binding of MgADP results in a reorientation of the light chain region toward the pointed end of the thin filaments.

Solution constituents

The solutions used for the in vitro studies of Whittaker et al. (1995) that demonstrated tilting of the light chain region upon MgADP binding differed from those used for Figs. 1–4 in ionic strength, free [Mg^{2+}], and [MgADP]. We tested the mechanical effects of adding MgADP using so-

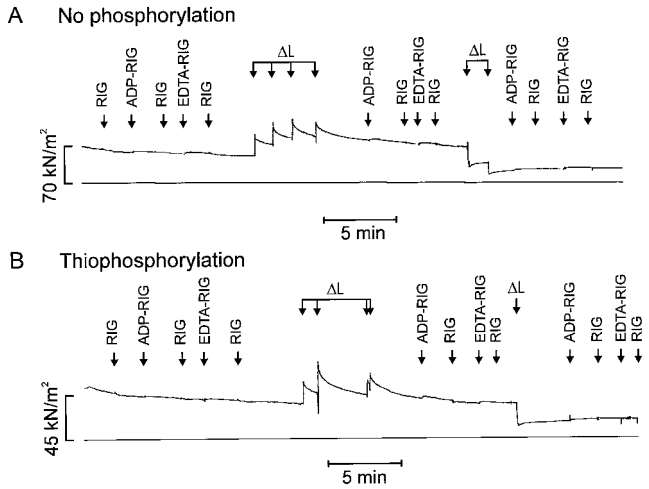

FIGURE 4 The effects of altered strain on MgADP-induced changes in rigor tension. The recordings came from two different strips of chicken gizzard muscle without (*A*) and with thiophosphorylation (*B*) of the regulatory light chain. The flat lines below each tension record represent the relaxed tension levels in 5.0 REL. The solution exchanges and changes in length (ΔL) are indicated with arrows. In each sequence, fiber length was held constant for the first addition of MgADP. The ADP was washed out by brief incubations in RIG, EDTA-RIG, and RIG again. The strip was then stretched several times to increase the rigor tension. When the tension stabilized at the new level, MgADP was again introduced (ADP-RIG). The same protocol was repeated after the fiber was released to obtain rigor tension approximately half that of the isometric level. Strip dimensions: (*A*) cross-sectional area = 9035 μm^2, length = 2.9 mm; (*B*) cross-sectional area = 8185 μm^2, length = 2.21 mm.

lutions of the same composition as used in their study. Our nomenclature for these solutions is as follows: rigor solutions are designated with an R and ADP solutions with a D (Table 1). A number indicates the ionic strength in mM, and a second number represents the [Mg^{2+}]. For example, R-50-2 is a rigor solution with 50 mM ionic strength and 2 mM [Mg^{2+}]. In this convention, the rigor solution primarily used by Whittaker et al. (1995) is R-14-2, and the ADP solution, containing 8 mM MgADP, is D-50-4. Exchange of the standard rigor solution (RIG or R-200-1) bathing a gizzard muscle strip for R-14-2 caused the rigor force and stiffness to increase by 40 ± 2% (mean ± SEM, n = 8). A subsequent change to D-50-4 caused tension to decrease to 80 ± 3% (n = 13) and stiffness to decrease to 80 ± 2% relative to their values in R-14-2 (Table 2 and Fig. 5 *A*). From solution D-50-4, removal of the ADP with R-50-4, a solution matched in ionic strength and [Mg^{2+}], resulted in a small decrease in tension (<2%) and a 3–5% increase in stiffness (Fig. 5 *A*).

Experiments illustrated in Fig. 5, *B* and *C*, distinguish the contributions made by changes in the ionic strength and [Mg^{2+}] to the drop in force observed in Fig. 5 *A*. When the solution was changed from R-14-2 to R-50-4 in Fig. 5 *B*, matching the ionic strength and free [Mg^{2+}] change of Fig. 5 *A* but without ADP, the force and stiffness dropped by approximately the same amount, with the change from R-14-2 to D-50-4. When the ADP was subsequently added by flowing through D-50-4 (Fig. 5 *B*), tension increased slightly (1.5%), while stiffness decreased (4%). Removal of ADP and reduction of Mg^{2+} from 4 to 2 mM resulted in a partial recovery of tension and stiffness toward the R-14-2 value. Finally, a decrease in ionic strength from 50 to 14 mM returned the rigor force and stiffness back to their original values.

The effects of increasing ionic strength (R-14-2 → R-50-2, Fig. 5 *C*) and [Mg^{2+}] (R-50-2 → R-50-4) were additive. Thus increasing ionic strength and/or [Mg^{2+}] decreased rigor tension and stiffness, whereas adding [MgADP] to solutions matched in ionic strength and [Mg^{2+}] caused an increase in tension and a decrease in stiffness of gizzard muscle.

The solutions used for the in vitro experiments were also tested in psoas muscle (Fig. 6). At a constant 200 mM ionic strength and 1 mM Mg^{2+}, the introduction of 2 mM MgADP (D-200-1) decreased force (see also Figs. 1 and 2). Exchange of R-14-2 for R-200-1 resulted in a 25% increase in force and a 5% increase in stiffness. When this low-ionic-strength solution was exchanged with the ADP solution, D-50-4, force and stiffness dropped 42% and 22% of their values in R-14-2. Thus solution ionic strength and free [Mg^{2+}] have similar effects on rigor tension and stiffness in psoas and gizzard muscle.

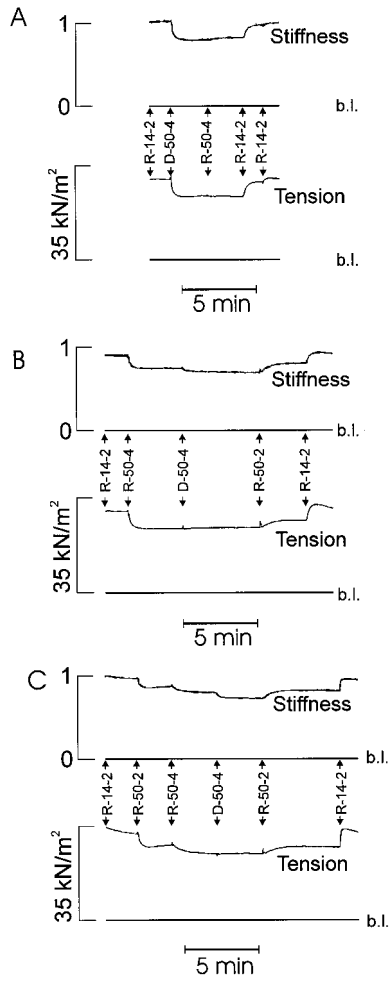

FIGURE 5 Comparison of mechanical responses of gizzard muscle in rigor, due to changes in ionic strength, free [Mg^{2+}], and [MgADP]. The upper and lower sets of traces in each panel are recordings of the stiffness and tension with their corresponding baselines. In each panel, the arrows indicate solution changes. The arrow labels indicate the solution constituents. Rigor is designated by R, MgADP-rigor by D. The two numerical values in each solution label indicate the ionic strength and free [Mg^{2+}] in mM. *A* illustrates the responses to the solutions used by Whittaker et al. (1995). *B* illustrates the responses to solutions that mimic those used by Whittaker et al. but do not contain MgADP. The addition of MgADP (D-50–4) causes tension to rise. *C* illustrates the isolated effects of increasing Mg^{2+} and ionic strength. Strip dimensions: cross-sectional area = 15,486 μm^2, length = 2.76 mm.

Muscle stiffness and series compliance

A final possible explanation we considered for the very small size of changes in force and stiffness with addition of MgADP to gizzard muscle is that the observed response is suppressed by mechanical compliance in series with the cross-bridges. We investigated the mechanical compliance of the smooth muscle strips by applying quick shortening and lengthening steps to relaxed, rigor, and active muscle strips. Transients measured in rigor and relaxation are shown in Fig. 7 *A*. In all three conditions, tension changed simultaneously with the length change, because of elastic components in the muscle, and then partially recovered because of viscoelasticity. Compared to rigor, the extent of this recovery was greater in the active contraction (data not shown).

T_1, the extreme tension deflection simultaneous with the length change, relative to the active tension, T_0, was plotted versus the length change as a percentage of the initial length (% L_0; Fig. 7 *B*). The solid horizontal axis in Fig. 7 *B*

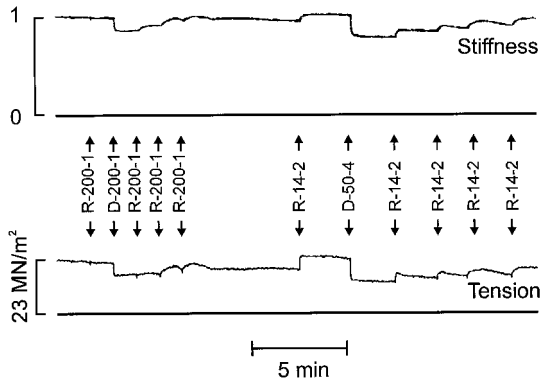

FIGURE 6 Comparison of mechanical responses of a skinned fiber of psoas muscle in rigor, due to changes in ionic strength, free [Mg^{2+}], and [MgADP]. The flat lines below the stiffness and tension show the relaxed level recorded in 0.1 REL. The solution exchanges are indicated by the labeled arrows as in Fig. 5.

indicates the zero tension level (i.e., at slack length), and the dashed horizontal line above it indicates the passive tension in relaxing solution at the muscle length of the experiment. Force-extension data in relaxing solution (*circles*) and rigor (*squares*) were fit by straight lines whose slopes provide values for the stiffness. The ratio of active to rigor stiffness was 1.55 ± 0.40 (mean ± SEM, $n = 5$). The strips were more compliant when relaxed (pCa 8.0), as expected. The results indicate that on average, a length release of 0.50 ± 0.11 ($n = 5$) and 0.78 ± 0.18% L_O ($n = 3$) would reduce force to zero from rigor and from the maximally active level, respectively.

To remove the effects of parallel elasticity, the active and rigor stiffness were corrected by subtracting the line fitted to the relaxed data. The rigor force-extension curve after subtraction of the passive curve is plotted as dashes (RIG_{corr}) in Fig. 7 *B*. For the corrected rigor data, the amount of shortening necessary to reduce rigor tension to zero (at slack tension) is shown by an arrow (y_0) and averaged as 0.94 ± 0.34% L_0 ($n = 5$). The corresponding intercept for active muscle was 1.11 ± 0.34% L_0 ($n = 3$). These values are greater than published data from skeletal muscle fibers, presumably because of compliance in series with the cross-bridges. The consequences of this series compliance for interpretation of the results from these experiments with ADP are discussed below.

DISCUSSION

Several recent papers have reported that the subfragment-1 head (S1) of smooth muscle (Whittaker et al., 1995; Gollub et al., 1996) and nonmuscle (brush border) myosin (Jontes et al., 1995, 1997) bound to actin filaments (AM), changes shape when MgADP binds to the active site (reversal of step 8, Scheme 1). This motion tilts the region of the head containing the regulatory light chain away from the barbed end of the actin filament, translating the C-terminus (the head-rod junction in whole myosin) a distance (Δx) of 3–4 nm. If this motion contributes to force generation in intact smooth muscle, force (F) would be expected to decrease when MgADP binds to rigor myosin heads by an amount given by $\Delta F = \Delta x \cdot S$, where S is the stiffness of the contractile apparatus. The results of the present study did not meet this expectation. As discussed below, neither the state of the smooth muscle nor the experimental conditions could have masked a tension decrease associated with MgADP binding. In fact, tension increased slightly when MgADP bound. Thus the function of the movement observed in smooth myosin S1 is not likely to be associated with force generation.

The present results that changes in [Mg^{2+}] and ionic strength alter rigor tension in gizzard muscle (Figs. 5 and 6), whereas changes in [ADP] have little affect, might at first be interpreted as a challenge to the conclusion of Whittaker et al. (1995) that binding of ADP causes tilting of smooth muscle myosin S1. We do not interpret our results this way because the observations of Whittaker et al. have been confirmed by cryoelectron microscopy (Hanein et al., 1998) and by other techniques (Gollub et al., 1996; Poole et al., 1997). Furthermore, Whittaker et al. did not detect any difference in the structure of smooth muscle S1-decorated actin in rigor when ionic strength was varied between 25 and 150 mM. Changes in rigor tension and stiffness in response to changes in ionic strength have been described previously for smooth and skeletal muscle (Fink et al., 1986; Arheden et al., 1988). It is not clear whether the effects of ionic strength and [Mg^{2+}] are localized in the myosin heads or other structures, such as the filament backbones.

Series compliance

If the increase in rigor tension observed with the addition of MgADP to the rigor smooth muscle is ignored because it is so small, then failure to observe the anticipated tension decrease might be attributed to high tissue compliance in smooth muscle preparations. If compliance were too high, a structural change would not be faithfully transmitted to the exterior of the muscle. Force-extension relationships were measured here for gizzard muscle strips to estimate the series compliance of this tissue. The results indicate that length releases of 0.50% L_0 and 0.78% L_0 would reduce force to zero from the rigor level (Fig. 7 *B*) and maximally active force, respectively. These values agree with earlier reports on smooth muscle elasticity (Mulvany and Warshaw, 1981; Pfitzer et al., 1982; Arheden and Hellstrand, 1991). For instance, Arheden and Hellstrand (1991) obtained corresponding values of 0.4% L_0 for rigor and 0.8% L_0 for active in guinea pig taenia coli. Corrected for parallel elasticity (Fig. 7), the present results indicate that length releases of 0.94% L_0 and 1.11% L_0 would reduce cross-bridge tension to zero from rigor and maximum activation, respectively.

To calculate the strain at the molecular level corresponding to the 1.1% L_0 macroscopic strain present in the active muscle would require knowledge of the number of thick and thin filament pairs (effective sarcomeres) arrayed in series along the muscle. In smooth muscle cells, dense bodies spaced along the muscle axis appear to function as anchor-

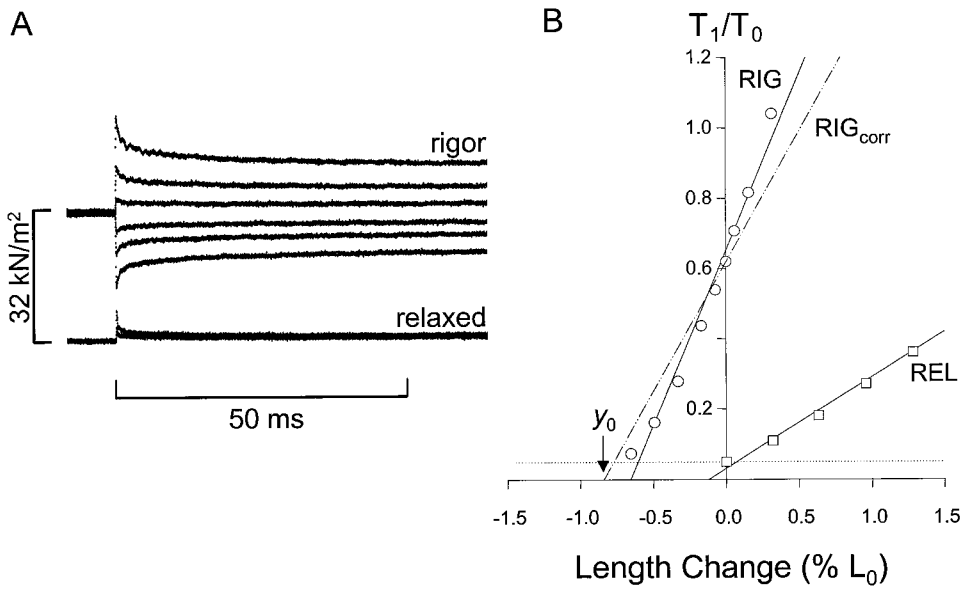

FIGURE 7 Compliance measurements on a strip of chicken gizzard muscle. *A* shows the tension transients recorded during length steps while the strip was relaxed (5.0 REL, *lower traces*) and in rigor (RIG, *upper traces*). The two traces shown for the relaxed strip were recorded during step stretches of 6.5 and 13 μm. Starting from the uppermost traces, the rigor length changes were stretches of 6.5, 3, and 1 μm and releases of 1.5, 3.5, and 7 μm. The extreme tension during the length change (T_1) relative to the active tension (T_0) for the strip is plotted against the length change. The solid lines indicate the linear regression fits to each set of data. The abscissa represents the slack tension, and the horizontal dashed line indicates the passive tension imposed on the strip at the beginning of the experiment ($0.05T_0$). For the strip in rigor, the force-extension curve intercepts the passive tension level at $y_0 = -0.60\%$ L_0. The dot-dashed line (RIG_{corr}) is the algebraic subtraction of the lines fitted to the rigor and relaxed data. Corrected for parallel compliance, $y_0 = -0.84\%$ L_0 based on slack (zero) tension. Strip dimensions: cross-sectional area = 26,453 μm^2, length = 2.04 mm.

ing points for thin filaments, analogous to the Z lines of striated muscle (Ashton et al., 1975; Bond and Somlyo, 1982; Kargacin et al., 1989). The spacing of the dense bodies is quite variable within a cell and among smooth muscle types, but has been observed to be as great as 6 μm (toad stomach muscle; Kargacin et al., 1989). If these irregularly spaced structures are analogous to the Z lines in striated muscle, then the effective half-sarcomere length in smooth muscle could be up to ~3.0 μm. The reduction of tension from maximum activation to zero then corresponds to 1.1% · 3.0 μm = 33 nm of total strain. If we further assume that when bearing full active tension, smooth muscle cross-bridge strain (C_c) is between 5 and 10 nm, then compliance in series with the cross-bridges could be has high as $C_s = 33\text{ nm} - C_c = 23\text{–}28$ nm. This estimate is probably an upper limit; the distance between dense bodies in chicken gizzard muscle has not been reported.

If the binding of MgADP to the rigor heads causes a 3-nm structural change (Whittaker et al., 1995), the rigor tension would decrease by $3/(3 + C_s) = 10\text{–}12\%$, an amount that would have been readily detected in our experiments. Therefore, excessive end compliance does not account for the lack of a decrease in rigor tension when MgADP is bound to the gizzard actomyosin.

Rigor tension was observed to decrease when [Mg^{2+}] or ionic strength was changed, indicating that series compliance did not completely suppress tension changes due to internal structural motions. More importantly, force increased slightly when MgADP was bound, a result that cannot be explained on the basis of tissue series compliance. Thus our results are inconsistent with the hypothesis that the myosin heads bearing rigor force in skinned gizzard muscle tilt axially by ~3 nm when MgADP is added to or removed from the bathing medium.

Residual ADP

The high affinity of smooth muscle actomyosin for MgADP (1–5 μM; Fuglsang et al., 1993; Khromov et al., 1995; Gollub et al., 1996; Cremo and Geeves, 1998) raised a concern that a significant proportion of myosin heads might have ADP bound in rigor. The rigor state of the cross-bridges was typically achieved by first activating the muscle, then removing the Ca^{2+} and ATP from the solution, possibly leaving residual ADP bound to the cross-bridges. In this case, addition of MgADP to the medium bathing the skinned gizzard muscle preparation would not sufficiently alter the proportion of cross-bridges with ADP bound.

We attempted to extract bound MgADP from the gizzard muscle strips in rigor by chelating Mg^{2+} with an EDTA-RIG solution (Table 1). Effectiveness of EDTA in accelerating removal of ADP bound to rabbit skeletal muscle fibers was shown previously by using a spectroscopic signal sensitive to bound ADP (Tanner et al., 1992). Here, preincubation with the EDTA-RIG solution accelerated the relaxation rate when ATP was added to skinned gizzard fibers in the standard rigor solution (RIG, Fig. 3), indicating that chelation of Mg^{2+} was successful in removing some bound ADP. Despite this result, the subsequent addition of

MgADP did not reduce rigor tension in smooth muscle strips pretreated with EDTA-RIG, but stiffness declined slightly when MgADP was added (Figs. 1, 2, 4, and 5). Even without the EDTA-RIG procedure, flow-through of ADP-RIG induced a small (4–10%) but reproducible decrease in stiffness (data not shown), whereas tension remained nearly constant. Thus ADP trapped within the fibers does not explain the lack of tension decrease with the addition of MgADP.

Additional controls

In previous in vitro studies, the S1 heads were not under mechanical strain, whereas in the present experiments they were mechanically strained. We varied the amount of mechanical strain on the cross-bridges by releasing or stretching the strips before the addition of the ADP-RIG. Small changes were observed (Figs. 2 and 4) that do not alter the conclusions. The possibility that the phosphorylation state of the myosin would change the mechanical response due to the introduction of MgADP was addressed in experiments where the regulatory light chain was thiophosphorylated. The phosphorylation state of the myosin did not alter the rigor tension response to the addition of MgADP. Changes in both $[Mg^{2+}]$ and ionic strength did have effects on tension (Fig. 5). However, these effects cannot account for the increased force upon ADP addition that we have observed.

Structural considerations

The fully assembled filament organization of the gizzard muscle could contribute to the apparent difference between our results and those with S1. The connection through S2 and LMM to the backbone of the thick filament would tend to limit motions of the heads produced by ADP binding. Tethering to the whole filament lattice might diminish the flexibility within the head, thereby limiting the motions of the head.

Attachment of both heads of a myosin molecule in rigor requires some distortion, possibly bending of the heads toward each other (Schmitz et al., 1996) or partial unraveling of S2 adjacent to the head-rod junction (King et al., 1995). The free energy associated with this strain affects the nucleotide affinity (Conibear and Geeves, 1998). In fact, higher ADP concentrations were required to cause tilting of an electron paramagnetic resonance probe bound to RLC in gizzard fibers (presumably with double-headed rigor attachments) than in S1 diffused into skeletal fibers (single-headed attachments; Gollub et al., 1998). Thus the different mechanical work term in the free energy for each of the myosin heads seems to modulate ADP binding. In the present experiments, changes in mechanical distortion due to preferential ADP binding to one of the myosin heads in a pair may cause the small mechanical changes observed.

Inasmuch as the addition of MgADP weakens the affinity of the attached heads for actin, our results could be explained by the detachment of a small population of highly strained heads. If negatively strained heads predominantly bind MgADP and detach, the net effect would be an increase in force and a decrease in stiffness upon ADP addition. Note, however, that energetic considerations (Cremo and Geeves, 1998; Gollub et al. 1998) suggest that negative cross-bridge strain would weaken ADP affinity.

Another possibility that should be considered is that the stiffness of S2 constrains motions of the heads induced by ADP binding to the azimuthal direction (around the actin filament axis). An azimuthal structural change could stretch S2, increasing force, and tilt S2 away from the filament axis, thereby decreasing transmission of cross-bridge stiffness to the external connections, consistent with the present results.

Relationship to force generation

The electron microscopic observations by Whittaker et al. (1995) and Jontes et al. (1995) of substantial tilting of the light chain domain when ADP bound to actomyosin seemed to provide direct support for the tilting cross-bridge theory. However, the simple interpretation that this tilting contributes to filament sliding during the cross-bridge power stroke is difficult to reconcile with other data. In skeletal muscle fibers force generation is most closely linked to release of P_i from AM·ADP·P_i (Scheme 1, steps 5 and 6) rather than release of ADP from AM·ADP (Scheme 1, step 8; Hibberd et al., 1985; Cooke and Pate, 1985; Dantzig et al., 1992), and addition of ADP to skeletal muscle fibers in rigor causes only small changes in force (Schoenberg and Eisenberg, 1987; Tanner et al., 1992; Takemori et al., 1995). Electron paramagnetic resonance spectroscopy (Gollub et al., 1996) showed no significant tilting of skeletal myosin on ADP binding, but tilting consistent with the EM data with smooth muscle myosin. Thus a postulated extra "throw" of the cross-bridge during ADP release would be a special feature of a subset of the myosin superfamily, including smooth muscle myosin II and brush border myosin I.

The dissociation constant for ADP binding to smooth muscle myosin attached to actin is ~1–5 μM (Fuglsang et al., 1993; Khromov et al., 1995; Gollub et al., 1996; Cremo and Geeves, 1998), whereas estimates of the concentration of ADP in the cytoplasm of a smooth muscle cell range from 18 to 100 μM (Clark et al., 1995; Butler and Davies, 1980). Under these circumstances, free energy is not liberated, but rather is absorbed, when ADP is released from the AM · ADP state. Therefore work cannot be derived directly from this transition, although the reaction will proceed in the forward direction because of the overall free energy liberated by ATP hydrolysis.

In a kinetic study, Cremo and Geeves (1998) found that actin binding reduces the affinity of myosin for ADP and accelerates ADP release much less in chicken gizzard myosin than in skeletal myosin. These differences are probably specializations of the myosin isoforms for their different physiological roles. They postulated that the AM state in smooth muscle myosin is similar to a transition state that limits the rate of ADP release from skeletal muscle actomyosin (see figure 7 of Cremo and Geeves, 1998). In this model, mechanical strain on the myosin heads in skeletal muscle affects the rate of ADP release, not the ADP affinity, whereas strain controls the affinity with smooth muscle myosin. The higher free energy of the AM state in smooth

muscle myosin causes tighter ADP binding, but prevents useful work production on ADP release. The present results showing no decrease (in fact, a tiny increase) of force when ADP is added to gizzard muscle in rigor provide strong evidence that ADP release is not energetically coupled directly to sliding of the filaments in this muscle.

Role of ADP release in the smooth muscle cross-bridge cycle

If the structural changes detected when ADP binds to and dissociates from actomyosin are not directly involved with energy transduction, what is their purpose? A role may be found in the reduction of the cross-bridge cycling rate during isometric contractions when the tissue maintains a high level of force. The rate of energy liberation is accelerated during isotonic contractions when the force on the muscle is lower than P_0 (maximum isometric tension), and the contractile filaments slide past one another, i.e., when the muscle does work. Thus the load on the cross-bridges influences their cycling rate.

Smooth muscle is adapted to the task of maintaining force for long intervals with high energetic economy. Strain dependence of the ADP release step would limit the rate at which cross-bridges release ADP in an isometric contraction and thereby reduce ATP consumption. This strain-dependent ADP release mechanism may account in part for the economy of force production in smooth muscle and may contribute to the physiological state of smooth muscle termed *latch*. During latch, dephosphorylated smooth muscle cross-bridges cycle slowly, providing force maintenance with very high economy (Dillon et al., 1981; Hai and Murphy, 1988). Under such conditions, the necessity for an additional stuctural movement to allow ADP release from cross-bridges may be a key element of the latch mechanism (Fuglsang et al., 1993; Nishiye et al., 1993; Khromov et al., 1995). Soleus muscle is also adapted to maintenance of tension with lower energy cost than psoas muscle. In the present experiments, the response of soleus muscle fibers to the addition of ADP was similar to that of gizzard strips rather than psoas fibers (Figs. 1 and 2). Both smooth muscle myosin and the β isoform of myosin II present in soleus muscle bind ADP more tightly than the fast myosin isoform of psoas muscle (Siemankowski and White, 1984). Structural changes on the addition of ADP to actin attached to β-myosin II have not been reported.

SUMMARY

The addition of MgADP to gizzard or soleus muscle causes a small increase in tension and a reduction of stiffness. Psoas muscle does exhibit a reduction of force upon binding ADP in rigor, but the motion is too small to contribute a substantial proportion of the power stroke. These observations, along with earlier biochemical data on the kinetics and energetics of ADP binding to smooth muscle actosubfragment 1, make it highly unlikely that structural changes observed in vitro upon binding of ADP to smooth muscle S1 attached to actin are related directly to force generation or energy transduction in the muscle. However, this structural change may contribute to force maintenance by slowing ADP release from strained heads, thereby increasing the dwell time of the attached cross-bridge.

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