Photolytic Release of MgADP Reduces Rigor Force in Smooth Muscle

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ABSTRACT Photolytic release of MgADP (25–300 μM) from caged ADP in permeabilized tonic (rabbit femoral artery-Rfa) and phasic (rabbit bladder-Rbl) smooth muscle in high-tension rigor state, in the absence of Ca^{2+}, caused an exponential decline (~1.5% in Rfa and ~6% in Rbl) of rigor force, with the rate proportional to the liberated [MgADP]. The apparent second-order rate constant of MgADP binding was estimated as $\sim 1.0 \times 10^6$ M^{-1} s^{-1} for both smooth muscles. In control experiments, designed to test the specificity of MgADP, photolysis of caged ADP in the absence of Mg^{2+} did not decrease rigor force in either smooth muscle, but rigor force decreased after photolytic release of Mg^{2+} in the presence of ADP. The effects of photolysis of caged ADP were similar in smooth muscles containing thiophosphorylated or non-phosphorylated regulatory myosin light chains. Stretching or releasing (within range of 0.1–1.2% of initial Ca^{2+}-activated force) did not affect the rate or relative amplitude of the force decrease. The effect of additions of MgADP to rigor cross-bridges could result from rotation of the lever arm of smooth muscle myosin, but this need not imply that ADP-release is a significant force-producing step of the physiological cross-bridge cycle.

INTRODUCTION

Establishing the relationship between biochemical states and the structural conformations of molecular motors continues to be a major challenge of modern biology, tantalizingly close to being met through the development and application of methods having high temporal (Gutfreund, 1995) and structural resolution (Rayment et al., 1993; Holmes, 1998; Houdusse et al., 2000). At the same time, the hypotheses developed through molecular approaches can and are also tested in vivo by probing the behavior of permeabilized cells and, in particular, muscle. A testable, structure-derived hypothesis was recently developed through the comparison of cryoelectron micrographs of actin-smooth muscle myosin S1 complexes, respectively, with and without bound ADP (Whittaker et al., 1995) and through electron paramagnetic resonance (EPR) studies of S1 introduced into muscle in rigor (Gollub et al., 1996). These studies showed that exogenous MgADP induced an ~23° rotation in the smooth muscle lever arm regions of myosin (Rayment et al., 1993; Dominguez et al., 1998), resulting in a reversal of the power stroke from the rigor position. The observation that the lever arm of the ADP-free rigor structure of skeletal myosin was in a position similar to that of the ADP-bound smooth muscle myosin was interpreted to suggest that ADP release in smooth, but not in skeletal, muscle provides an extra kick to the power stroke of the cross-bridge cycle (Whittaker et al., 1995). Implied in this interpretation is that loading nucleotide-free rigor bridges with MgADP should decrease rigor force. Indeed, MgADP reduces rigor force of skeletal muscle by ~10% (Dantzig et al., 1991; Lu et al., 1993), but curiously, MgADP-induced backward movement of the lever arm, although inferred from fiber x-ray diffraction of skeletal muscle (Takezawa et al., 1999), was not detectable in cryoelectron micrographs of acto-S1 (Whittaker et al., 1995).

Functional significance has been assigned to the AM.ADP cross-bridge formed by smooth muscle, particularly in tonic smooth muscles that are biochemically characterized by lacking a 7-amino-acid insert in the catalytic domain of myosin (present in the phasic isoform) and a high content of the more basic essential (LC_{17b}) light chains (reviewed in Somlyo, 1993). Myosin-II of smooth muscle, as well as non-muscle myosin-II, is activated by phosphorylation of serine-19 of the regulatory (LC_{20}) myosin light chain (reviewed in Hartshorne, 1987; Kamm and Stull, 1989; Somlyo and Somlyo, 1994) and inactivated by dephosphorylation of this residue by a specific smooth muscle myosin phosphatase (reviewed in Hartshorne et al., 1998; Somlyo and Somlyo, 2000). However, smooth muscles and, particularly, tonic smooth muscles, can also maintain force at relatively low levels of LC_{20} phosphorylation and at low ATP cost (ATPase activity) and slow shortening velocity (Somlyo and Somlyo, 1967; Dillon et al., 1981). The very high affinity of smooth muscle myosin for MgADP (Nishiye et al., 1993; Fuglsang et al., 1993; Gollub et al., 1996) and experiments showing that removal of ADP speeded (Fuglsang et al., 1993) whereas its addition slowed relaxation from both isometric contraction and rigor (Khromov et al., 1995) suggested that a strongly bound AM′.ADP state of dephosphorylated myosin contributed to force maintenance at low levels of phosphorylation These observations, attributed to the slow off-rate of ADP from cross-bridges conflicted, if not in fact, at least in appearance with the interpretation (Whittaker et al., 1995) that ADP release causes an increase, rather than a decrease, in force.

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The inference derived from cryoelectron microscopy (Whittaker et al., 1995) that smooth muscle myosin-II and, to an even greater extent, nonmuscle myosin-II (Yontes et al., 1995) is in a lower force state when loaded with exogenous ADP (AM.ADP state) rather than following ADP release, was readily testable by adding ADP to permeabilized smooth muscles in rigor. Surprisingly, however, when this test was performed by diffusing MgADP into the solution bathing smooth muscle, it caused not a decrease, but a small (~1%) increase in rigor force (Dantzig et al., 1999). Control experiments by these authors ruled out the possibilities that LC_{20} phosphorylation, mechanical strain, the effect of compliance, or ADP contamination could account for the unexpected change (increase) in force. Notwithstanding this carefully controlled study, previously we (Somlyo et al., 1988), in collaboration with one of the authors (Y. E. Goldman) of the above cited work (Dantzig et al., 1999), have observed that cooperative force developed by rigor bridges in smooth muscles was clearly detectable under Ca^{2+}-free conditions, when micromolar ATP was released by photolysis of caged ATP, but not when ATP was diffused into fibers. Therefore, we thought that diffusional delays could have also obscured the small changes in rigor force induced by ADP and tested the hypothesis that flash photolysis of caged ADP may reveal such small changes in force that were undetectable by a kinetically less well resolved, diffusion-limited method, without prejudice, however, that a reduction of rigor force by photolytic release of exogenous MgADP would necessarily support the hypothesis that ADP release is a force-producing step in the physiological cross-bridge cycle. Preliminary results have been published (Khromov et al., 2000).

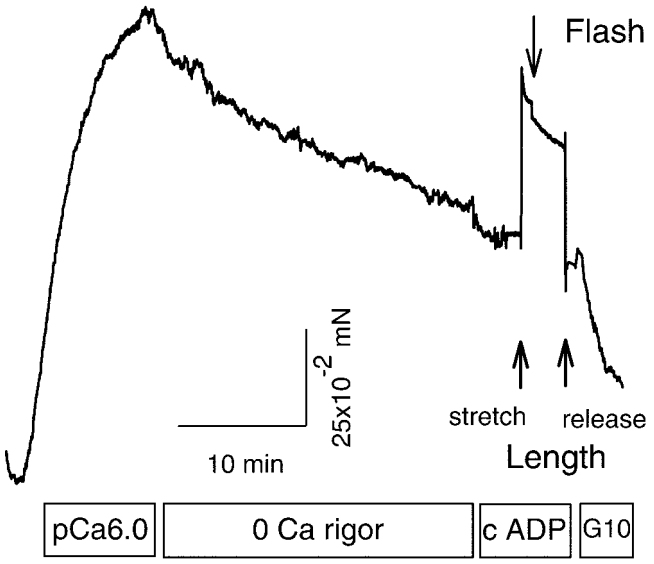

FIGURE 1 The experimental protocol is shown for a rabbit bladder (Rbl) smooth muscle strip permeabilized with α-toxin (130 U/ml for 45 min) and then activated with pCa 6.0 solution in the presence of exogenous creatine phosphate CP (10 mM) and creatine phosphokinase CK (100 U/ml). After the force had reached a plateau, the strip was placed into 0 Ca high-tension rigor state by washing it in ATP- and Ca-free solution for 30–40 min with constant stirring and then transferring it into the photolysis trough containing 1 mM caged ADP for a 5-min incubation period. The muscle was stretched before photolysis (flash). At the end of the experiment the strip was returned to its original length and relaxed in G10 solution, and the photolysis solution was collected for determination of ADP content.

MATERIALS AND METHODS

Dissection of smooth muscles and preparation of strips of rabbit femoral artery (Rfa) and rabbit bladder (Rbl) were described earlier (Fuglsang et al., 1993). In the present study the smooth muscle strips (160–200 μm wide, 2.5–3.2 mm long) were tied and, in addition, glued (Super Bonder 416, Locktite Corp., Newington, CT) to the hooks of the measuring apparatus. The protocol of the experiment is illustrated in Fig. 1. After permeabilization with α-toxin (List Biological Laboratories, Campbell, CA; 130 U/ml for 1 h) or Triton X-100 (0.5% for 15 min) in 0 Ca^{2+} rigor solution (to avoid accumulation of excessive ADP during permeabilization in ATP containing solutions; Table 1), the strips of Rfa or Rbl were relaxed in a Ca^{2+}-free solution containing 10 mM EGTA (G10, Table 1) solution for 10–15 min. Ca-activated contraction was initiated by transferring the strips into pCa 6.0 Ca-activating solution containing 10 mM creatine phosphate (CP), followed by induction of a high-tension rigor state by removal of MgATP (30 min in 0 Ca rigor solution with constant stirring) from the maximally activated smooth muscles (Arner et al., 1987; Somlyo et al., 1988). Alternatively, the strips were first thiophosphorylated (pCa 6.0, in the presence of 2 mM ATPγS and 1 μM calmodulin for 10 min), contracted in Ca-free solution by 4 mM MgATP, and finally, a high-tension rigor state was induced by removal of MgATP as described above. Under these conditions, 80–90% of the regulatory light chains are thiophosphorylated (Khromov et al., 1998). In some cases, the strips were also washed in EDTA rigor solution during the MgATP removal (2 mM EDTA, 10 mM EGTA for 3 min), followed by washing in regular 0 Ca rigor solution for 30–40 min to facilitate removal of the remaining MgADP (Dantzig et al., 1999). Finally, the strips were incubated for 3–5 min with caged ADP in a 30-μl trough with a quartz window. The extent of ADP release was varied by changing either laser energy, by varying the number of glass slides inserted in the optical path between the laser and the muscle trough, or the concentration of caged ADP. Calibration of photolysis efficiency was performed at [caged ADP] = 1mM and various energies illuminating the strips. Following photolysis, the trough solution was analyzed by HPLC and the amount of ADP released quantitated (Fuglsang et al., 1993). The force signal was digitized (sampling rate 10–20 kHz) and stored in the computer with the aid of Acquire Data 3.0 program (National Instrument Corp., Austin, TX). The in-phase stiffness of the strip (determined as the force response in phase with the sinusoidal length oscillations at 1 kHz and amplitude of less than 0.5% L_o) was monitored simultaneously with force. The in-phase and 90°-phase components of the stiffness were separated by a lock-in amplifier (Stanford Research System SR830DSP) in the auto-mode (the amplifier automatically minimized the phase shift between the input and the reference sinusoidal signals before photolysis of caged ADP, thus maximizing the in-phase component of the signal). The stiffness of the strip in relaxing solution was subtracted automatically from the stiffness in rigor by engaging the offset mode of the lock-in amplifier. The time resolution of the stiffness measurements was adjusted to ~10 ms as a compromise between the amplitude of the input signal (which is limited by the amplitude of the length oscillations, ~0.5%, of the strip length) and the level of noise at the output of the low-pass filter of the lock-in amplifier (which is higher at high time resolution). The force transients induced by the photolysis of caged ADP (within the time

TABLE 1 Composition of solutions (total mM) at pH 7.1

	Na_2ATP	$MgMs_2$	CP	Pipes	KMs	EDTA	EGTA	CaEGTA
Relaxing (G10)	4.6	6.07	10	30	70	0	10	0
Ca-activating (pCa 6.0)	4.5	6.4	10	30	50	0	2.11	7.88
0 Ca rigor	0	2.7	0	30	112.9	0	10	0
EDTA rigor	0	0	0	30	110	2	10	0

CP, creatine phosphate; $MgMs_2$, magnesium methanesulfonate; KMs, potassium methanesulfonate.

range of 0–0.2 s) were subjected to a two-exponential (decaying and rising) fit with the aid of Sigma Plot 4.0 software (Jandel Scientific, San Rafael, CA) according to the equation $F = A \exp(-k_1 t) + B[1 - \exp(-k_2 t)]$, where A, k_1, and B, k_2 represent the amplitudes and rate constants of the falling and rising phases, respectively. Within the short time range 0–0.05 s, records were fitted to a single exponential. After photolysis of caged ADP and collecting the data (~1 min), the strips were transferred into 0 Ca rigor solution, and all nucleotides (ADP, ATP, and caged ADP) were washed out (20–30 min in 0 Ca rigor solution under constant stirring). Usually two to three photolysis experiments were performed on a single strip. The ability to develop Ca-activated force was checked after photolysis in each strip; the final force was only 20–30% lower than initial after a few photolysis trials (total time of the experiment, ~40–50 min). Although the amplitude of Ca-activated force was higher (~4 times) in α-toxin-permeabilized strips in comparison with those permeabilized with Triton X-100, the kinetic parameters of ADP-induced effects were not significantly different between the two groups of strips, and the data have been pooled.

Composition of solutions

The composition of the solutions is shown in Table 1. All solutions had an ionic strength of 0.2 M and pH buffer capacity of 30 mM PIPES. The pH was adjusted to 7.1 at 20°C. The photolysis solution contained 20 mM glutathione (GSH) (with proportional reduction in potassium methanesulfonate) for neutralizing possible effects of the photolysis by-product, nitroso-ketones.

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO), except for caged nucleotides (Molecular Probes, Eugene, OR) and ATPγS (Boehringer-Mannheim, Mannheim, Germany). Caged ADP was cleaned by apyrase treatment (100 μg/ml) before use, as described previously (Nishiye et al., 1993).

Statistics

Data are presented as means ± SE; n is the number of measurements. The comparison of data was performed using non-paired Student's t-test, and $p < 0.05$ was taken as significant.

RESULTS

Photolysis of caged ADP in muscles in rigor

The force and stiffness traces demonstrating the main events following photolysis of 2 mM caged ADP in Rbl smooth muscle are shown in Fig. 2. Release of ~200 μM MgADP produced a rapid (complete in ~20 ms) ~5% decrease in rigor force. The stiffness signal immediately after photolysis was not reliable for quantitative analysis, due to the limited time resolution (see Materials and Methods). During the first ~10 ms the signal often showed a sharp burst or drop, followed by recovery to a new level. After this transient artifact, the in-phase stiffness consistently decreased, following the decrease in rigor force. The force-to-stiffness ratio did not significantly change after photolysis of caged ADP: 1.02 ± 0.3. The fast decrease in rigor force was followed by a relatively slow (rate constant 2–6 s^{-1}) small recovery phase, reflecting, probably, a recoil of the elastic elements. The results of fitting to a single exponential (Fig. 2 *A*) slightly overestimated the rate constants and underestimated the amplitudes (within 10–20%); therefore, fitting to two exponentials has been used. No recovery in force or stiffness was observed in the strips that had been prestretched before photolysis (for summary of experiments, see below).

Over a longer time of observation (5–10 min) the amplitude of initial reduction in force slowly recovered, probably reflecting cooperative cycling through resynthesis of ATP from ADP by myokinase and/or ADP hydrolysis by the ecto-ADPase (Trinkle-Mulcahy et al., 1994). Within the first 2 min after photolysis of MgADP (200 μM), rigor force in Rbl smooth muscle recovered 51 ± 2.5% and 62 ± 3.6% ($n = 5$) of the initial reduction in force in the presence and absence of 250 μM Ap_5A (P,P′-diadenosine-5′ pentaphosphate-inhibitor of myokinase activity), respectively (Fig. 2 *B*). In Rbl smooth muscle containing thiophosphorylated regulatory light chains, recovery of rigor force was more pronounced (90 ± 4%; $n = 5$); however, the inclusion of 250 μM Ap_5A reduced it to 62 ± 4% ($n = 5$) as in dephosphorylated muscles (Fig. 2 *B*). However, Ap_5A did not affect the force transient during the first few seconds following photolysis, indicating that resynthesis of ATP from ADP by myokinase did not affect the rapid force relaxation induced by MgADP, which is the subject of our study; therefore, in view of the much lower rates of ATP generation in comparison with the rate of MgADP-induced decrease in rigor force, we did not add the Ap_5A to subsequent solutions.

Control experiments

To identify and distinguish the real effect of MgADP binding from possible artifacts (mechanical jerk due to laser

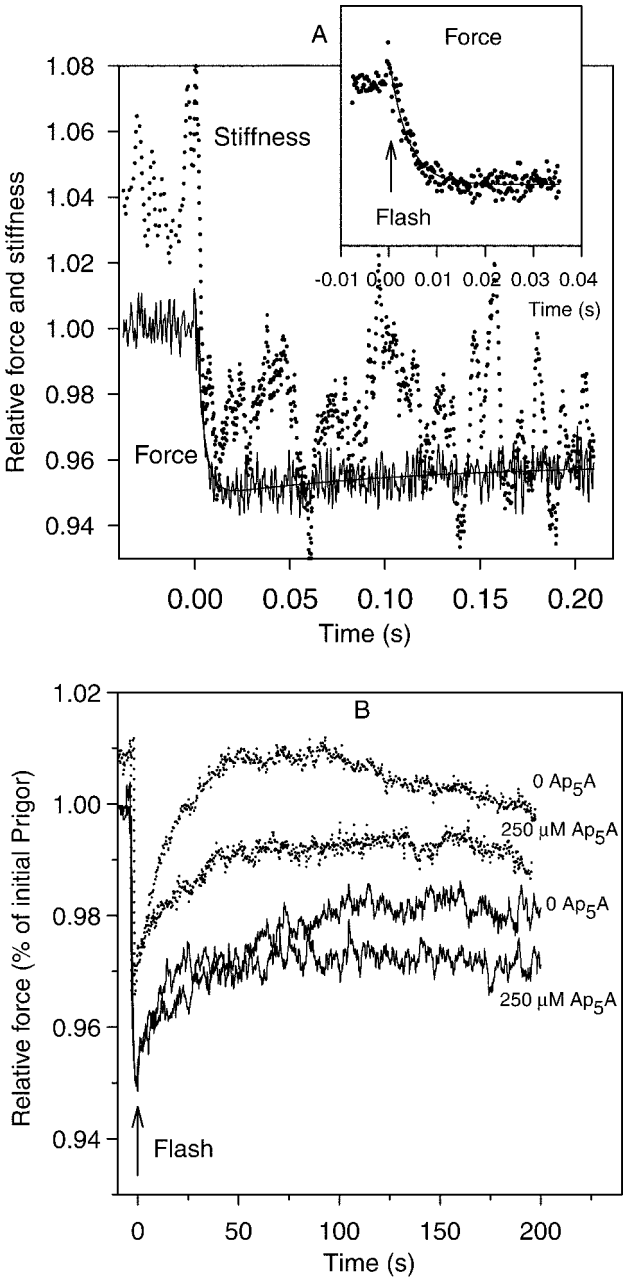

FIGURE 2 (*A*) Original records showing the decrease in rigor force and stiffness following photolysis of caged ADP in rabbit bladder smooth muscle in the 0 Ca high rigor state following the standard protocol (see Fig. 1). The muscle was stretched up to 0.7 P_{max} before photolysis of 2 mM caged ADP, producing ~0.2 mM MgADP. The relative decreases in rigor force and stiffness were ~5% and ~6%, respectively. The data were normalized to values before photolysis. The trace of the stiffness signal was artificially lifted by ~0.4 units for reasons of clarity. The inset shows the initial ~30 ms of force decrease. The results of fitting to two exponentials over 0.2 s shown as lines were $K_1 = 200\ s^{-1}$ for rate and 5.2% for the amplitude. Fitting to the first 50 ms (*inset*, *solid line*) with a single exponential gave a rate $K_1 = 238\ s^{-1}$and amplitude 5%. (*B*) The long time scale records, showing the effect of 250 μM Ap_5A on the recovery of force, following photolysis of caged ADP in Rbl smooth muscle having unphosphorylated (*solid lines*) or thiophosphorylated LC_{20} (*dotted lines*). The traces from smooth muscle having thiophosphorylated LC_{20} have been artificially lifted by 0.01 unit for reasons of clarity.

flash and effects of the photolysis by-product, etc.), we performed control experiments using two caged compounds, DM-nitrophen and caged ADP, that yield different photolysis byproducts: iminodiacetic acid with a nitrosoacetophenone-substituted iminoacetic acid and nitrosoketone, respectively (Kaplan and Ellis-Davies, 1988). We also made the reasonable assumption that, if binding of MgADP (but not ADP) to rigor cross-bridges is required for decreasing rigor force and stiffness, then photolysis of caged ADP in the absence of Mg^{2+} will not reproduce the mechanical effects of MgADP. Alternatively, a rapid increase of $[Mg^{2+}]$ by photolysis of Mg^{2+}-loaded DM-nitrophen in the presence of sufficient free [ADP] should produce effects similar to photolysis of caged ADP in the presence of excess free $[Mg^{2+}]$. In the first type of experiment, the strips were washed in EDTA rigor solution (0 Ca, 0 Mg) only and loaded with caged ADP. As shown in Fig. 3 *A*, photolysis of caged ADP had no effect on rigor force in the absence of Mg^{2+}, except for a very short reversible force transient (jerk) immediately after the laser flash, whereas in the presence of Mg^{2+} it induced relaxation of rigor force (Fig. 2 *A*).

For the control experiments designed to produce rapid changes in $[Mg^{2+}]$ in the presence of constant [ADP], the

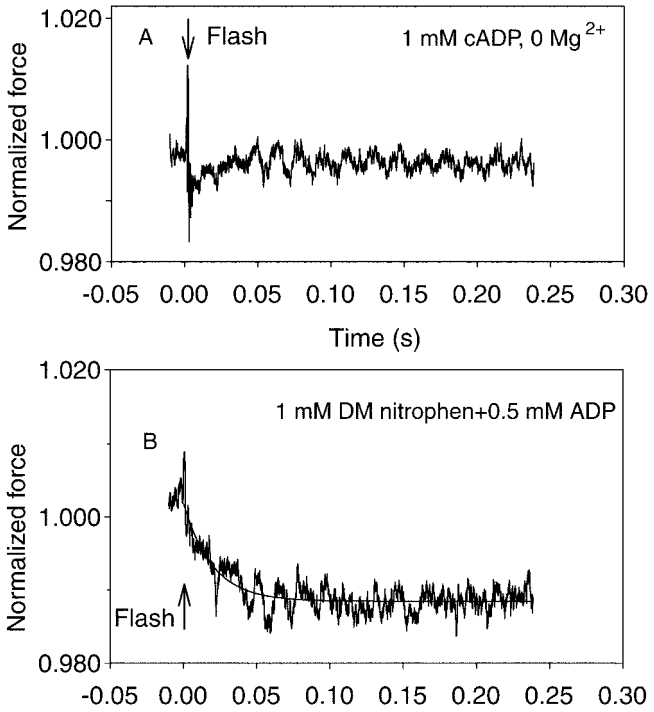

FIGURE 3 (*A*) Control experiments showing the effect of photolysis of 1 mM caged ADP in the absence of free Mg^{2+}. The Rbl strip was taken through the rigor protocol (Fig. 1) and incubated in 0 Mg, 0 Ca rigor solution (2 mM EDTA) for 5 min and subsequently loaded with 1 mM caged ADP. The strip was stretched ~1% of L_o before photolysis. (*B*) Photolysis of 1 mM DM-nitrophen loaded with Mg^{2+} (caged Mg) in the presence of 0.5 mM ADP. Calculated [MgADP] was 1–2 μM before and 40–50 μM after photolysis.

Rbl strips undergoing the rigor protocol (see above) were equilibrated with low Mg^{2+} rigor solution (two changes of 0.5 mM Mg^{2+} rigor solution for 15 min) and, after that, loaded with 1 mM DM-nitrophen (3–5 min), followed by incubation in 0.5 mM Na_2ADP. Under these conditions free [Mg^{2+}] is estimated to be less than 10 μM (based on published dissociation constants of Mg^{2+} binding to DM-nitrophen and ADP (Kaplan et al., 1988; McCray et al., 1992; Zucker, 1994)). The affinity of DM-nitrophen for Mg^{2+} decreases upon photolysis from 0.8–2.5 μM to 3 mM, so that at an estimated ~20% efficiency of photolysis, ~40–50 μM MgADP is produced. The result of this experiment is shown in Fig. 3 *B*. The rate constant and the amplitude of the decrease in rigor force, ~50 s^{-1} and ~2%, respectively, are similar to those observed with photolytic release of ~50 μM MgADP in the presence of 2 mM Mg^{2+}. Photolysis of DM-nitrophen loaded with Mg^{2+} in the absence of ADP did not noticeably change rigor force, but did cause a small decrease in stiffness (data not shown).

Independence of strain

In view of the possibility that the strain imposed on cross-bridges may affect the kinetics of MgADP association/dissociation (Huxley, 1957; Huxley and Simmons, 1971; Dantzig et al., 1991) and the substantial variability in the amplitudes of rigor force (25–50% of maximally Ca-activated force) found in the present study, we further examined the kinetics of mechanical transients using two approaches. In the first series, [MgADP] was held constant at ~70 μM and the degree of external strain was optimized such that the rates and amplitudes of the MgADP-induced force decrease would be relatively insensitive to the variability of strain. External strain was imposed on the strips by stretching (increase strain) or releasing (decrease strain) its length over a range of ~1–2% of the initial length L_o. Photolysis of caged ADP was performed ~30–60 s after the imposed length change. In a second series, the concentration of MgADP was varied and the MgADP-induced force decrease was recorded in the predetermined range of imposed strain. The second-order rate constant of MgADP binding to the smooth muscle cross-bridges was determined in muscles containing either dephosphorylated or thiophosphorylated regulatory light chain (LC_{20}; results given below).

The absolute amplitudes of MgADP-induced decrease in rigor force increased with strain, but the relative amplitudes of the decrease were independent of strain in both muscles (Fig. 4) ($p > 0.05$), except at very low strain (less than 20% of maximally Ca activated), where there was a tendency for the amplitudes to decrease (data not shown). Similarly (Fig. 4), the rates of the decrease in rigor force (after release of ~70 μM MgADP) were also not significantly ($p > 0.05$) dependent on externally imposed strain (normalized to maximally Ca-activated force).

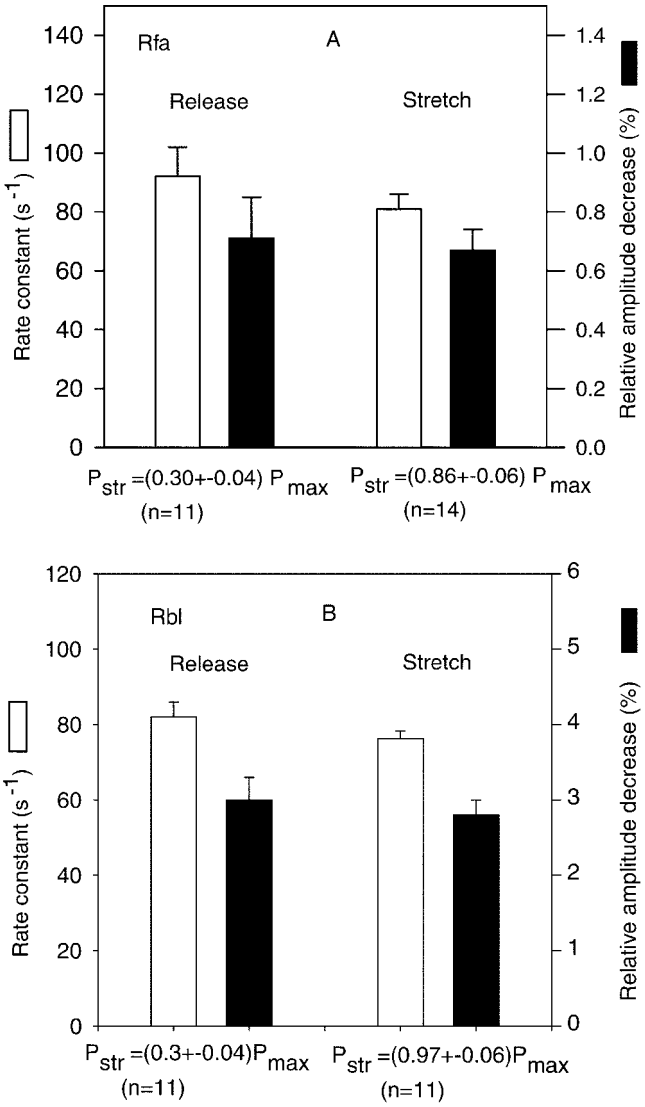

FIGURE 4 The independence of the rate constants (*open bars*) and amplitudes (*closed bars*) of the MgADP-induced decrease in rigor force on the average strain imposed on rabbit femoral artery (*A*) and rabbit bladder (*B*) smooth muscle strips. The strain (expressed as percent of the post-stretch, P_{str}, rigor force relative to maximally Ca activated force, P_{max}) in the strip was increased by stretching (strain greater than 0.45 P_{max}, $\bar{x}$ = 0.97 ± 0.06 P_{max}) or decreased (strain less than 0.45 P_{max}, $\bar{x}$ = 0.3 ± 0.04 P_{max}) by releasing the length of the strip. The mean values ±SEM of P_{str} for the stretches or releases are shown on the abscissa. The experiments were performed with ~70 μM MgADP released by photolysis.

We also tested in greater detail the effects of MgADP on rigor force in the presence of an increased population of negatively strained cross-bridges. The relative proportion of negatively strained rigor cross-bridges is expected to increase transiently after a rapid release of muscle length (Somlyo et al., 1988; Dantzig et al., 1991). We determined the effects of negative strain in Rbl, in view of the larger amplitudes of force decrease in this muscle. First, we performed an experimental test for the presence of negatively

strained cross-bridges: the strips were rapidly released until rigor force fell to near zero, and after ~100 ms, caged ATP (1 mM) was photolyzed to ~100–150 μM MgATP. Photolysis of caged ATP caused an increase in rigor force (Fig. 5 *A*), that was accompanied by a decrease in a stiffness (probably reflecting detachment of negatively strained cross-bridges) as described earlier (Somlyo et al., 1988; Dantzig et al., 1991). In contrast, photolytic release of the same amount of ATP in pre-stretched strips (proportion of positively strained cross-bridges is increased) resulted in the expected decrease in rigor force and stiffness (relaxation) due to detachment of positively strained cross-bridges (data not shown). Caged ADP was photolyzed under the identical experimental conditions (magnitude of length release and time delay) as in the experiment with caged ATP on negatively strained cross-bridges. The force and stiffness signals (Fig. 5 *B*) were, in general, qualitatively not different from the force transients obtained from the strips at initial (not released) length (Fig. 2 *A*). Rigor force decrease was followed by a small force recovery. The amplitudes of the decrease in force and rate constants were not significantly different from the pooled data presented in Fig. 4 *B*; at average force (0.1 $\pm$ 0.02 of P_{max}), the amplitude decrease and rate constants were 3.7 $\pm$ 0.4% and 97 $\pm$ 9 s^{-1} (n = 4). We conclude from these experiments that although at low strain the relative proportion of negatively strained cross-bridges was transiently increased (Fig. 5 *A*), negative strain of cross-bridges did not detectably affect either the kinetics or the direction of ADP-induced force transients.

MgADP-concentration dependence of the rate and amplitude of the decrease in rigor force and the effect of thiophosphorylation

The dependence of the rate and amplitude of force decrease from high-tension rigor state on [MgADP] was determined on strips from both Rfa and Rbl stretched so that rigor force was 0.6–1.2 of maximal Ca-activated force. In both muscles the rate constant of force decay increased monotonically with [MgADP] (Fig. 6). The amplitude of force reduction was almost independent of [MgADP] for Rfa, within the range of 0.075–0.3 mM, but increased in Rbl with the increase in [MgADP]. At a constant [MgADP] = 0.28 $\pm$ 0.02 mM, the amplitude of force reduction was 1.4 $\pm$ 0.1% (n = 4) and 5.9 $\pm$ 0.5% (n = 4) for Rfa and Rbl, respectively. The MgADP concentration dependence is consistent with a two-step reaction pathway:

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

and

$$AM + \text{MgADP} \xrightarrow{k_2/k_{-2}} \text{AM.MgADP},$$

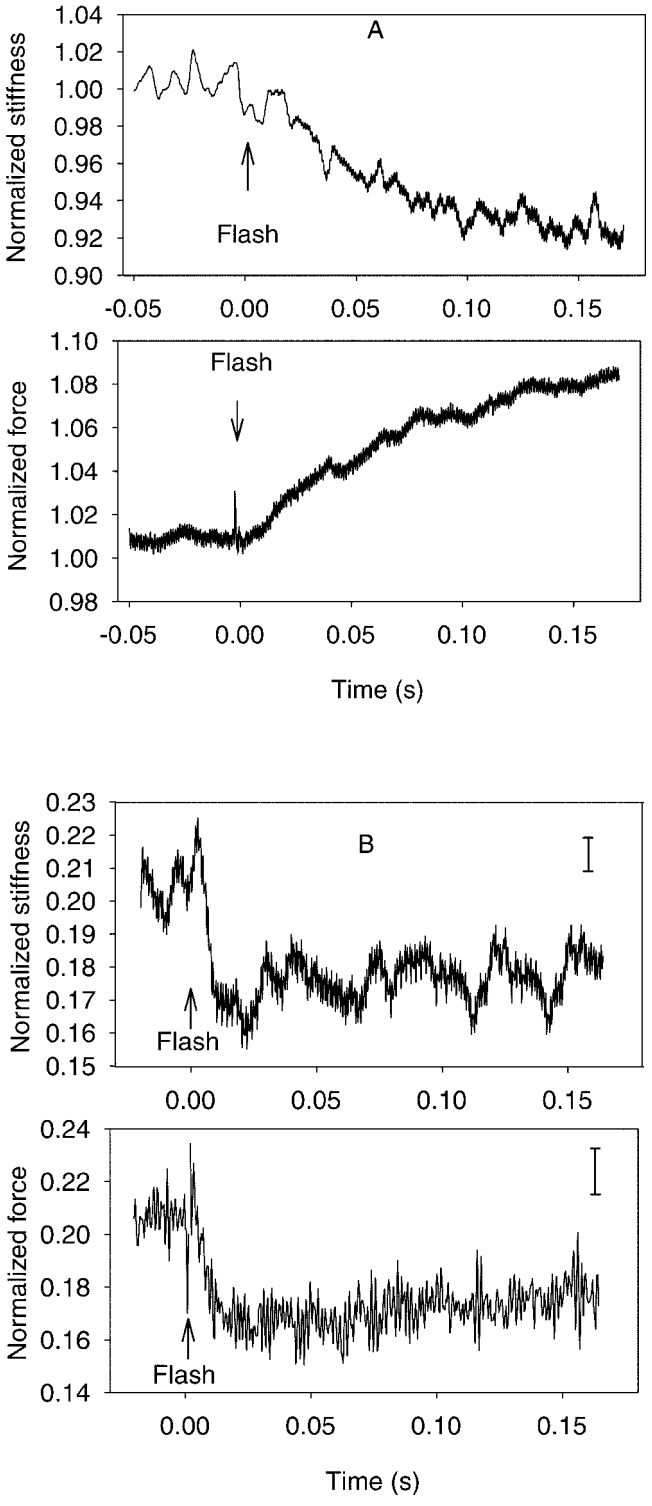

FIGURE 5 The effect of photolysis of caged ATP (*A*) or ADP (*B*) on negatively strained cross-bridges. The time course of rigor force and stiffness after photolysis of caged ATP (*A*) and caged ADP (*B*) in the pre-released (~100 ms before photolysis) Rbl smooth muscle. Calibration bars (*B*) represent 1% of rigor stiffness and 2% of rigor force for the upper and lower panels, respectively.

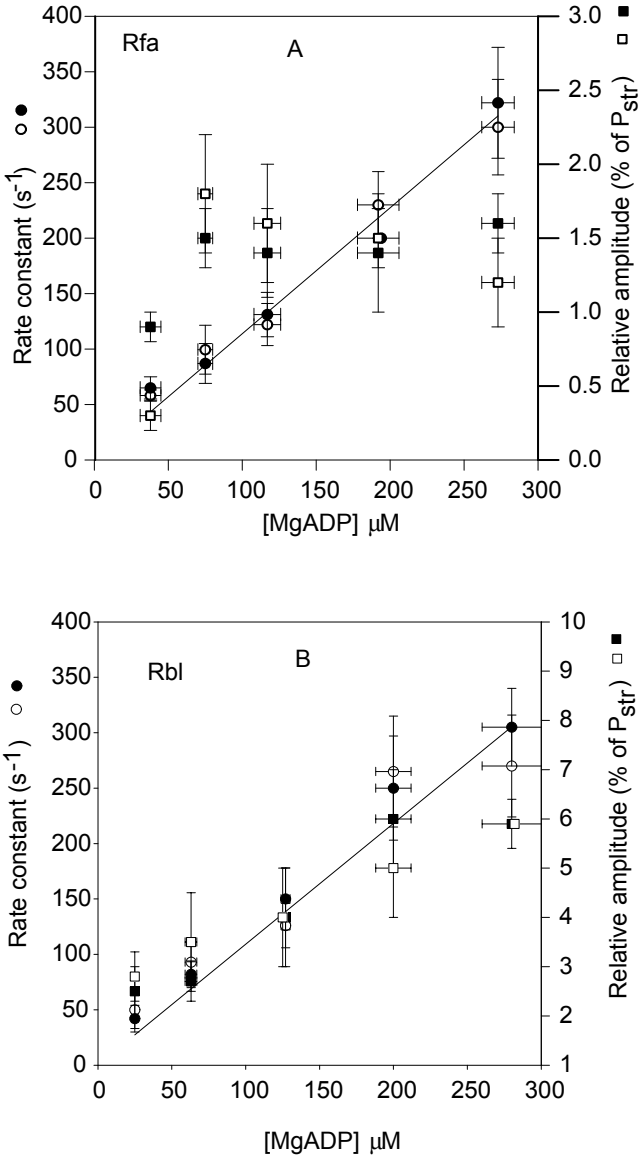

FIGURE 6 The [MgADP] dependence of the rate constants (●) and amplitudes (■, expressed as percent of post-stretch rigor force (P_{str}) of MgADP-induced decrease in rigor force in Rfa (*A*) and Rbl (*B*) smooth muscle. The strips were stretched up to 0.6–1.0 of P_{max} before photolysis of caged ADP. ○ and □, rate constants and amplitudes of force responses to photolysis of caged ADP in Rfa and Rbl strips, having thiophosphorylated LC_{20}. The number of experiments at each [MgADP] was $n = 4–7$. The lines are drawn for the rate constants and are constrained to pass through the origin.

where the first step represents the photolytic release of ADP with a rate constant k_1 and the second one describes MgADP binding to cross-bridges with rate constants k_2/k_{-2}. The observed reaction rate is expected to be a linear function (pseudo-first order) of [MgADP] at low [MgADP], but limited by k_1 at high [MgADP]. The amplitude of force decrease, being proportional to [AM.MgADP], should demonstrate hyperbolic dependence on [MgADP] (Woledge et al., 1985).

The second-order rate constants of MgADP binding to rigor (AM) cross-bridges, determined from the slope of the plot over the range of 0–0.2 mM [MgADP] (Fig. 6, *A* and *B*), were not significantly different in the two muscles ($1.05 \pm 0.12 \times 10^6$ M^{-1} s^{-1}, $R^2 = 0.96$, $n = 18$ and $0.94 \pm 0.25 \times 10^6$ M^{-1} s^{-1}, $R^2 = 0.81$, $n = 17$ for Rfa and Rbl, respectively) and were not significantly influenced by thiophosphorylation of LC_{20}, as shown in Fig. 6, *A* and *B* (shown as open circles): $1.09 \pm 0.1 \times 10^6$ M^{-1} s^{-1} ($n = 16$) and $1.07 \pm 0.12 \times 10^6$ M^{-1} s^{-1} ($n = 14$) for Rfa and Rbl, respectively.

The amplitudes of MgADP-induced decrease in rigor force at constant [MgADP] = 0.28 ± 0.02 mM were not significantly affected by thiophosphorylation of MLC_{20} in either muscle, being 5.9 ± 0.5% ($n = 4$) and 1.2 ± 0.3% ($n = 4$) in Rbl and Rfa, respectively. However, the amplitude of MgADP-induced decrease in force saturated at lower [MgADP] in Rfa than in Rbl smooth muscle (Fig. 6 *A*).

DISCUSSION

The effect of MgADP binding to cross-bridges of striated muscles was thoroughly explored earlier and is still a focus of contemporary studies (Rodger and Tregear, 1974; Dantzig et al., 1991; Lu et al., 1993), but investigations of the effects on smooth muscle have been, until recently, limited (Arner et al., 1987; Fuglsang et al., 1993; Nishiye et al., 1993; Khromov et al., 1998a; Rhee and Brozovich, 2000). Interest in these effects of MgADP has been revived by observations (see Introduction) showing the effect of MgADP on the structure of the head domain of smooth muscle myosin attached to actin (Whittaker et al., 1995) and the high affinity of smooth muscle actomyosin for MgADP (Fuglsang et al., 1993; Nishiye et al., 1993; Gollub et al., 1996; Cremo and Geeves, 1998).

The present study showed a significant reduction (~6% in phasic smooth muscle) in rigor force induced by MgADP in smooth muscles and can be interpreted as resulting from a conformational change in cross-bridges involving rotation of the light chain binding domain (Whittaker et al., 1995). Our finding contrasts with a previous study in which MgADP produced an opposite, albeit very minor (~1%) increase in rigor force (Dantzig et al., 1999). The reason for this difference is probably related to the different experimental protocols used. Dantzig et al. (1999) added ADP to the solution bathing gizzard smooth muscle, whereas we photolytically liberated ADP from caged ADP. The true value of [MgADP] inside the core of the smooth muscle strip may be affected by various ATP/ADP degrading/resynthesis processes (i.e., ecto-ATP/ADPases; Trinkle-Mulcahy et al., 1994), even in the presence of substantial concentrations of inhibitors (in general, nonspecific), and small mechanical transients may also be obscured by time averaging during diffusion of MgADP. Photolysis, on the other hand, results in a high rate of synchronous and ho-

mogeneous liberation of ADP throughout the volume of the smooth muscle strip, exceeding the rates of ATP/ADP degrading/resynthesis processes. This was demonstrated in control experiments, in which inclusion of a myokinase inhibitor (250 μM Ap_5A) in the photolysis solution did not change significantly the kinetic parameters of MgADP-induced force decrease over a short time scale (0.2 s), but noticeably modified the response of the strip during the 2–5 min following caged ADP photolysis; in the absence of inhibitor, rigor force recovered slowly to the initial level (Fig. 2 *B*).

We cannot exclude the possibility that the different results of the two studies were due to the different smooth muscles investigated but consider this unlikely, because both gizzard (Dantzig et al., 1999) and rabbit bladder (present study) smooth muscles contain myosins having an N-terminal, 7-amino-acid insert in the heavy chain and a high proportion of the acidic fast LC_{17a} isoforms (Malmquist and Arner, 1991; Kelley et al., 1993; White et al., 1993; Matthew et al., 1998; and unpublished data; reviewed in Somlyo, 1993).

The rates of MgADP binding to myosin in both (tonic and phasic) smooth muscles were within the range estimated previously from an ATP/ADP competition study ($0.4–2.2 \times 10^6$ M^{-1} s^{-1} (Nishiye et al., 1993)) and of the same order of magnitude as the values in solution for gizzard acto-S1 (4.4×10^6 M^{-1} s^{-1} (Cremo and Geeves, 1998)) and the constant for skeletal muscle (0.5×10^6 M^{-1} s^{-1} (Lu et al., 1993)). The similarity between the second-order rate constants of MgADP binding in, respectively, phasic and tonic smooth muscles implies that the large difference in the MgADP dissociation constants (K_d) between these smooth muscles (Fuglsang et al., 1993) reflects, not surprisingly, the difference in MgADP off-rates. In contrast to the ADP binding rates, the amplitudes of MgADP-induced decrease in rigor force were almost fourfold different in the two muscles. Based on reasonable assumptions about the compliance of smooth muscle and the distance between dense bodies, the expected amplitude of MgADP-induced decrease in rigor force resulting from a ~23° rotation of the lever arm has been estimated (Dantzig et al., 1999) to be 10–12% of initial rigor force. The amplitude of the decrease in rigor force in phasic Rbl smooth muscle (~6%; present study) may be considered close to the above theoretical estimate (taking into account the uncertainty in the distances between dense bodies representing the mini-sarcomere unit (Ashton et al., 1975; Bond and Somlyo, 1982) and the unknown distribution of the total compliance between linear and nonlinear parts. The significantly smaller amplitude of MgADP-induced decrease in rigor force in the tonic, Rfa smooth muscle could be due to the different magnitudes and components of compliance in, respectively, Rfa and Rbl smooth muscles. A larger part of the structural changes in cross-bridges produced by MgADP may be absorbed by nonlinear series elastic component (SEC), which is larger in Rfa. However, although the instantaneous stiffnesses of Rfa and Rbl smooth muscle in rigor are significantly different (20–25% higher in Rbl (Khromov et al., 1998b; unpublished results)), this difference is insufficient to account for the almost fourfold difference in the amplitudes of MgADP-induced force decrease in the two muscles. A more likely explanation of the smaller than calculated reduction in rigor force is that only some of the nucleotide-free cross-bridges are in the appropriate conformation to be tilted by MgADP (Gollub et al., 1999), and this fraction is lower in the more compliant femoral artery (Fuglsang et al., 1993). The larger than predicted difference may also be related to differences in ultrastructural organization of the contractile apparatus (Ashton et al., 1975) and/or different myosin isoform contents, such as the absence of the insert in the heavy chains and a higher ratio of basic to acidic LC_{17} in Rfa compared with Rbl. The amplitude of the decrease in rigor force saturated at lower concentrations of MgADP in Rfa than in Rbl (Fig. 6). It is tempting to ascribe this higher sensitivity of Rfa to the higher ADP affinity of cross-bridges in tonic, than in phasic, smooth muscle (see above), but this conclusion must be mitigated by recognizing that, given the small absolute amplitude of response of Rfa, we were measuring small changes over a very limited dynamic range.

Rigor stiffness, considered to be proportional to the number of attached cross-bridges, is not expected to be affected by MgADP, if it affects the conformation of cross-bridges without causing their detachment. We consider it unlikely that photolysis of caged ADP caused detachment of the cross-bridges, because the decrease in stiffness (Fig. 2 *A*) that accompanied the MgADP-induced decrease in force (Fig. 2 *A*) was no greater than the changes expected due to the nonlinear (exponential, not cross-bridge-related) part of the total compliance. Due to the nonlinearity, any change in force will be accompanied by a proportional change in total stiffness in the same direction, even if the linear component remains constant. This interpretation is consistent with MgADP not dissociating acto-S1 in solution (Cremo and Geeves, 1998).

The effects of strain and (lack of) effect of LC_{20} phosphorylation on the mechanical parameters

Neither positive nor negative strain imposed on the smooth muscle strip significantly affected the kinetic parameters of MgADP-induced rigor force decrease (Fig. 4). Studies of striated muscle indicate only small differences in orientation of the lever arm in different (relaxed, rigor, or contracted) states (Corrie et al., 1999) and no significant effect of lengthening on ADP release from cross-bridges (Shirakawa et al., 2000). Lengthening also fails to affect the spectroscopic signals reporting axial rotation of regulatory light chain in smooth muscle (Gollub et al., 1999). In one study of striated muscle the changes induced in the x-ray diagram by, respectively, MgADP and stretch had the same effect

(Takezawa et al., 1999). If such were also the case in smooth muscle, we would not expect to detect an effect of lengthening on the response to MgADP.

Increasing the population of negatively strained cross-bridges did not produce a response to MgADP opposite (increase of force) to that of positively strained cross-bridges (decrease in force). In such a case, an increase in the rate and decrease in the amplitude (due to truncation) of the MgADP-induced drop in rigor force could be expected. Neither of these expectations was confirmed experimentally, ruling out a substantial contribution of negatively strained cross-bridges to the MgADP-induced force transient. In addition, unlike photorelease of ATP, the release of ADP did not cause an increase in force attributable to detachment of negatively strained cross-bridges; which is consistent with MgADP having affected the conformation of cross-bridges, rather than detaching them.

The lack of effect of LC_{20} thiophosphorylation on the MgADP-induced force transients (present study) is unlike the four- to sevenfold increase in K_d observed following thiophosphorylation in two previous studies (Nishiye et al., 1993; Gollub et al., 1999). The simplest explanation of these divergent results is that the present study probed the on-rate of MgADP binding to nucleotide-free cross-bridges, whereas the studies showing a significant effect of thiophosphorylation employed methods that were not a direct measure of ADP binding (Gollub et al., 1999) or estimated an apparent affinity (Nishiye et al., 1993) largely dependent on the off-rate of ADP from cross-bridges.

The MgADP-induced effects on rigor force and its relation to smooth muscle mechanics

Interpretation of the mechanism(s) of MgADP-induced reduction in rigor force (present study) may not be directly applicable to actively contracting smooth muscle. The [MgADP]-induced slowing of relaxation that follows dephosphorylation of the regulatory light chain of myosin in tonic smooth muscle (Khromov et al., 1995, 1998) can be most readily reconciled by the existence of a high-energy, strongly bound AM′.ADP state that maintains force during a catch-like (latch) state. Whereas the reduction in rigor force induced by MgADP (present study) is consistent with a conformational change in cross-bridges resulting in partial reversal of the power stroke and with the important structural finding that indicated flexibility within S1 (Whittaker et al., 1995), it does not necessarily support the hypothesis that ADP release supplies mechanical energy for an extra kick of the physiological cross-bridge cycle. The possibility that ADP release is a force-producing event seems unlikely on thermodynamic grounds, in view of the relatively high MgADP concentration in normal smooth muscle and the high affinity of cross-bridges for MgADP, conditions under which ADP release would have to occur against a gradient and would absorb, rather than release, energy (Gollub et al., 1996). Furthermore, the binding of ADP added to nucleotide-free actomyosin (AM) does not result in a cross-bridge state equivalent to that produced through ATP hydrolysis by a cycling cross-bridge (Sleep and Hutton, 1980), and the state reached by adding ADP to a rigor bridge is not considered to be a reversible state of the cycle. If ADP release is an irreversible step of the cycle (Sleep and Hutton, 1980), then the state induced by the binding of added ADP cannot be assumed a priori to represent reversal to the state or structure preceding the irreversible step. Therefore, we do not believe that the mechanical (present study) or structural (Whittaker et al., 1995; Gollub et al., 1996, 1999) effects of exogenous MgADP on nucleotide-free cross-bridges necessarily suggest that the ADP release step makes a significant contribution to force maintenance in smooth muscle.

SUMMARY

MgADP induced a decrease in rigor force of smooth muscle that was consistent with a structural change in the myosin motor opposite in direction to that of the power stroke. The relative amplitude of force decrease was in reasonable agreement with the theoretical one for phasic (Rbl), but not for tonic (Rfa), smooth muscle.

Thiophosphorylation of the regulatory light chains (MLC_{20}) had no effect on the rate and amplitude of MgADP-induced rigor force decrease in either muscle.

The second-order rate constant of MgADP binding to the cross-bridges, $\sim 1.0 \times 10^6\ M^{-1}\ s^{-1}$, was similar in the two muscles.

The totality of evidence available is, however, insufficient to conclude that ADP release from physiologically cycling cross-bridges produces significant force in smooth muscle.

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