The Role of MgADP in Force Maintenance by Dephosphorylated Cross-bridges in Smooth Muscle: A Flash Photolysis Study

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ABSTRACT The effect of [MgADP] on relaxation from isometric tension, initiated by reducing free [Ca^{2+}] through photolysis of the caged photolabile Ca^{2+} chelator diazo-2, was determined at 20°C in α-toxin permeabilized tonic (rabbit femoral artery, Rf) and phasic (rabbit bladder, Rb) smooth muscle. In Rf, the shape of the relaxation curve was clearly biphasic, consisting of a slow "plateau" phase followed by a monotonic exponential decline with rate constant k. The duration of the plateau (d = 44 ± 4 s, mean ± SEM, n = 28) was well correlated (R = 0.92) with the total $t_{1/2}$ of relaxation that was 66 ± 3 s (n = 28) in the presence of 20 mM creatine phosphate (CP), and was prolonged in the absence of CP ($t_{1/2}$ = 83 ± 3 s, n = 7); addition of 100 μM MgADP further slowed relaxation ($t_{1/2}$ = 132 ± 7 s, n = 14). In Rb, a plateau was not detectable and $t_{1/2}$ (= 15 ± 2 s, n = 6) was not affected by 100 μM MgADP. In Rf the Q_{10} between 20°C and 30°C was 4.3 ± 0.4 for d^{-1} and 2.8 ± 0.3 for k (n = 8; p = 0.006). The regulatory myosin light chain (MLC_{20}) in Rf was dephosphorylated at 0.07 ± 0.02 s^{-1}, from 42 ± 3% before to 20 ± 2% after photolysis of diazo-2, reaching basal values at a time when force had fallen by only 40%. We conclude that, in the presence of ATP, as during rigor, the affinity of dephosphorylated cross-bridges for MgADP is significantly higher in tonic than in phasic smooth muscle and contributes to the maintenance of force at low levels of phosphorylation. The MgADP dependence of the post-dephosphorylation phase of relaxation is consistent with its being rate-limited by the slow off-rate of ADP from cross-bridges that were dephosphorylated while in force-generating ADP-bound (AM*D) cross-bridge states. The fourfold faster off-rate of ADP from AM*D in the phasic, Rb, compared to tonic, Rf, smooth muscle is a major determinant of the different kinetics of relaxation in the two types of smooth muscle.

INTRODUCTION

Contraction of smooth, like that of striated, muscle is mediated by chemomechanical transduction of energy derived from actin-activated ATP hydrolysis (Marston and Taylor, 1980; reviewed in Hibberd and Trentham, 1986; Hartshorne, 1987) into mechanical activity by cross-bridges (A. P. Somlyo et al., 1973; A. V. Somlyo et al., 1988) on myosin filaments. The major differences between the contractile processes of striated and smooth muscles reside in the different regulatory mechanisms and the significantly slower mechanical and chemical kinetics of smooth muscle. Photolyzable caged compounds that eliminate diffusional delays (Kaplan and Somlyo, 1989; McCray and Trentham, 1989; Somlyo and Somlyo, 1990; Adams and Tsien, 1993; Zucker, 1994) have been valuable for investigating the kinetics of the cross-bridge cycle in both types of muscle. Experiments with caged ATP (Arner et al., 1987a; Somlyo et al., 1988) showed, in agreement with solution studies (Marston and Taylor, 1980), that detachment of rigor bridges by ATP does not rate-limit the cycle. Similar studies of smooth muscles, in which the rate-limiting, regulatory step of myosin light chain (20 kDa, MLC_{20}) phosphorylation had been bypassed by thiophosphorylation, revealed the significantly faster rate of force development by phasic, than by tonic, smooth muscles (Horiuti et al., 1989), and showed the high affinity of rigor bridges for MgADP in tonic smooth muscles (Nishiye et al., 1993; Fuglsang et al., 1993). Consequently, we had suggested (Fuglsang et al., 1993; Somlyo, 1993) that this high MgADP affinity could prolong the lifetimes of force-generating, phosphorylated, as well as nonphosphorylated and cooperatively (Somlyo et al., 1988) attached cross-bridges, and so contribute to the ability of smooth muscles to maintain force with high economy at low rates of cycling ("latch"). We further proposed that such an effect of MgADP is due to isoformic differences in the respective myosins (Somlyo, 1993) and is expected to be more pronounced in tonic than in phasic smooth muscles (Nishiye et al., 1993; Fuglsang et al., 1993). In these studies the effects of MgADP were evaluated in the absence of ATP, and its (high) affinity was estimated, based on its effect on the amplitude of the rapid phase of relaxation from rigor, in muscles in which MLC_{20} was dephosphorylated. Consequently, the results of these experiments reflected, in large part, the affinity of the dephosphorylated rigor and cooperatively cycling cross-bridges for MgADP.

The purpose of the present study was to determine whether MgADP, at the submillimolar concentrations thought to be present in smooth muscle (Krisanda and Paul, 1983; Hellstrand and Paul, 1983; Hardin et al., 1992), could affect the kinetics of cross-bridge cycling during physiological relaxation from isometric contraction, and, if so, whether such an effect was, as predicted, greater in tonic than in phasic smooth muscles. Previous studies have

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shown that dephosphorylation of MLC_{20} can be significantly faster than relaxation (Butler et al., 1983; Driska et al., 1989; Hai and Murphy, 1989). Therefore, we also wanted to test the hypothesis that this late phase of relaxation, after dephosphorylation, reflects the detachment of dephosphorylated cross-bridges and is rate-limited by the slow off-rate of ADP bound with high affinity to dephosphorylated cross-bridges.

Upon photolysis, the Ca^{2+}-affinity of the "caged" Ca^{2+} chelator, diazo-2, increases rapidly (>2000 s^{-1}), with K_d decreasing from 2.2 μM to 73 nM (Adams et al., 1989), sufficient to induce relaxation in frog skeletal muscle with a half-time of 60 ms (Ashley et al., 1991). Therefore, we used diazo-2 to rapidly initiate relaxation, and followed the kinetics of relaxation in the presence of varying concentrations of endogenous and/or exogenous MgADP. We also probed the effect of MgADP on the cross-bridge cycle by photolysis of caged ADP, after initiating relaxation from isometric force by [EGTA] jumps. The time course of dephosphorylation after photolysis was monitored by rapid freezing and two-dimensional gel electrophoresis. We found that the effects of MgADP on relaxation were consistent with a physiological regulatory role during latch and relaxation in tonic smooth muscle. A preliminary report of some of these findings has been presented (Khromov et al., 1994).

MATERIALS AND METHODS

Muscle fiber preparation

Small strips (150 μm wide, 2–2.5 mm long) of rabbit femoral artery (Rf) or bundles of rabbit bladder (Rb; detrusor muscle) were dissected. The internal elastic lamina and endothelium were peeled off the Rf strips, to facilitate transmission of near-UV light into the muscle during laser illumination. The ends of the strip were tied with monofilament silk (40 μm diameter), and one end was connected to the hook of a force transducer (Akers AE801) and the other to a fixed hook in the muscle trough system. The cell membrane was permeabilized with *Staphylococcus aureus* α-toxin (List Biological, Campbell, CA) at 130 U/ml in 50 μl of G0 solution (Table 1) for 40 min with stirring. An estimate of MgADP diffusion through permeabilized smooth muscle was obtained by determining the diffusion of fluorescent molecules (fluorescein or fluo-3) from smooth muscle into the surrounding solution. The $t_{1/2}$ (36 ± 3 s) indicated that the extent of permeabilization achieved was sufficient to ensure the passage of molecules of this size. A23187 (10 μM) was added to deplete Ca^{2+} stores. After permeabilization, the preparations were washed several times in G1 solution (Table 1). Unless stated otherwise, experiments were conducted at 20°C.

All solutions contained 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 2 μg mitochondrial blockers/ml oligomycin, 1 mM KCN, and 1 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. Glutathione (40 mM) was included in the Ca-activating solutions to protect cellular proteins from damage by photolysis by-products. Diadenosine pentaphosphate (Ap_5A) (50 μM) was added to the solution as indicated. Inasmuch as MgADP, rather than free ADP, is the ligand affecting cross-bridges (Kerrick and Hoar, 1987; Lu et al., 1993), all concentrations are given as [MgADP], calculated assuming an association constant of $6.24 \times 10^2\ M^{-1}$. Creatine kinase in Rf and rabbit psoas muscle was measured with the Sigma diagnostic kit (catalog no. 47-10) (Sigma, St. Louis, MO) at 30°C.

The set-up used for laser flash photolysis has been described previously (Somlyo et al., 1988; Nishiye et al., 1993). Diazo-2 was photolyzed with a frequency-doubled ruby laser (Lumonics, Warwickshire, England), delivering 50-ns pulses of near-UV light at 347 nm. The energy of the beam was controlled by insertion of glass slides in the light path. The laser light was focused on the front quartz window of the 15-μl trough.

Mechanical measurements

The time course of isometric tension was monitored on a pen recorder and stored digitally, at a sampling frequency of 100 Hz, via an A-D converter on an IBM PC computer and on videotape. Relaxation time courses were fitted by exponential functions using Sigma Plot (Jandel Scientific, San Rafael, CA) software. Data are presented as mean ± SEM. Statistical significance was evaluated by Student's *t*-test. For graphical presentation, the force was normalized to the force value immediately before relaxation except in [EGTA] jump experiments (see below).

Measurements of the 20 kDa myosin light chain (MLC_{20}) phosphorylation

Muscle strips were frozen between small copper hammers precooled in liquid nitrogen, mounted on the muscle trough apparatus. This freezing device, known as "flash and smash," was designed by Dr. Yale Goldman with software written by Mr. Marcus Bell (University of Pennsylvania, Philadelphia). Just prior (2.8 s) to freezing, the muscle trough was lowered and the hammers triggered to fall at the desired time point (±10 ms) after photolysis. To avoid evaporative cooling and dehydration, the muscle temperature was maintained at 20°C by a stream of humid air. Phosphorylation of MLC_{20} was measured with two-dimensional SDS-gel electrophoresis, as described previously (Kitazawa et al., 1991). One strip contained sufficient MLC_{20} for its detection on the gel.

Protocol

The experimental protocol is shown in Fig. 1. After permeabilization was complete, the muscle strip was relaxed in G1 solution. After several washes in G0 solution (pCa 5.9) to remove EGTA completely, the preparation was transferred into solution G0 plus diazo-2 and sufficient Ca^{2+} to generate 30–40% or 15–30% of the maximal force by Rf and Rb, respectively. The $[Ca^{2+}]_{free}$ in the solutions (containing 2 mM $[Mg^{2+}]_{free}$) before photolysis

TABLE 1 Composition of solutions (mM) at pH 7.1

	NaATP	MgMs*	KMs	CP*	EGTA	Pipes	GSH*	$[Ca^{2+}]_{free}$	$[Mg^{2+}]_{free}$
G0 + 20 CP	4.5	7.5	11.3	20	0	30	40	pCa 5.9	2
G0 + 0 CP	4.5	6.0	69.6	0	0	30	40	pCa 5.9	2
G1 + 20 CP‡	4.5	7.6	48.2	20	1	30	0	pCa 8	2
G1 + 0 CP	4.5	6.1	106.5	0	1	30	0	pCa 8	2

*Ms, methane sulfonate; CP, creatine phosphate; GSH, glutathione.

‡G1 + 20 CP was used during α-toxin permeabilization; otherwise G1 + 0 CP was the relaxing solution. In [EGTA] jump experiments both solutions were used as noted.

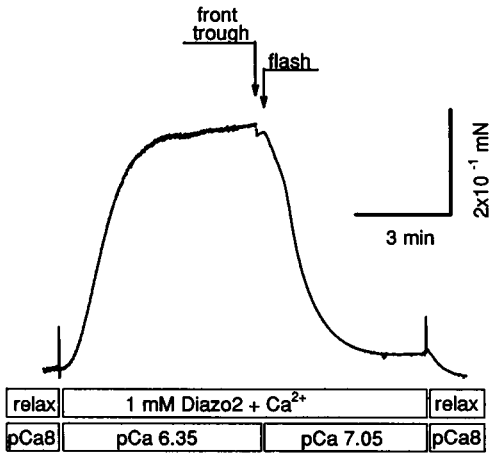


FIGURE 1 Force recording showing the experimental protocol. The permeabilized strip was first transferred from the relaxing to the Ca^{2+}-activating solution containing 1 mM diazo-2 and 100 μM total Ca, followed by transfer to the front trough with the transparent (quartz) window; at the second arrow the laser was flashed. After the relaxed level came to a plateau, the strip was transferred to the relaxing solution. The first sharp deflection marks washes in G0 (see Materials and Methods).

was calculated to be 0.25–0.30 μM (Rf) and 0.7–0.8 μM (Rb), respectively; measured in Rf, by using fluo-3 (10 μM) mixed with solutions containing Ca-diazo-2, $[Ca^{2+}]_{free}$ was 0.46 ± 0.06 μM (pCa 6.35 ± 0.05) before and 0.09 ± 0.01 μM (pCa 7.05 ± 0.03) after the photolysis. These $[Ca^{2+}]$ were chosen based on the lower Ca^{2+} sensitivity of phasic than tonic smooth muscles (Gong et al., 1992), and because the relaxation induced by photolysis of diazo-2 from higher initial activating $[Ca^{2+}]$ was incomplete.

When isometric force had reached a plateau, the strip was transferred into the front trough equipped with a transparent quartz window containing 15 μl of the same activating solution, and the laser was flashed. Once relaxation had reached a steady level of isometric tension (5–10 min), the strip was transferred into the relaxing solution G1, to compare the basal levels of tension before and after photolysis. The solution from the front trough was collected and frozen for high-performance liquid chromatography (HPLC) analysis of nucleotide content and Ca measurements. Relaxation kinetics evoked by photolysis of 1, 2, or 5 mM diazo-2 were not significantly different, provided that free $[Ca^{2+}]$ was adjusted to be the same in each solution before photolysis. The optimal concentration of diazo-2 was found to be 1–1.5 mM.

In experiments in which MgADP was generated by photolysis, relaxation was induced by "[EGTA]-jump," by transferring muscles from activating solution containing no EGTA to G1 (1 mM EGTA) with 0.5–0.8 mM caged ADP (the P^2 1-(2-nitrophenyl)ethyl ester of ADP synthesized as for the corresponding caged ATP; Walker et al., 1989), but no CP. The transfer caused a further rise in force (about 25% of existing tension) over 20 s in Rf. Experimental records (Fig. 4) and data collection of relaxation began from this peak, which was also observed in the absence of caged compounds, after similar transfer, between pCa 4.5 and pCa 8 solutions, and may be due to a slight depressant effect of higher $[Ca^{2+}]$ on force (Zimmermann et al., 1995). To avoid mechanical artifacts due to the intense laser flash in these experiments, the number of glass slides in the light path was increased, and the loss of light energy was compensated for by producing multiple (2–4) flashes in 1–2 s.

Photolysis of 1–1.5 mM diazo-2 induced complete relaxation (>90%) from (submaximal) isometric tension in permeabilized Rf and Rb. Compared to the usual method of relaxing by substituting the activating with the relaxing solution, relaxation induced by photolysis of the Ca-chelator has the advantage of eliminating diffusional delays that result in spatially and temporally inhomogeneous inactivation of myosin light chain kinase. The transient rise in tension, noted above, after substitution ([EGTA] jump) of the solutions, was not seen when relaxation was induced by photolysis of diazo-2 (Fig. 1). Relaxation of Rf was significantly faster when initiated by photolysis than by [EGTA] jump ($t_{1/2}$ = 66 ± 3 s versus 88 ± 7 s, n = 28, $p < 0.05$). Therefore, except in the experiments requiring photolysis of caged ADP, relaxation was induced by photolysis of diazo-2.

The rate of ADP production during relaxation was measured in permeabilized strips transferred into a 50-μl trough containing relaxing (G1) solution; aliquots of 5 μl were taken at 0, 20, and 40 min. The volume of the muscle strip (0.05 μl) was sufficiently small relative to the volume of solution. The nucleotides were separated and analyzed by HPLC. Four microliters of solution were injected and HPLC (Beckman, System Gold) was run under the following conditions: 87% 0.25 M ammonium phosphate $(NH_4)H_2PO_4$ + $(NH_4)_2HPO_4$ (pH 5.4) with 13% of MeOH at 3 ml/min, Radial-Pak Cartridge type 8PSAX 10 μ (Waters). Elution over 20 min was sufficient for separation of all the nucleotides. The area under the UV absorption peak (254 nm) is proportional to the concentration and was used to quantitate ATP, ADP, and AMP. The ADP measured is expressed in the Results as [MgADP], except in Table 3, where it is expressed as ADP. Contaminant ADP due to 4.5 mM commercial ATP was 30 μM.

RESULTS

Relaxation of isometric tension initiated by photolysis of diazo-2 in α-toxin-permeabilized Rf and Rb

In Rf the time course of photolytically initiated relaxation was resolved into two parts: an initial "plateau" and an exponential "tail" (Fig. 2). Three parameters were measured for quantitative analysis of the relaxation kinetics: the $t_{1/2}$ of relaxation, the duration (d) of the plateau, defined as the time between photolysis and the inflection point in the

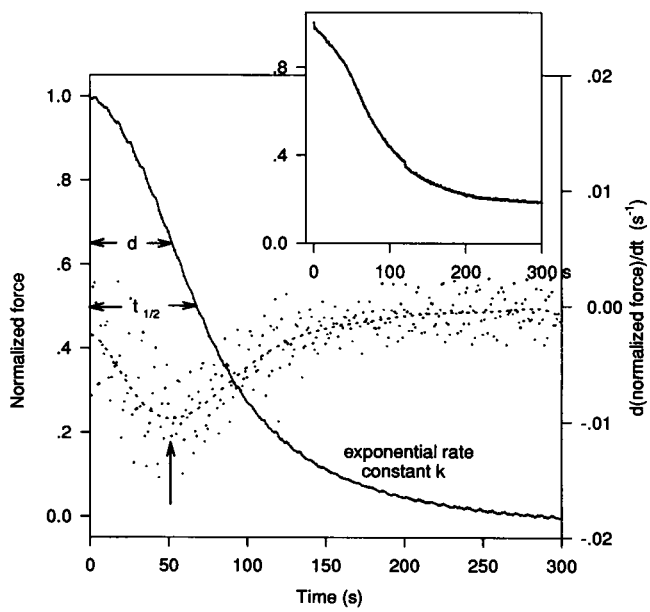


FIGURE 2 Traces of relaxation initiated in Rf by photolysis of 1 mM diazo-2. The inset shows the time course of relaxation after photolysis of 1 mM diazo-2 in an Rf strip immersed in silicon oil. Experiments were carried out in the presence of 20 mM CP and no added MgADP. The dotted line shows smoothed time course of d (normalized force)/dt of force decay. Arrow indicates the inflection point and hence determines d.

tension curve, and the rate constant (k) of the second phase of relaxation, estimated from a nonlinear least-square fit to a single exponential. In Rb the time course of relaxation was well described by a single exponential and the plateau was not sufficiently well defined (or present) for accurate measurement.

The plateau phase of relaxation observed in the presence of high [Ca^{2+}] could have been due, at least in part, to spatially inhomogenous illumination of the trough during photolysis of diazo-2. Thus, although the surrounding solution may have been illuminated sufficiently, self-screening by diazo-2 within the strip may have significantly reduced the photon flux, resulting in a smaller decrease in [Ca^{2+}]. In such a case, the relaxation kinetics would depend in part on diffusion of Ca^{2+} from the inside of the strip into the surrounding solution. Therefore, we also performed photolysis experiments on Rf strips activated at pCa 6.35 and transferred into silicon oil immediately before the laser flash (Fig. 2, *inset*). The time course of the relaxation had the same characteristic biphasic features and was indistinguishable from the results of experiments conducted in aqueous solutions. This result indicates that the biphasic character of relaxation from high force corresponding to high [Ca^{2+}] was not due to less efficient intracellular photolysis and diffusion. Relaxation in oil sometimes was not complete (Fig. 2), probably because of MgATP hydrolysis within the volume of the strip and transition of the muscle into rigor, with accumulation of MgADP within the muscle contributing to the maintenance of force-generating cross-bridges (see below).

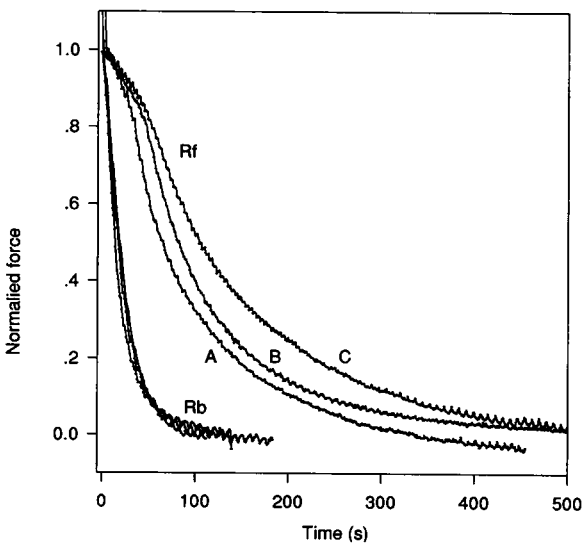


FIGURE 3 Relaxation of tonic (Rf) and phasic (Rb) smooth muscle preparations after photolysis of diazo-2 under different conditions: (*A*) 20 mM CP, 0 exogeneous ADP; (*B*) 0 CP, 0 exogenous ADP; (*C*) 0 CP, 100 μM MgADP. The records from these three conditions overlie one another in Rb.

The effect of MgADP and creatine phosphate

To determine the effects of MgADP on the kinetics of relaxation, experiments were carried out under three different conditions in which the MgADP concentrations inside the fiber were expected to be, respectively, either A) low (in the presence of added CP (20 mM) and without exogenous ADP); B) intermediate (endogenous ADP generated in the absence of both CP and exogenous ADP); and C) high (in the absence of CP and with 100 μM MgADP added). Fig. 3 shows typical tension records during photolytically initiated relaxation of Rf and Rb under the above three experimental conditions. The results, summarized in Table 2, show that the rate of relaxation in Rb was about four times faster than the exponential phase of Rf. Furthermore, the above variations in [MgADP] had significant effects on relaxation in the tonic (Rf), but not in the phasic (Rb), smooth muscle. In Rb very high ($\geq$500 μM) [MgADP] was required to slow relaxation, implying significantly lower affinity of MgADP for cross-bridges in Rb then in Rf. Therefore, the majority of further experiments were conducted on Rf.

In Rf there was a strong correlation ($R = 0.92$) between d and $t_{1/2}$, with the plateau duration accounting for about 70% of the total $t_{1/2}$ under all experimental conditions shown in Table 2. The amplitude of the tension decline during the plateau phase also depended on [MgADP] and [CP]: the relative tension decrease was less, 0.25 $\pm$ 0.02 versus 0.33 $\pm$ 0.02 ($n = 14$, $p < 0.009$) in the presence of 20 mM CP, compared to that in the presence of 100 μM MgADP (no CP). Addition of MgADP (100 μM) in the absence of CP doubled the $t_{1/2}$ of relaxation (132 s versus 66 s) compared to having 20 mM CP present. At intermediate [MgADP] the kinetic parameters of relaxation were also intermediate (Table 2). In Rb, in contrast to Rf, these kinetic parameters of relaxation were not significantly different under these three experimental conditions (Table 2).

To investigate the effect of a larger range of [MgADP] in Rf, two additional types of experiments, designed to reduce

TABLE 2 Parameters of relaxation initiated by photolysis of diazo-2 in α-toxin-permeabilized rabbit femoral artery and bladder (mean $\pm$ SEM)

Conditions	n	$t_{1/2}$ (s)	d (s)	k (s^{-1})
Rabbit femoral artery				
20 mM CP, 0 MgADP,* 100 U/ml CK	12	56 $\pm$ 3	38 $\pm$ 2	0.020 $\pm$ 0.002
20 mM CP, 0 MgADP*	28	66 $\pm$ 3	44 $\pm$ 4	0.013 $\pm$ 0.001
0 CP, 0 MgADP*	7	83 $\pm$ 4	60 $\pm$ 4	0.011 $\pm$ 0.002
0 CP, 100 μM MgADP	14	132 $\pm$ 6	88 $\pm$ 8	0.007 $\pm$ 0.002
20 mM CP, 0 MgADP‡	7	70 $\pm$ 5	50 $\pm$ 4	0.018 $\pm$ 0.002
Rabbit bladder				
20 mM CP, 0 MgADP*	6	14 $\pm$ 1.5	—	0.047 $\pm$ 0.001
0 CP, 0 MgADP*	6	15 $\pm$ 2.0	—	0.047 $\pm$ 0.001
0 CP, 100 μM MgADP	5	14 $\pm$ 1.5	—	0.051 $\pm$ 0.001

d, duration of plateau phase between photolysis and the inflection of the tension curve; k, rate constant of the exponential phase of relaxation; CK, creatine kinase.

*No added ADP.

‡$[Mg^{2+}]_{free}$ reduced to 0.5 mM (cf. Table 1).

the endogenous [MgADP], were performed: in the first, 100 U/ml of exogenous CK was added to Ca-activating solution also containing 20 mM CP. Because the molecular size cutoff (<1000 D; Ahnert-Hilger and Gratzl, 1988) by pores (2–3 nm) in α-toxin permeabilized cell membranes does not permit CK to penetrate into the cell, the effect of exogenous CK was largely due to its ability to decrease [MgADP] in the surrounding solution and to the resulting diffusion of MgADP from smooth muscle. Addition of 100 U/ml CK significantly reduced the $t_{1/2}$, shortened the plateau duration, and increased the rate of the exponential phase (Table 2).

Because MgADP, rather than free ADP, is the species affecting cross-bridges (Kerrick and Hoar, 1987; Lu et al., 1993), in the second type of experiment we reduced the endogenous [MgADP] approximately twofold by decreasing free [Mg^{2+}] from 2.0 to 0.5 mM in the presence of 4 mM MgATP and 20 mM CP without added MgADP. This decrease in free [Mg^{2+}] increased k by about 40% but had no significant effect on $t_{1/2}$ and d (Table 2).

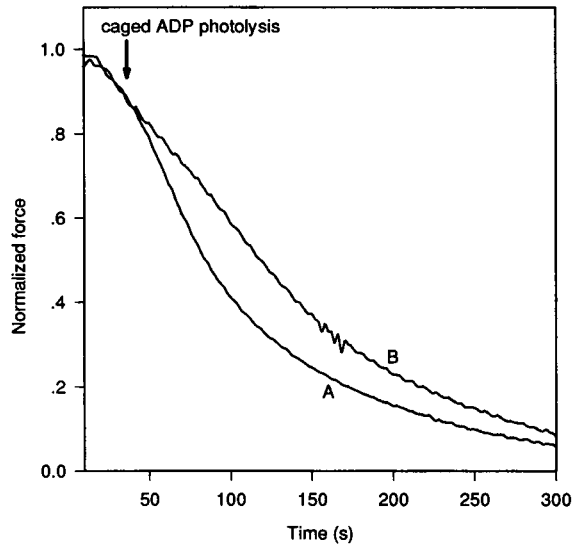


FIGURE 4 The effect of photolytically liberated MgADP on relaxation of Rf (*B*) compared to control (*A*). At the time marked by the arrow, 30 s after the peak of the tension transient caused by [EGTA] jump, 120 μM MgADP was photolytically liberated in the Rf strip.

The effect of photolysis of caged ADP during relaxation of isometric tension

The effects of MgADP on the kinetics of relaxation in tonic and phasic smooth muscle were also probed by photolytic liberation of different amounts of ADP at different times during isometric tension decay. In these experiments the Rf strips were activated with solutions containing CP at pCa 6.0 and Rb at pCa 5.5, and relaxation was initiated by [EGTA] jump. Activating and relaxing solutions contained identical amounts of caged ADP (0.5–0.8 mM) that was photolyzed either during the plateau (20–30 s after the force peak following the[EGTA] jump; see Materials and Methods) or during the exponential decline of tension (1 min after the onset of relaxation). The effects of MgADP photolytically released 30 s after the peak are shown in Fig. 4. The slowing in relaxation of Rf was well defined, provided CP was absent in the relaxing solution.

The introduction of 120 μM MgADP by photolysis during the plateau phase of relaxation slowed the total $t_{1/2}$ of isometric force relaxation by significantly prolonging d (72 ± 9 s versus 47 ± 5 s, $n = 7$, $p < 0.05$) and decreasing k (0.0062 ± 0.0006 s^{-1} versus 0.009 ± 0.001 s^{-1}, $p < 0.05$). Liberation of the same amount of ADP during the monotonic phase of relaxation also decreased the rate constant k (0.005 s^{-1} versus 0.009 s^{-1}), but produced no visible plateau phase.

Photolysis of caged ADP (to yield 100 μM MgADP) during relaxation of Rb strips (3 s after the force peak after the [EGTA] jump) had no significant effect on any of its kinetic parameters. However, in this case the rate of relaxation may have been limited by diffusion of EGTA, and a small effect of MgADP could have been undetectable.

The rate of MLC_{20} dephosphorylation

The rate of MLC_{20} dephosphorylation induced by photolysis of diazo-2 was also determined in α-toxin permeabilized Rf strips frozen at different times during the force decay (see Materials and Methods). The levels of MLC_{20} phosphorylation and normalized force during relaxation in the presence of 20 mM CP (no added ADP) are shown in Fig. 5. Upon photolysis of diazo-2, the initial level of MLC_{20} phosphorylation (42 ± 3%, $n = 8$) decayed to 20 ± 2%

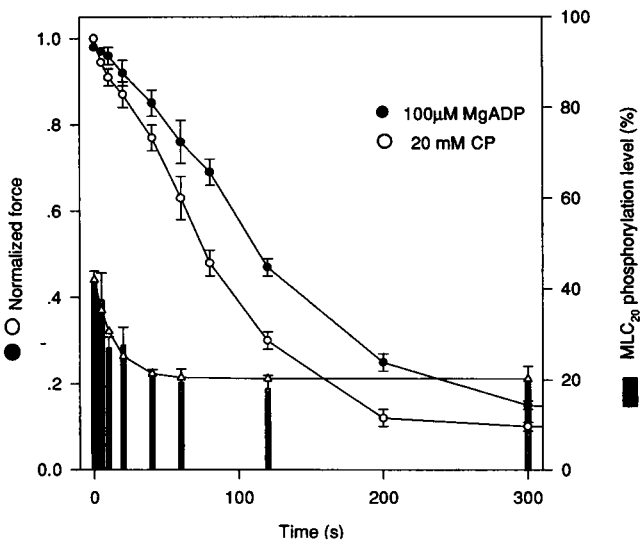


FIGURE 5 Time course of MLC_{20} dephosphorylation (*bars*) and normalized force decay during relaxation in α-toxin-permeabilized Rf in the presence of 20 mM CP (O) or 100 μM MgADP (no added CP) (●). Error bars show SEM. Triangles show the fit of MLC_{20} dephosphorylation to a single exponential with $k_{deph} = 0.07\ s^{-1}$.

with a rate constant $k_{deph} = 0.07 \pm 0.02\ s^{-1}$ (fitting to a single exponential). This rate of dephosphorylation was significantly faster than in previous studies (Gong et al., 1992) in which the reduction of Ca^{2+} was diffusion limited and the time course of dephosphorylation was determined both from higher initial levels and in the presence of ML9 (1-(5-chloronapthalene-1-sulfonyl)-homopiperazine; Calbiochem., La Jolla, CA). The basal level of MLC_{20} phosphorylation, before activation of the Rf strips (16 ± 3%, $n = 5$), was not significantly different from the level at 60 s (20 ± 2%) or 120 s (18 ± 3%) after initiating relaxation.

Neither ADP nor the myokinase inhibitor, Ap_5A, added to these solutions had a significant effect on the rate of dephosphorylation: the initial level of MLC_{20} phosphorylation and the rate constant were, respectively, 40 ± 4% ($n = 3$) and $k_{deph} = 0.10 \pm 0.03\ s^{-1}$ in the presence of 110 μM MgADP and/or 50 μM Ap_5A.

The dependence of the time course of relaxation on the initial amplitude of force

To determine whether the shape and/or kinetic parameters of relaxation were affected by the amplitude of force immediately before photolysis, we performed experiments on α-toxin-treated Rf strips activated with different free $[Ca^{2+}]$, while maintaining the same (1 mM) diazo-2 concentration. The activating conditions were adjusted so that the amplitude of force ranged from 4% to 80% of the maximal force developed at pCa 6.0. Although relaxation of the strips activated with higher Ca^{2+} (pCa < 6.4) was incomplete, the kinetic parameters and the biphasic shape of the force decay were undistinguishable, except after low levels of activation (pCa 6.8–6.9), when the initial level of force was less than 10% of the maximal force at pCa 6.35. Above this level of force, the $t_{1/2}$ of relaxation was not significantly different: 52 ± 3 s and 64 ± 6 s for, respectively, forces with amplitudes higher and lower than 40% of maximal force ($p = 0.15$, $n = 13$). When the initial level of force was less than 10% of the maximum, the time course of relaxation became monophasic with a significantly reduced plateau phase (Fig. 6). The value of k at low levels of force (0.011 ± 0.002 s^{-1}, $n = 7$) was not significantly different from k (0.013 ± 0.001 s^{-1}, $n = 28$) of the monoexponential phase of relaxation under control conditions (pCa 6.35, cf. Table 2, 20 mM CP, zero exogenous ADP).

The effect of temperature on the kinetic parameters of Rf relaxation

To compare the temperature sensitivity of the different phases of relaxation in Rf, experiments were also carried out at 30°C. Experimental solutions were the same as in the control conditions at 20°C, containing 20 mM CP and no exogenous ADP, and pH was adjusted to 7.1 at 30°C (the difference was about 0.1 pH unit from that at 20°C). The

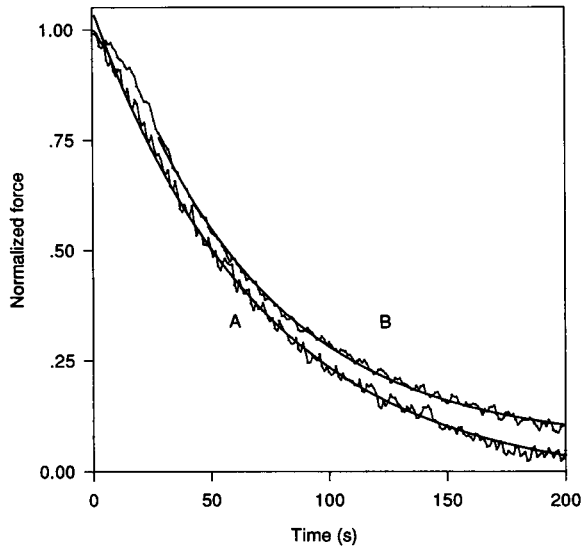


FIGURE 6 The time course of relaxation in α-toxin-permeabilized Rf activated at pCa 6.8–6.9 (*A*) and pCa 6.35 (*B*). The initial amplitude of force was 10% (*A*) relative to the force in (*B*). Smooth solid lines are exponential fit to the post-shoulder component of relaxation (in *B*) and to the whole time course of relaxation in *A*.

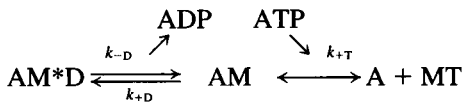
dissociation constant of the carboxymethyl derivative of BAPTA (photolyzed diazo-2) for Ca^{2+} depends only weakly upon pH (Adams et al., 1989; Tsien, 1980). $[Ca^{2+}]_{free}$ in activating solutions at 30°C was adjusted so that the amplitudes of force at 30°C and 20°C did not differ by more than 10%. The experiments were performed with a "bracketed protocol" on a given strip: measurements were made at 20°C, then at 30°C, and again at 20°C or, in two experiments, at 30°C, 20°C, and 30°C. The temperature coefficient Q_{10} was determined as the ratio of the averaged data for the appropriate parameters: d^{-1} (d in reciprocal form to give dimensions of rate constant) and k, measured at 30°C and 20°C.

Increasing the temperature from 20°C to 30°C accelerated the time course of force decay with coefficients of $Q_{10} = 4.3 \pm 0.4$ for the reciprocal of the plateau duration, d^{-1}, and $Q_{10} = 2.8 \pm 0.3$ for the exponential rate constant, k. The values of Q_{10} were significantly different from one another (paired t-test, $t = 3.89$, $p = 0.006$, $n = 8$).

The effect of [MgATP]

In this section we consider the influence of [MgATP] on relaxation kinetics. Before describing the experimental data, we formulate the relationship between [MgADP] and [MgATP] in terms of Scheme 1, in which the cross-bridges are at their basal level of MLC_{20} phosphorylation. By the time the plateau is over (time d), the phosphorylation of MLC_{20} had essentially returned to the basal level of relaxed Rf (cf. Table 2, Fig. 5). The kinetics of the force decay during the exponential phase, therefore, were modeled with

cross-bridges initially dephosphorylated and containing bound ADP:



Scheme 1

Scheme 1 thus describes the part of the cross-bridge cycle in which the release of ADP from force-generating dephosphorylated cross-bridges (AM*D states) is followed by ATP binding and detachment of cross-bridges. Assuming that [AM] $\ll$ [AM*D] + [MT] (resulting in the steady-state approximation), the dependence of k, the rate constant of the exponential phase of relaxation, on [MgADP] and [MgATP] is as follows:

$$\frac{1}{k} = \frac{1}{k_{-D}} + \frac{k_{+D}[\text{MgADP}]}{k_{-D}k_{+T}[\text{MgATP}]} \quad (1)$$

where k_{+D}, k_{-D}, and k_{+T} are the rate constants of ADP association and dissociation and ATP association, respectively (Scheme 1; Nishiye et al., 1993).

To examine the possible competition between MgADP and MgATP for the nucleotide binding sites on cross-bridges, experiments were conducted in the presence of different concentrations of MgATP, under conditions where MgADP was expected to be A) high (0 CP + 100 μM MgADP); B) low (20 CP+100 U/ml CK); or C) intermediate (20 CP and no added ADP). In general, the time course of relaxation retained the same characteristic features and could clearly be resolved into two parts: plateau and mono-exponential. As [MgATP] was reduced, relaxation became slower under all conditions. The durations of the plateau, d were 50 $\pm$ 10%, 66 $\pm$ 10%, and 43 $\pm$ 10% of the $t_{1/2}$ values of relaxation at 2, 4, and 10 mM MgATP, respectively. The influence of MgADP on relaxation kinetics was consistent with competition between MgADP and MgATP for AM cross-bridge states. (Fig. 7). The fast component that follows ATP binding during relaxation from rigor (Nishiye et al., 1993) was not seen in our experiments, probably because of the small population of AM complexes during isometric contraction and/or the masking effect of the initial (plateau) phase of relaxation.

Competition between MgADP and MgATP was further explored by evaluating the kinetic data in terms of Eq. 1 over the second phase of the relaxation. To do this, double-reciprocal plots of k versus [MgATP] were drawn at the three [MgADP]. The [MgADP] measured with HPLC after completion of relaxation (10 min) in the relaxing solutions at conditions A, B, and C was, respectively, 100 $\pm$ 20 μM, <2 μM, and 45 $\pm$ 10 μM. In condition A contamination of ADP in commercial ATP might be expected to influence the [MgADP]. In practice the maximum contamination would be at 10 mM MgATP when [MgADP] would be 140 μM rather than 100 μM. In view of the HPLC measurements we have ignored any corrections which, in any event (see next paragraph), would give rise to errors less than the error range estimated for k_{+D}/k_{+T}.

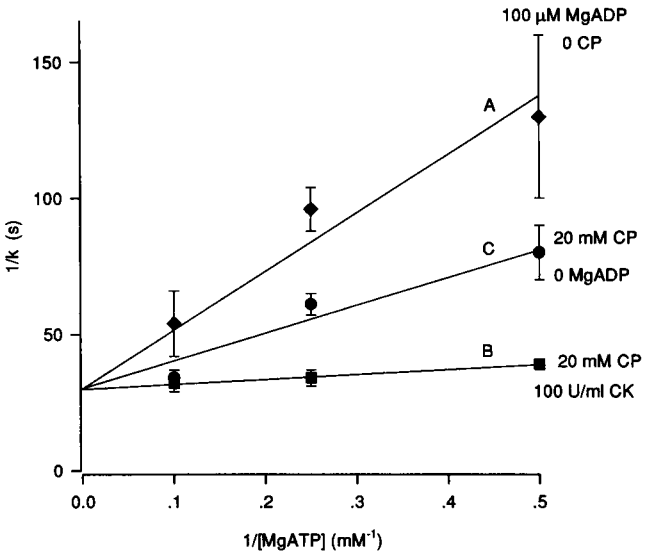


FIGURE 7 Plot of $1/k$ versus 1/[MgATP]. Lines were drawn with a common ordinate intercept, as described in the text. Error bars show SEM.

The data in Fig. 7 show that in condition B there is very little dependence of k on [MgATP] and hence these data provide the best estimate of k_{-D} (= 0.033 s^{-1}). Equation 1 predicts the ordinate intercepts of each data set (i.e., conditions A, B, and C) to be the same. Hence the best-fit straight lines associated with the data sets A and C were drawn through the ordinate intercept estimated from the data set of condition B (Fig. 7). From the line associated with condition A in which [MgADP] was 100 $\pm$ 20 μM, k_{+D}/k_{+T} = 71 $\pm$ 15 (from Eq. 1), and for condition C in which [MgADP] was 45 $\pm$ 10 μM, k_{+D}/k_{+T} = 75 $\pm$ 17.

The fact that k is only weakly dependent on [MgATP] under condition B is consistent with low [MgADP]. However, Eq. 1 predicts no dependence of k on [MgATP] at zero [MgADP]. The weak dependence of k on [MgATP] that is observed may reflect a breakdown of the model (Scheme 1) in terms of Eq. 1. For example, at low [MgADP] it may not be valid to assume that the concentration of a force generating AM state is negligible, in which case the weak dependence of k on [MgATP] indicates that the apparent K_m of MgATP is 150 μM. Another explanation of the weak dependence of k on [MgATP] under condition B may be because [MgADP] is not zero within the muscle strip (see comment below and Eq. 2 about gradient), even though zero [MgADP] was detected in the HPLC assay. If Eq. 1 holds, and from the above analysis k_{+D}/k_{+T} = 73, then [MgADP] = 3.4 μM within the muscle strip—a reasonable concentration for the experimental conditions.

In any event, the small value of k_{-D} (0.033 s^{-1}), coupled with the high value of the k_{+D}/k_{+T}, suggests a very high affinity of MgADP for cross-bridges in their force-generating dephosphorylated state. That this ratio is so high may not be surprising, because the process controlled by k_{+T}

comprises at least two steps in contrast to that controlled by k_{+D}. The rate constant of ATP-induced cross-bridge dissociation in guinea pig portal vein is in the range 0.4–2.3 × 10^4 M^{-1} s^{-1} (Nishiye et al., 1993). Using this value for k_{+T}, the ADP association rate constant to AM, k_{+D}, is 0.3- 1.7 × 10^6 M^{-1} s^{-1} ($k_{+D} \geq 3 \times 10^6$ M^{-1} s^{-1} fast twitch skeletal muscle actomyosin; Geeves, 1989). From this we infer the dissociation constant of MgADP to Rf cross-bridges during relaxation to be in the range 0.02–0.11 μM. The reason relaxation occurs at 100 μM MgADP, in spite of its high affinity for cross-bridges, is that millimolar ATP competes effectively with 100 μM MgADP for cross-bridges in the AM state (see Scheme 1). It should be noted that this conclusion of a very high affinity constant of MgADP for cross-bridges during relaxation depends on the assumptions that ADP interaction with cross-bridges is noncooperative and that ADP dissociation is the only process before ATP association. The complex time course of relaxation suggests that these assumptions may not be valid during the plateau phase.

In smooth muscles permeabilized with *S. aureus* α-toxin, ecto-ATPases can also make a significant contribution to ADP production (Hoar et al., 1991; Trinkle-Mulcahy et al., 1994). Therefore, we determined whether ADP production by ecto-ATPase (Table 3) could significantly affect our results.

The net ADP produced in femoral artery by basal nucleotidase (including ecto-ATPase) activity was 1.0 ± 0.1 mM/min, significantly lower than found in portal vein by Trinkle-Mulcahy et al. (1994), whereas the activity in bladder, a phasic smooth muscle, was higher. Therefore, as a result of ecto-nucleotidase activity in femoral artery (typically 0.05 μl in volume), by the end of a 10-min interval of relaxation [MgADP] in the 15-μl trough could increase by approximately 20 μM in the absence and 12 μM in the presence of CP. These values are within the experimental errors of our measurements and would have no significant impact on our conclusions.

Finally, considering the myosin- and ecto-ATPases as a continuous source generating an ADP gradient during relaxation, we can also calculate an upper limit of [MgADP] in the core of the strip [$C(r)$] at the end of 10 min of relaxation (Cooke and Pate, 1985; Chase and Kushmerick, 1995) using Eq. 2, assuming a homogeneous distribution of the resting ATPase throughout the cross-section of the strip:

$$C(r) = C_o + a^2k'/4D \quad (2)$$

where C_o = [MgADP] in the surrounding solution, a (= 75 μm) is the radius of a cylinder (i.e., the strip), k' (= 1.0 mM/min) is the rate of ADP production, and D (= 150 $\mu m^2/s$) is the diffusion coefficient for ADP (Peterson, 1982; Chase and Kushmerick, 1995).

For example, we can estimate the maximum [MgADP] at the core of an Rf strip under condition A of the experiment described in Fig. 7. After 10 min of relaxation, C_o = 100 μM. It then follows from Eq. 2 that $C(r)$ = 186 μM (taking into account the MgADP association constant). This value is probably an overestimate of intracellular [MgADP], because 1) ADP produced by ecto-ATPase is generated on the extracellular surface of the cell membrane; 2) the calculated [MgADP] in the fiber is based on the highest level (after 10 min) reached in solution; 3) the good fit of the later phase of relaxation to a single exponential argues against a significant perturbation due to changing levels of MgADP; and 4) in experiments where CP was added, the CK-catalyzed reaction would further serve to equilibrate [MgADP] inside and outside the fiber. Nevertheless, even assuming the highest value of [MgADP] in the core of the muscle (i.e., 186 μM), the lower value of k_{+D}/k_{+T} (= 40) is still remarkably high. (Note that Eq. 2 is not applicable in the presence of exogenous creatine kinase, as in this condition the rate of net ADP production within the strip is not known.)

TABLE 3 The rate of basal ADP formation in α-toxin-permeabilized rabbit femoral artery and rabbit bladder (mean ± SEM)

Conditions	n	Rate of ADP formation (mM/min)
Rabbit femoral artery		
0 CP, 0 ADP	6	1.0 ± 0.1
20 mM CP, 0 ADP	6	0.6 ± 0.1
Rabbit bladder		
0 CP, 0 ADP	6	5.2 ± 0.4

DISCUSSION

The major findings of this study are that a) the slowing effect of MgADP on relaxation is much more prominent in tonic than in phasic smooth muscle; b) this effect of MgADP is due to its competition with MgATP for binding to cross-bridges, causing inhibition of ADP dissociation and an increase in the population of force-producing, AM*D states; c) the slow rate of ADP release from dephosphorylated AM cross-bridges is the rate-limiting step of relaxation from isometric force; and d) the affinity of MgADP for cross-bridges during relaxation, as during rigor (Fuglsang et al., 1993), is higher in tonic than in phasic smooth muscle.

Photolysis of diazo-2 and the resultant rapid fall in [Ca^{2+}] caused relaxation of tonic smooth muscle with a transient consisting of two prominent phases: a plateau and a subsequent monoexponential decline. The very rapid ($k \geq$ 2000 s^{-1}) fall in [Ca^{2+}] that followed photolysis and the rapid (3.5 s^{-1}; Kasturi et al., 1993) dissociation of calmodulin from myosin light chain kinase could not readily account for the duration of the plateau that ranged from 38 to 88 s. The mechanisms responsible for the two phases also seem to be different. The initial, plateau phase appears to be the expression of somewhat complex processes. We suggest that it is due to continued cycling of cross-bridges that remain phosphorylated after photolysis of diazo-2, as the

result of the relatively (compared to the drop in $[Ca^{2+}]$) slow rate ($0.07 \pm 0.02\ s^{-1}$) of dephosphorylation. In addition, cooperative cycling of nonphosphorylated cross-bridges may contribute to the plateau (Somlyo et al., 1988; for striated muscle, see Thirlwell et al., 1994, and references therein). We do not believe that structural disorder, similar to that giving rise to the shoulder in relaxation observed in striated muscle (Edman and Flitney, 1982; Huxley and Simmons, 1973), was a major cause of the shoulder in smooth muscle, because a) the Q_{10} of d^{-1} was very much higher than would be expected from a simple mechanical effect; b) the effect of [ADP] on d^{-1} cannot be accounted for by any known effect of the nucleotide on structural organization, and c) the very modest structural order of mini-sarcomeres (Ashton et al., 1975) in smooth muscle is unlikely to be subject to significantly greater disordering with major consequences on mechanical properties.

The temperature dependence of the reciprocal of the plateau phase of relaxation had a higher Q_{10} than its later, monoexponential component and can be accounted for by the (at least) three processes operating with differing temperature dependences: dephosphorylation ($Q_{10} = 5.1$; Mitsui et al., 1994), cross-bridge attachment during the plateau (assumed to be $Q_{10} \approx 2.0$), and detachment ($Q_{10} = 2.8$). Given these different processes, relaxation is not expected to have the Q_{10} of any single component. Thus, although the Q_{10} of d^{-1} is expected to be most closely related to the Q_{10} of dephosphorylation, as indeed was the case (Q_{10} of $d^{-1} = 4.3 \pm 0.4$), the two are not expected to be identical. It has recently been reported that the Q_{10} of relaxation and dephosphorylation are nearly identical (Mitsui et al., 1994), but, rather surprisingly, these authors determined the Q_{10} of relaxation in a phasic (portal vein) and that of dephosphorylation in a relatively tonic (femoral artery) smooth muscle. Myosin light chain phosphatase activity is higher (Gong et al., 1992), and the affinity of cross-bridges for MgADP is lower in phasic than in tonic smooth muscle (Fuglsang et al., 1993; present study). Therefore, the reported (Mitsui et al., 1994) very close relationship between the temperature dependences of relaxation and dephosphorylation may represent a fortuitous case.

The second phase of relaxation occurred after MLC_{20} phosphorylation had returned to baseline levels (Fig. 5), and could be well fitted by a single exponential with an MgADP-dependent rate constant. Indeed, the kinetics of this phase of relaxation could be accounted for by its being rate-limited by the off-rate of ADP from cross-bridges. This off-rate, $k_{-D} = 0.033\ s^{-1}$, is comparable to that calculated from the slow phase of relaxation from rigor ($0.1 \pm 0.05\ s^{-1}$; Fuglsang et al., 1993) and somewhat faster than the resting rate of ADP exchange ($0.004\ s^{-1}$; Vyas et al., 1994) in the more phasic, portal vein smooth muscle. Our results indicate that the properties of the cross-bridge cycle of tonic smooth muscle and, specifically, the slow off-rate of ADP from cross-bridges, are sufficient to explain the slower rate of relaxation than dephosphorylation (Butler et al., 1983; Hai and Murphy, 1989; Driska et al., 1989; present study), without having to invoke other regulatory mechanisms, although clearly not excluding the possibility of their existence.

The much higher affinity of smooth than striated muscle myosin for MgADP was first demonstrated through the inhibitory effect of MgADP on ATP-induced relaxation from rigor (Nishiye et al., 1993; Fuglsang et al., 1993). The present results show that in tonic smooth muscles, MgADP is also a high-affinity competitive inhibitor of MgATP during relaxation from isometric contraction. The K_d (= 1.1 μM in Rf) of cross-bridges for MgADP detected by measuring the amplitude of the rapid phase of cross-bridge detachment from rigor (Fuglsang et al., 1993) represented the affinity of a state (rigor, AM) that is only minimally populated in the presence of millimolar ATP during the physiological cross-bridge cycle. On the other hand, the effects of MgADP, with somewhat lower apparent affinity, on the slow phase of detachment from rigor (figure 7 in Fuglsang et al., 1993) probably represent competition with 1–1.5 mM photolytically released ATP for a state of (cooperatively or phosphorylated) cycling cross-bridges. Reports of somewhat lower affinities of smooth muscle myosin for MgADP probably reflect not only differences in experimental conditions, but also the fact that such studies were conducted on phasic smooth muscles during unloaded shortening (Arner et al., 1987b) or obtained through motility assays of phasic smooth muscle myosin (Warshaw et al., 1991). However, even myosin isolated from phasic (gizzard) smooth muscle has higher affinity for MgADP than striated muscle myosin (R. J. Barsotti, cited in Siemankowski et al., 1985; Drew et al., 1992), in agreement with the effects of MgADP on relaxation from rigor (Nishiye et al., 1993) and from isometric contraction (present study).

Our present findings support the notion (Nishiye et al., 1993; Fuglsang et al., 1993) that the high-affinity binding of MgADP to dephosphorylated cross-bridges, possibly in conjunction with their cooperative cycling (Somlyo et al., 1988), contributes to the maintenance of force at low levels of MLC_{20} phosphorylation and shortening velocity (catch-like state, Somlyo and Somlyo, 1967; latch, reviewed in Rembold and Murphy, 1993) and accounts for the slower rate of relaxation than dephosphorylation. A more quantitative assessment of these contributions will require detailed knowledge of the physiological intracellular milieu and, in particular, of the concentration of free MgADP. Unfortunately, chemical measurements of free ADP are subject to contamination by bound ADP released from actin, whereas the sensitivity of NMR is insufficient for directly measuring MgADP. Indirect NMR values are based on the assumption that the creatine kinase reaction is at equilibrium and, like the chemical measurements, are estimates of bulk concentrations that may differ from [MgADP] at the cross-bridges. These reservations notwithstanding, and in view of the lower ATP content (1–2 mmol/kg wet wt) of smooth than striated muscle (Butler and Davies, 1980), estimates of free

ADP in smooth muscle (up to about 120 μM) are in the range that, according to the present study, is expected to modulate the cross-bridge cycle in tonic smooth muscle. Furthermore, the concentrations of creatine phosphate (2–4 mM; Butler and Davies, 1980) and creatine kinase [1–47 IU/g wet wt; reviewed in Table 1 of Clark, 1994; Clark et al., 1994; 61 ± 3 IU/g wet wt, femoral artery, measured here (see Materials and Methods)] are extremely low in tonic smooth muscle, particularly when compared to striated muscles (CP 20 mM, CK 1270 IU/g per wet weight in the cat soleus; McFarland et al., 1994; CK 4620 ± 150 IU/g wet wt in rabbit psoas; present study) in which creatine kinase regenerates ATP, while at the same time buffering free ADP at very low levels. Chemically measured ADP significantly increases during contraction in a tonic (porcine carotid artery; Krisanda and Paul, 1983) but not in a phasic (portal vein; Hellstrand and Paul, 1983) smooth muscle, and the concentration of cellular free ADP estimated in porcine carotid artery (44–123 μM; Krisanda and Paul, 1983) would certainly be sufficient to prolong the lifetime of strongly bound AM*D cross-bridge states (Fig. 7). Differences in the ATP affinities of the two types of smooth muscle, if such occur, could also influence cross-bridge kinetics, and this possibility is currently being explored.

The physiological mechanism of the regulatory function of ADP proposed by us is one in which the slow off-rate of ADP from dephosphorylated, strongly bound cross-bridges prolongs the lifetimes of force-generating states and slows the rate of cross-bridge detachment and, hence, relaxation. The same mechanism is also thought to be rate-limiting maximum shortening velocity (Siemankowski et al., 1985), albeit probably at different, strain-dependent rates during, respectively, unloaded shortening and isometric relaxation. It is further implied (Fuglsang et al., 1993) that transition into a "catch-like state" or "latch" occurs when a sufficient number of cross-bridges are dephosphorylated before ADP release, resulting in a significant population of strongly bound, dephosphorylated AM*D states. This hypothesis identifies a specific strongly bound molecular species (AM*D) that, when dephosphorylated, results in the "latch" state (Rembold and Murphy, 1993). It also indicates the relationship between this "catch-like state" (Somlyo and Somlyo, 1967) and molluscan catch, which is also associated with a high affinity of ADP for myosin (Takahashi et al., 1988). We had suggested that the affinity of smooth muscle myosin for MgADP is modulated by differences in the isoformic composition of their 17-kDa light chains (LC_{17}) and/or heavy chains (reviewed in Somlyo, 1993). Consistent with this conclusion is the fact that bladder smooth muscle that contains only the LC_{17a} isoform and a heavy-chain message containing the insert near the catalytic site (reviewed in Somlyo, 1993) is relatively insensitive to MgADP both during rigor (Fuglsang et al., 1993) and during relaxation from isometric force (present study).

The affinity of a phasic (bladder) smooth muscle myosin for MgADP during physiological cycling is relatively low (present study), and only unphysiologically high concentrations of MgADP or its nonmetabolizable analog, MgADPβS, appear to affect contractility of phasic smooth muscles (Kerrick and Hoar, 1987; Nishimura and van Breemen, 1991; present study). Phasic smooth muscles can maintain only a very modest level of force after being dephosphorylated (Himpens et al., 1988), and it seems unlikely that MgADP plays a significant role in their regulation under physiological or near-physiological conditions.

The basal level of phosphorylation (approximately 18%) in relaxed muscle was significant. This has been noted by several laboratories, and possible contributory mechanisms have been discussed (reviewed in Hai and Murphy, 1989). A true basal phosphorylation in these tonic smooth muscles may result from the activity of the Ca^{2+}-independent myosin light chain kinase combined with relatively low myosin light chain phosphatase activity (Gong et al., 1992; Tokui et al., 1995). The mechanism through which similar levels of phosphorylation give rise to force in phasic (Kitazawa et al., 1991) but not in tonic smooth muscle remains to be determined but, in any case, does not affect the conclusions of the present study.

CONCLUSIONS

1. During isometric relaxation, as during rigor, the affinity of cross-bridges for MgADP is higher in tonic than in phasic smooth muscle.

2. The slow rate of ADP dissociation from dephosphorylated cross-bridges can account for the exponential phase of relaxation being slower than MLC_{20} dephosphorylation.

3. High-affinity binding of MgADP to dephosphorylated (AM*D) cross-bridges contributes to force maintenance.

4. MgADP probably has a much lesser role in modulating contractility in phasic than in tonic smooth muscle, and the lower affinity of MgADP for phasic than tonic smooth muscle myosin can largely account for the different kinetics of relaxation and shortening velocity in these two types of smooth muscle.

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