Myosin Regulatory Light Chain Phosphorylation and Strain Modulate Adenosine Diphosphate Release from Smooth Muscle Myosin

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ABSTRACT The effects of myosin regulatory light chain (RLC) phosphorylation and strain on adenosine diphosphate (ADP) release from cross-bridges in phasic (rabbit bladder (Rbl)) and tonic (femoral artery (Rfa)) smooth muscle were determined by monitoring fluorescence transients of the novel ADP analog, 3′-deac-eda-ADP (deac-edaADP). Fluorescence transients reporting release of 3′-deac-eda-ADP were significantly faster in phasic (0.57 ± 0.06 s^{-1}) than tonic (0.29 ± 0.03 s^{-1}) smooth muscles. Thiophosphorylation of regulatory light chains increased and strain decreased the release rate ~twofold. The calculated (k_{-ADP}/k_{+ADP}) dissociation constant, K_d of unstrained, unphosphorylated cross-bridges for ADP was 0.6 μM for rabbit bladder and 0.3 μM for femoral artery. The rates of ADP release from rigor bridges and reported values of P_i release (corresponding to the steady-state adenosine triphosphatase (ATPase) rate of actomyosin (AM)) from cross-bridges during a maintained isometric contraction are similar, indicating that the ADP-release step or an isomerization preceding it may be limiting the adenosine triphosphatase rate. We conclude that the strain- and dephosphorylation-dependent high affinity for and slow ADP release from smooth muscle myosin prolongs the fraction of the duty cycle occupied by strongly bound acto-myosin.ADP state(s) and contributes to the high economy of force.

INTRODUCTION

A distinctive property of the contractile mechanism of smooth muscle is the very high affinity of smooth muscle myosin for adenosine diphosphate (ADP) (Cremo and Geeves, 1998; Gollub et al., 1999; Fuglsang et al., 1993; Nishiye et al., 1993) that gives rise to important physiological properties. The rate of dissociation of ADP from actomyosin (AM) is correlated with the maximal shortening velocity of muscle (Siemankowski et al., 1985), and tight binding of ADP to smooth myosin results in a strongly bound, AM.ADP state that contributes to force maintenance at low levels of regulatory light chain (RLC) phosphorylation (Fuglsang et al., 1993; Khromov et al., 1995, 1998; Nishiye et al., 1993).

The purpose of this study was to determine directly the rate of ADP release from myosin as a function of 1), RLC phosphorylation and 2), the strain on cross-bridges. We also compared the ADP-release rates from tonic and phasic smooth muscle myosins because their affinities for ADP and, hence, the effects of ADP on force maintenance in the two types of muscles are quantitatively different (Fuglsang et al., 1993; Khromov et al., 1995, 1996, 2001).

We used fluorescent ATP and ADP analogs bound to myosin to determine rates of product release from both isolated myosin and from myosin filaments in smooth muscle. We established that the fluorescent ATP analog is a suitable substrate for smooth as for skeletal (Webb and Corrie, 2001) muscle myosin, determined that the fluorescent ADP analog binds to the myosin containing A-bands of striated muscle, and recorded the time courses of fluorescence change resulting from displacement of fluorescent ADP by non-fluorescent ATP or ADP released from caged precursors (reviewed in McCray and Trentham, 1989; Somlyo and Somlyo, 1990). Our major findings show that both RLC phosphorylation and strain affect ADP-release kinetics and modulate the ability of smooth muscle to maintain force through strongly bound AM.ADP cross-bridges.

METHODS

Experimental apparatus

The experimental set up for flash photolysis consisted of a computer controlled muscle trough system for solution exchange and a quartz window for photolysis, a force transducer, length adjusting device, and 30-ns pulse 347-nm frequency doubled ruby laser (Lumonics, Warwickshire, England) as described in detail previously (Khromov et al., 1998; Nishiye et al., 1993).

Fluorescence was observed with an epifluorescence microscope (Olympus BX30, Olympus, Tokyo, Japan) mounted above the quartz photolysis trough similar to the apparatus described in (He et al., 1998). Excitation was at 425 nm with a halogen lamp (Philips, Eindhoven, The Netherlands, No. 7724, 100 W), and collection of the emitted light at 475 nm was through a 40× water immersion objective (0.8 NA, Olympus, I-UM568). For separation of exciting and emitted light, a dichroic mirror (CT-91017) and filter (CT-31001, Opelco, Dulles, VA) were used together with the additional optical filters (L390, Schott, Duryea, PA), placed before the photomultiplier (PMT) for elimination of scattered light originating from the 1-ms laser pumping flash light. Emitted fluorescence was detected by the PMT (ORIEL Straftord, CT, No. 77348, 800-V cathode voltage) mounted on the microscope head and connected to the data collecting system via a transimpedance amplifier (A1, Thorn EMI, Geneom, Fairfield, NJ). Fluorescence and force signals were digitized (sampling rate 1000 Hz) and simultaneously collected using the Labview 4.0 data acquisition program

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(National Instruments, Austin, TX). The time course of fluorescence change after photolysis was analyzed to determine its rate constant by nonlinear least squares fitting to single or double exponential functions using Sigma Plot 4.0 software (Jandel Scientific, San Rafael, CA).

Photolysis of 1-(2-nitrophenyl)ethyl phosphate esters of adenine nucleotides (NPE-caged nucleotides, ATP, and ADP) yields nucleotides at a rate limited by the rate of decay of the *aci*-nitro intermediate (Walker et al., 1988), which has a broad absorption band with a peak close to 406 nm, thus affecting the excitation and emission intensities of the observed fluorescence. The contribution of the decay of the *aci*-nitro intermediate to the fluorescence signal was determined experimentally by photolyzing caged nucleotide in solution and rearranging the halogen light source and the CT-91017 dichroic mirror and filters used for excitation from above to below the muscle trough. Consequently, the light at 425 nm passed directly to the PMT through the transparent quartz bottom of the trough and the capillary filled with the fluorophore and NPE-caged compound. At 20°C photolysis of caged ADP caused a sharp decrease in the intensity of detected light resulting from absorption at 406 nm by the *aci*-nitro intermediate, recovering to the initial level with the rate of the *aci*-nitro decay 50–75 s^{-1}.

Fluorescence measurements of smooth and skeletal muscle fibers

The coumarin-labeled nucleotide 3′-deac-eda-ADP (deac-edaADP) used in these experiments was synthesized as described previously with a purity of 94% (Webb and Corrie, 2001). The rate of ADP dissociation from myosin was estimated by following the fluorescence transient resulting from displacement of the fluorescent diphosphate bound to myosin with a large excess of nonfluorescent natural ligand: ATP or ADP released by laser flash photolysis at 347 nm from, respectively, NPE-caged ATP or NPE-caged ADP (Molecular Probes, Eugene, OR). To maintain a constant total concentration of fluorophores in the smooth muscle during the time of observation, the loaded muscle strips were transferred into the trough with a quartz front window and prefilled with silicone oil (Dow Corning, Midland, MI, Fluid No. 200, 10 centistokes) that had low absorption and low intrinsic fluorescence within the range of 260–500 nm. The laser beam was focused into an ellipse of 4 mm × 1 mm with a cylindrical quartz lens to illuminate the strip. A predetermined number of glass slides were inserted into the light pathway to vary, as needed, the laser energy reaching the preparation and hence the nucleotide photolysis yield. Photolysis yields of ADP or ATP, used as displacing ligands, were estimated by high-performance liquid chromatography (HPLC) analysis of the postphotolysis solutions of caged compounds at the same concentration and conditions of illumination (area and energy) as used in these experiments. The yields were 15–20% for both caged ADP and caged ATP, at 1.0 mM caged ADP or caged ATP.

ADP released from caged ADP rather than ATP from caged ATP was generally used for displacing deac-edaADP from smooth muscle, avoiding potential problems due to dissociation of myosin from actin and the presence of ATP (Conibear and Bagshaw, 1996). Caged nucleotides were cleaned by apyrase treatment (100 μg/ml) as described previously (Nishiye et al., 1993).

Tissue preparation and mechanical measurements

Rabbit bladders (Rbls) and femoral arteries (Rfas) were dissected from New Zealand white rabbits anesthetized with halothane and exsanguinated as approved by the Animal Care and Use Committee at the University of Virginia. Glycerinated rabbit psoas fibers were prepared as previously detailed (Thirlwell et al., 1994). Small strips of smooth muscle 150–200-μm wide and 2–3-mm long were tied to the hooks of the force transducer (AE801, AME, Horten, Norway) and to the length adjusting device in the experimental trough system filled with HEPES buffered Krebs solution (Fuglsang et al., 1993). After permeabilization (0.5% Triton X-100 for 15 min in G10 solution; Table 1), the strips were activated with pCa 6.0 solution (Table 1) containing 1 μM added calmodulin and, at the plateau of developed force, transferred into Ca-free rigor solution (0CaR; Table 1) (high rigor protocol).

TABLE 1 Composition of the solutions used for the experiments (mM)

	Na_2ATP	$MgMes_2$	PIPES	EGTA	KMes	CaEGTA
Relaxing (G10)	4.6	6.07	30	10	70	0
Rigor (0CaR)	0	2.7	30	10	112.9	0
pCa 6.0	4.5	6.4	30	2.11	50	7.88

pH 7.1, ionic strength 0.2 M, magnesium methanesulfonate ($MgMes_2$), potassium methanesulfonate (KMes) stock solutions were prepared from methanesulfonate acid and MgO or KOH. For the photolysis solutions only, 20 mM KMes was substituted by an equal amount of glutathione to eliminate the effects of the photolysis by-products (nitroso-ketones).

In some cases, smooth muscles were thiophosphorylated as described earlier (Nishiye et al., 1993). The extent of regulatory myosin light chain (MLC_{20}) thiophosphorylation was determined by two-dimensional gel electrophoresis (Kitazawa et al., 1991). Thiophosphorylated smooth muscles were activated with 4 mM MgATP in the absence of Ca and high rigor developed by transferring into 0CaR solution as described above. When in rigor, the residual ATP and/or ADP were removed from the strips by intensive washing (30 min in 0CaR solution while stirring and with four to five solution changes). The strips were loaded for 5 min with fluorescent nucleotide and caged ADP in 0CaR solution, transferred into the photolysis trough (25-μl volume) prefilled with silicone oil. Finally, the trough was covered with a glass cover slip to maintain a constant optical pathway. Mounting the single skeletal muscle fibers, loading with deac-edaADP, and UV photolysis were conducted as described for smooth muscle.

After photolysis of caged ADP and data collection, the muscle strips were transferred into G10 relaxing solution for 30 min, and nucleotides were washed out before the next experimental cycle, until no fluorescence was detected by the PMT. The kinetics of displacement obtained with a single strip were unchanged after up to three to four experimental cycles, indicating that there was no significant loss of adenosine triphosphatase (ATPase) activity due to UV irradiation.

Maximal velocity of unloaded shortening was determined by the slack test (Edman, 1979) as described earlier (Khromov et al., 1998): three different releases (7–15% of initial length, at a release rate > 200 muscle length/s), were applied at the plateau of the developed isometric force with a servo motor (model 6800, Cambridge Technology, Watertown, MA) operating in length control mode, and the velocity was estimated as the slope of release length to time to onset of force (slack length versus time). Experiments were conducted at 20°C.

Protocol for modifying the strain on cross-bridges

The strain imposed on the muscle was varied by stretching in small steps (5% increase of rigor force in 5 min) starting from zero (basal) level until force reached 25–30% of maximal force (P_{max}; increased strain) or by releasing the muscle length in small steps (decreased strain). Using this protocol the rigor force after the 5 min of stabilization was maintained with less than a 10% relative decrease within 20 s of observation. The measurements for decreased strain were conducted at zero force (unstretched muscle) achieved by releasing the muscle in the high rigor state to a slack length. Negative strain was not used, to avoid the effects of movement artifacts on the fluorescence signal during force recovery.

Localization of deac-edaADP

Skeletal fibers loaded with deac-edaADP were imaged at 470–490 nm using two-photon excitation at 760 nm in the W. M. Keck Center for Cellular

Imaging of University of Virginia. Single rabbit psoas fibers were mounted on glass slides by fixing T-clips to the ends and attaching the clips to double stick tape. The fibers were washed, loaded using procedures identical to that in the mechanical experiments with or without exposure to a 50-ns 347-nm laser pulse, mounted in silicone oil and the preparation covered by a glass coverslip and imaged. The distribution of deac-edaADP fluorescence was compared before and after photolysis of caged ADP.

Myosin purification and fluorescence measurements with smooth muscle myosin and skeletal heavy meromyosin

The preparation of smooth muscle myosin and skeletal muscle heavy meromyosin (HMM), ATP hydrolysis, and single turnover measurements using the 2-amino-6-mercapto-7-methylpurine riboside (MESG)/purine nucleoside phosphorylase (PNP) system as described by Webb (1992) as well as the method for eliminating damaged myosin molecules (dead heads) and contaminant actin (Baker et al., 2003) are described in detail online (see Supplementary Material).

Smooth myosin regulatory light chains (RLC_{20}) were thiophosphorylated as described earlier (Ikebe and Hartshorne, 1986; Zhang et al., 1997). Before the displacement experiments, the proteins were subjected to overnight dialysis at 4°C in ATP- and Ca-free high ionic strength (350 mM K methanesulfonate) 1,4-piperazinediethanesulfonic acid (PIPES) buffer (30 mM, pH 7.0). The extent of thiophosphorylation of smooth myosin RLCs was determined by its separation on urea gels (Facemyer and Cremo, 1992).

Deac-edaADP release from smooth muscle myosin and skeletal HMM in solution

For deac-edaADP displacement experiments, smooth muscle myosin or skeletal HMM at concentrations of 2–5 μM in high salt (350 mM K methanesulfonate) Ca-free buffer were mixed stoichiometrically with deac-edaADP together with 1 mM caged ADP and 10 mM glutathione (GSH) and introduced into a thin glass capillary (0.5-mm inner diameter, 1.5-mm outer diameter) and placed in the focal plane of the fluorescence microscope in the beam path of the UV laser. The vertical position of the microscope was adjusted for maximal observable fluorescence before photolysis. The displacement of deac-edaADP was effected by caged ADP photolysis.

RESULTS

Deac-edaATP as a substrate for smooth muscle contraction

The basic fluorescence properties of deac-edaATP excitation and emission maxima (430 nm and 477 nm), fluorescence quantum yield in free solution (0.038) and on skeletal subfragment 1 (S1) (~0.012), and a K_d of 1.3 μM for binding to skeletal subfragment 1 have been reported (Webb and Corrie, 2001). Steady-state ATPase measurements and the nucleotide release rates were similar to those reported for the natural nucleotide (Webb and Corrie, 2001).

We examined the ability of deac-edaATP to serve as a substrate, compared with ATP, for supporting contraction of permeabilized smooth muscle. As deac-edaATP is not a good substrate for MLC_{20} kinase or for creatine phosphokinase (CK) (data not shown), we used muscle strips (Rbl) containing thiophosphorylated RLC_{20} without an ATP regenerating system. Velocity of unloaded shortening, an index of smooth muscle actomyosin ATPase activity, was determined at 1.0 mM and 2.0 mM MgATP or Mg-deac-edaATP. At 1.0 mM and 2.0 mM, deac-edaATPase activity was 0.8 of that of ATPase activity, which in turn was 0.4 of that of the ATPase in the presence of a backup ATP regenerating system. These results suggest that deac-edaATP is a satisfactory substrate for smooth muscle contraction.

Optimization of deac-edaADP loading

Unlike a single skeletal fiber, the thicker smooth muscle strips scatter and absorb more excitation light (inner filter effect). This, combined with the three to five times lower myosin content of smooth muscles, reduces drastically the magnitude of the observed changes in fluorescence intensity.

To estimate the contribution of the absorption of the excitation light to the observable fluorescence changes during displacement of deac-edaADP in muscles and thus optimize loading conditions, we measured fluorescence intensity as a function of deac-edaADP concentration in permeabilized smooth and skeletal muscles at different intensities of excitation light. When muscle strips were loaded with fluorophore in the range 0.015–8 μM, immersed in oil, and illuminated with the same excitation light intensity, the observed fluorescence intensity was not proportional to the concentration of deac-edaADP (Fig. 1). At maximal excitation light intensity (*open symbols*), the deviation from linearity started in Rbl at 0.25 μM loaded fluorophore, and

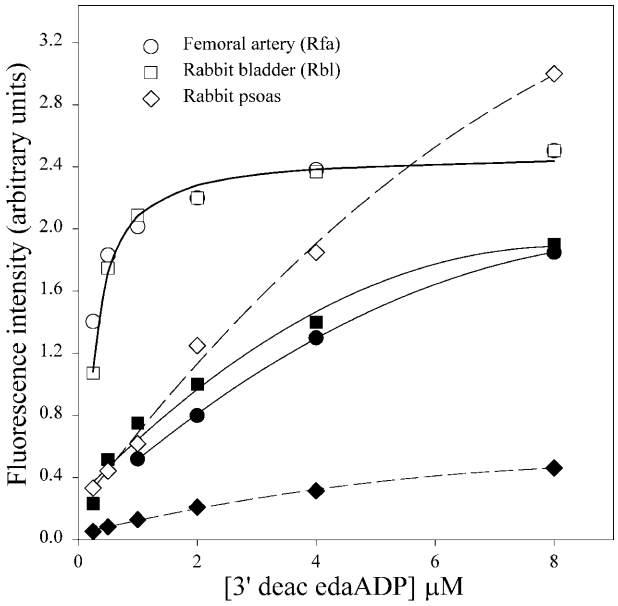

FIGURE 1 Dependence of the intensity of fluorescence as a function of [deac-edaADP] measured in skeletal fibers (*diamonds*), Rbl (*squares*), and Rfa (*circles*) at high (*open symbols*) and low (*solid symbols*) excitation light intensity.

fluorescence intensity remained constant at higher deac-edaADP concentrations. In single rabbit psoas fibers (*diamond symbols*) the fluorescence intensity was approximately linear over a much broader range of deac-edaADP concentrations (up to 8 μM). Decreasing the intensity of excitation light (~twofold) for Rbl and Rfa smooth muscle increased the linear range of fluorophore concentration dependence up to 5 μM but reduced sensitivity (~threefold). Thus, an optimal fluorophore concentration (2–4 μM) and intensity of excitation light (40–60% of maximum) was chosen as a compromise between linearity and sensitivity of detection of the fluorescence signals.

Fluorescence transients of isolated nonphosphorylated and thiophosphorylated smooth muscle myosin and skeletal HMM

After photorelease of ADP (~50–100 μM), the intensity of fluorescence exponentially increased (for skeletal HMM) or decreased (for smooth muscle myosin), as shown in Fig. 2. The relative amplitudes and rates of these changes were significantly different for the two myosins: positive ~15% at ~3 s^{-1} for skeletal HMM and negative ~6% at ~1–2 s^{-1} for smooth muscle myosin, respectively. Large, but short (~10 ms), fluorescence intensity transients, observed immediately with the laser pulse, were artifacts probably resulting from the 1-ms xenon lamp flash, required to produce the laser flash, scattered by the solution and/or capillary, since they were also observed in the absence of myosin in a capillary. Photorelease of 50 μM ADP completely displaced 2–4 μM bound deac-edaADP from turkey gizzard myosin, as no fluorescence change was induced by a second laser flash. Increasing the concentration of smooth muscle myosin from 2 μM to 5 μM led to an increase in the amplitude of fluorescence decay (~twofold) with a similar rate of fluorescence decay (~1 s^{-1}). The fluorescence traces of smooth muscle myosin were fitted better (chi-square decreased twofold) by two exponentials, having rate constants and amplitudes of ~7–8 s^{-1} and ~0.5 s^{-1} and ~60% and ~40%, respectively, rather than by a single exponential having rate constant ~1–2 s^{-1}. The sources of these two components were evaluated in additional experiments where the fast phase was found to be simply due to actin contamination or dead heads.

Thiophosphorylation of gizzard smooth muscle myosin MLC_{20} increased the myosin ATPase rate to 0.04 s^{-1} vs. 0.007 s^{-1} for unphosphorylated myosin, but did not significantly change either the amplitudes or the rate constants of the fast and slow components of the fluorescence decrease after displacement of deac-edaADP (fit to two exponentials; Table 2).

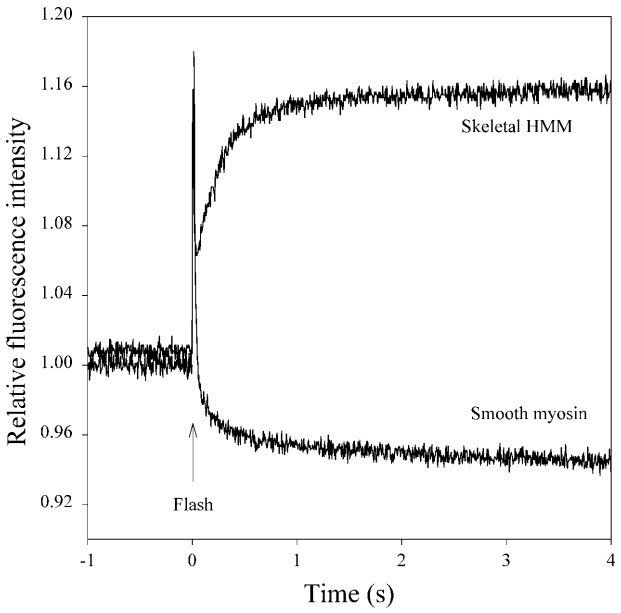

FIGURE 2 The time courses of fluorescence change of the rabbit skeletal HMM and unphosphorylated turkey gizzard smooth muscle myosin in solution loaded with deac-edaADP after photolysis of 1 mM caged ADP. Fluorescence change was normalized relative to initial intensity before photolysis.

Fluorescence transients in skeletal muscle fibers

After photolysis of caged ADP, the release of deac-edaADP from cross-bridges in skeletal muscle fibers was accompanied by an exponential increase in fluorescence (similar to that seen with skeletal HMM) as shown in Fig. 3, with amplitude (relative to fluorescence before photolysis of caged ADP) and rate constant of ~25% and ~40 s^{-1}, respectively, similar to previously reported values (Millar et al., 1999). The initial sharp decrease in fluorescence intensity followed by its recovery (with the rate constant ~50 s^{-1}) was the result of absorption of the excitation light

TABLE 2 Kinetic parameters of fluorescence change after displacement of deac-edaADP from gizzard myosin and rabbit skeletal HMM

	Fluorescence		Rate constant	
Specimen	Direction ±	Amplitude* % mean ± SE	(s^{-1}) mean ± SE	*n*
Skeletal HMM	+	~15	~3.0	3
Smooth muscle myosin	–			
(Unphosphorylated)	–	6.0 ± 0.2	7.0 ± 0.8 (0.4 ± 0.1)†	12
(Without dead heads)	–	5.0 ± 0.2	0.51 ± 0.07	11
(Thiophosphorylated)	–	6.0 ± 0.3	8.8 ± 1.2 (0.5 ± 0.1)†	15
(Without dead heads)	–	5.0 ± 0.3	0.4 ± 0.1	10

*Change in fluorescence intensity relative to that before photolysis of caged ADP.
†Data in parenthesis are for slow component of fluorescence decrease.

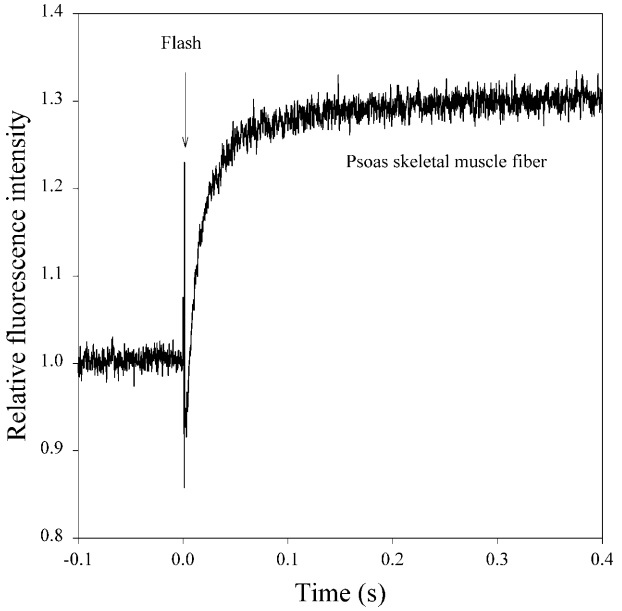

FIGURE 3 The time course of fluorescence increase (relative to fluorescence before photolysis) in a single rabbit psoas muscle fiber loaded with deac-edaADP (80 μM) after photolysis of 5 mM caged ADP. Rate constant of fluorescence increase was estimated as $\sim$48 s^{-1}.

(425 nm) by the intermediate *aci*-nitro group generated during the photolysis process of caged ADP (McCray and Trentham, 1989; Walker et al., 1988).

Imaging of deac-edaADP in single skeletal muscle fibers

The location of deac-edaADP in single rabbit psoas fibers before and after photolysis of caged ADP was imaged by two-photon excitation of deac-edaADP fluorescence at 760 nm and imaging at 470–490 nm. The fluorescent striation pattern initially observed (Fig. 4 *A*) disappeared after laser flash photolysis of caged ADP (Fig. 4 *B*), supporting the interpretation that deac-edaADP, bound to myosin in A-bands before photolysis, was displaced by the photo-released nonfluorescent ADP.

Fluorescence transients in phasic and tonic smooth muscle, the effects of thiophosphorylation and strain

The major focus of this study was to determine the effects of strain, imposed on smooth muscle rigor cross-bridges and of thiophosphorylation of RLC_{20} on the kinetics of ADP release monitored by deac-edaADP fluorescence transients. The signal due to the *aci*-nitro decay that followed photolysis of caged ADP was much faster ($\sim$50–75 s^{-1}), than the rate of fluorescence change observed due to deac-edaADP release in smooth muscle ($\sim$1 s^{-1}) and was subtracted before further data processing.

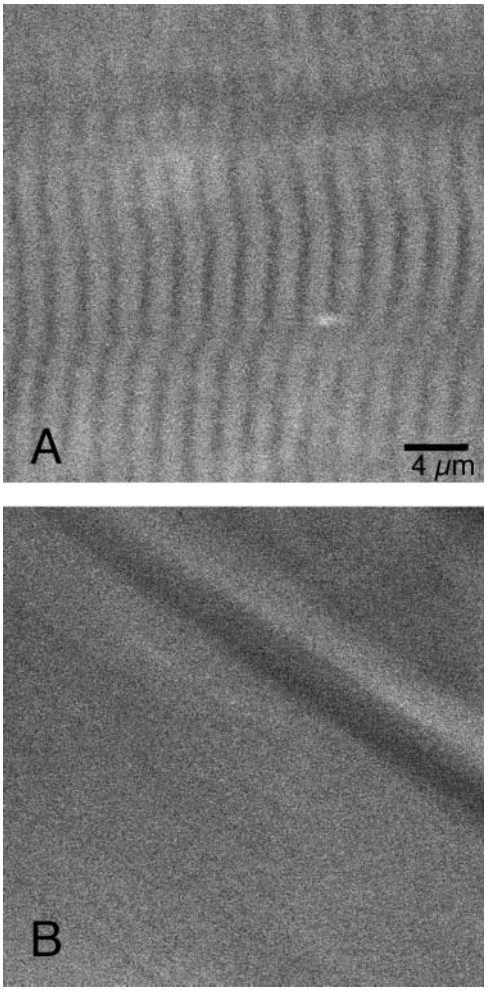

FIGURE 4 The two-photon microscopy images of a single rabbit psoas muscle fiber loaded with 20 μM deac-edaADP before (*A*) and after (*B*) photolysis of 2 mM caged ADP. The two-photon excitation (760 nm) of the deac-edaADP fluorescence imaged at 470–490 nm. A striation pattern with an $\sim$2-μm repeat was observed before photolysis. The ridge along the longitudinal axis of the fiber in *B* reflects a fold in the surface. The fluorescence after photolytic release of ADP indicates displacement of the fluorophore from myosin to give a homogeneous distribution.

The effects of strain were determined at two extremes, i.e., in unstretched muscles (zero strain) and in muscles stretched up to $\sim$25–30% of P_{max}.

At zero imposed strain in both tonic and phasic smooth muscles, the intensity of fluorescence decreased monotonically, after photolysis of caged ADP (Fig. 5 *A*), similar to that observed for smooth muscle myosin in solution. Both the rate constants and amplitudes of the fluorescence signal change differed from those in skeletal muscle (25 $\pm$ 10% and 40.0 $\pm$ 5 s^{-1}, respectively) by an order of magnitude with the lowest value measured in strained tonic smooth muscle being 0.14 $\pm$ 0.09 s^{-1}, amplitude 2 $\pm$ 1%, and the highest value measured in unstrained thiophosphorylated phasic muscle 1.40 $\pm$ 0.20 s^{-1}, amplitude 4 $\pm$ 1%. The magnitude of the fluorescence change was maximum at 2–4 μM

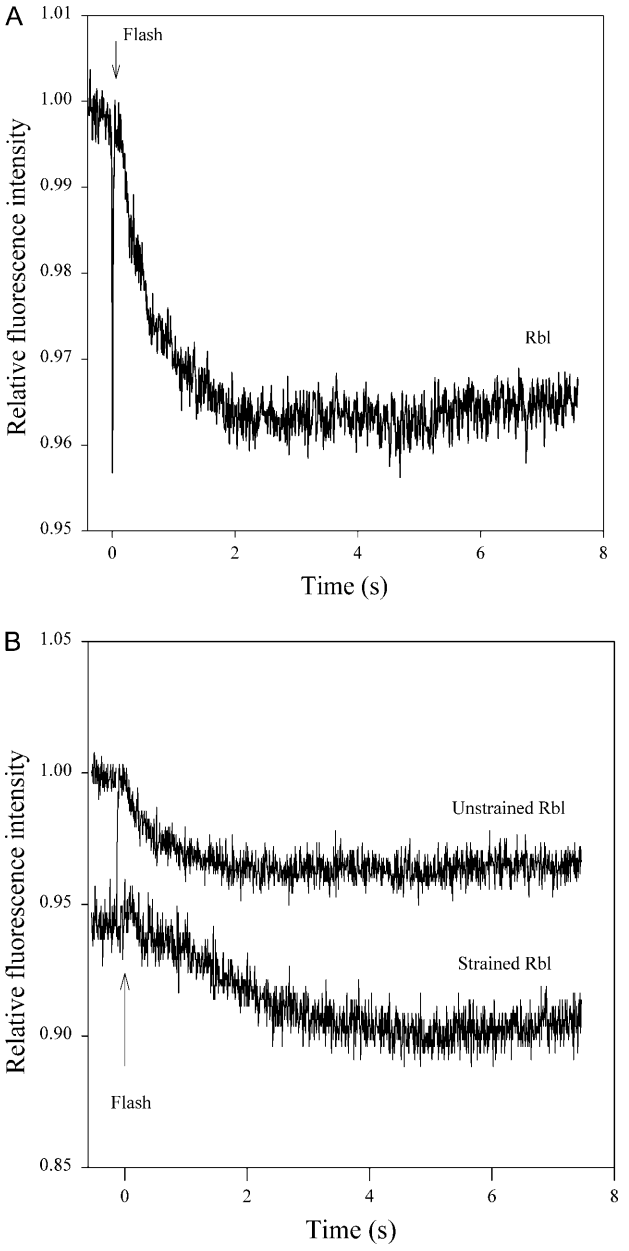

FIGURE 5 (*A*) Typical record of relative decrease in fluorescence (relative to fluorescence before photolysis) of smooth muscle (*Rbl*) loaded with deac-edaADP (4 μM) initiated by photolysis of 2 mM caged ADP. (*B*) The time course of relative fluorescence decrease after photolysis of caged ADP in the unstrained or strained (up to ~30% of P_{max}) Rbl smooth muscle. For clarity the strained trace was shifted down by 0.05 units.

deac-edaADP, consistent with the nonlinear dependence of the fluorescence intensity upon fluorophore concentration (Fig. 1). No significant change in rigor force accompanied the fluorescence transients when photolysis of caged ADP was used to produce the displacing ligand, ADP. The absence of detectable changes in rigor force after photolysis of caged ADP was consistent with most of the cross-bridges containing bound nucleotides: deac-edaADP (before photolysis) or ADP (after photolysis), in contrast to the mechanical perturbations induced by ADP binding to nucleotide-free cross-bridges (Khromov et al., 2001).

Under the same conditions (with respect to strain), the rate of fluorescence decrease was significantly faster in phasic (Rbl) than in tonic (Rfa) smooth muscle: at zero strain the rates were 0.57 ± 0.06 s^{-1} ($n = 18$) for Rbl and 0.29 ± 0.03 s^{-1} ($n = 16$) for Rfa. Both rates, however, were reduced significantly (twofold) by positive strain (~25–30% of P_{max}) imposed on the muscles: 0.3 ± 0.2 s^{-1} and 0.14 ± 0.09 s^{-1} for phasic and tonic smooth muscles, respectively (Table 3; Fig. 5 *B*).

Photolysis of caged ADP in smooth muscles containing thiophosphorylated RLCs under zero strain also showed a decrease in fluorescence with a rate significantly (twofold) faster in both phasic (1.4 ± 0.2 s^{-1}) and tonic (0.63 ± 0.08 s^{-1}) smooth muscles than in the case of unphosphorylated RLCs (Table 3). This result was different from that obtained with smooth myosin in solution in the absence of actin, in which thiophosphorylation did not affect ADP-release kinetics (Table 2).

To summarize, external strain decreased (by twofold) the rate of ADP release in muscles containing both unphosphorylated and thiophosphorylated RLC_{20} in tonic and in phasic smooth muscles.

Relationship between the time course of fluorescence transients and cross-bridge detachment

We also examined whether the deac-edaADP fluorescence transients correlated with the slow phase of ATP-induced

TABLE 3 Kinetic parameters of the fluorescence change after displacement of deac-edaADP in skeletal and smooth muscles

	Fluorescence		Rate constant	
Specimen	Direction ±	Amplitude* % mean ± SE	(s^{-1}) mean ± SE	*n*
Skeletal fiber	+	25 ± 10	40 ± 5	3
Unstrained smooth muscle Rfa				
(Unphosphorylated)	–	2 ± 1	0.29 ± 0.03	16
(Thiophosphorylated)	–	2 ± 1	0.63 ± 0.08	12
Strained smooth muscle Rfa				
(Unphosphorylated)	–	2 ± 1	0.14 ± 0.09	10
(Thiophosphorylated)	–	2 ± 1	0.43 ± 0.04	10
Unstrained smooth muscle Rbl				
(Unphosphorylated)	–	4 ± 1	0.57 ± 0.06	18
(Thiophosphorylated)	–	4 ± 1	1.4 ± 0.2	16
Strained smooth muscle Rbl				
(Unphosphorylated)	–	4 ± 1	0.3 ± 0.2	15
(Thiophosphorylated)	–	3 ± 1	0.5 ± 0.1	9

*Change in fluorescence relative to fluorescence before photolysis of caged ADP.

relaxation from rigor. The time course of rigor force relaxation in both skeletal and smooth muscles consists of a fast component (due to ATP binding to nucleotide free cross-bridges in the AM state and rapid detachment from actin) and a slow component rate limited by ADP release from cross-bridges in AM.ADP state (Goldman et al., 1984; Somlyo et al., 1988).

Permeabilized smooth muscle (Rfa) in high rigor was loaded with fluorescent nucleotide (1 μM) and caged ATP (10 mM), instead of caged ADP as in the protocol described above, to induce muscle relaxation and simultaneously monitor fluorescence and force. As shown in Fig. 6, after a delay, fluorescence decreased exponentially with the rate ($\sim$0.15 s^{-1}) similar to the rate of the slow phase of force relaxation ($\sim$0.13 s^{-1}), both saturating at $\sim$1 min after photolysis. No fluorescence change was observed during the fast ($\sim$30 s^{-1}) phase of force relaxation. These findings support the interpretation that the fluorescence decrease reflects dissociation of ADP from rigor cross-bridges in AM.ADP state(s) (Fuglsang et al., 1993; Nishiye et al., 1993; Somlyo et al., 1988; Thirlwell et al., 1994). The delay in the fluorescence signal was variable and not studied further, but may reflect a change in optical properties due to cross-bridge detachment or redistribution of strain on the population of attached heads with bound deac-edaADP.

Control experiments

A control experiment was conducted on smooth muscle (Rbl) preloaded with fluorescent nucleotide and caged ADP together with an excess of nonfluorescent ADP (1 mM), such that displacement of deac-edaADP by ADP would be expected to occur before photolysis. Under these conditions the laser flash caused no change in fluorescence apart from short transients due to the *aci*-nitro intermediate (Fig. 7, *trace 1*).

Ecto-ATPase, -ADPase activity, even though diminished by Triton X-100 permeabilization (He et al., 1998; Trinkle-Mulcahy et al., 1994), remains high in smooth muscle and could have contributed to the fluorescence signal observed in smooth muscle, although its affinity for ADP is much lower than that of myosin ($K_D \sim$ 1–2 mM vs. 1–5 μM; Nishiye et al., 1993). To verify whether binding to ectonucleotidase activity could corrupt the fluorescence signals that arise upon release from myosin, experiments were performed on intact smooth muscle, where the extracellularly introduced fluorophore was not able to enter the cell. After addition of identical concentrations of deac-edaADP (4 μM) and caged ADP (2 mM) to intact as used for permeabilized muscles, photolytic release of ADP produced no change in fluorescence. An $\sim$1% or less decrease in fluorescence (Fig. 7, *trace 2*) was observed upon photolysis of 5 mM caged ADP in the presence of a large excess of deac-edaADP ($\sim$50 μM) to enhance fluorophore binding to ecto-ATPase(s). However, when the same strip was permeabilized, followed by loading with a much lower concentration of a fluorescent nucleotide analog ($\sim$1 μM), photolysis of 2 mM caged ADP caused the

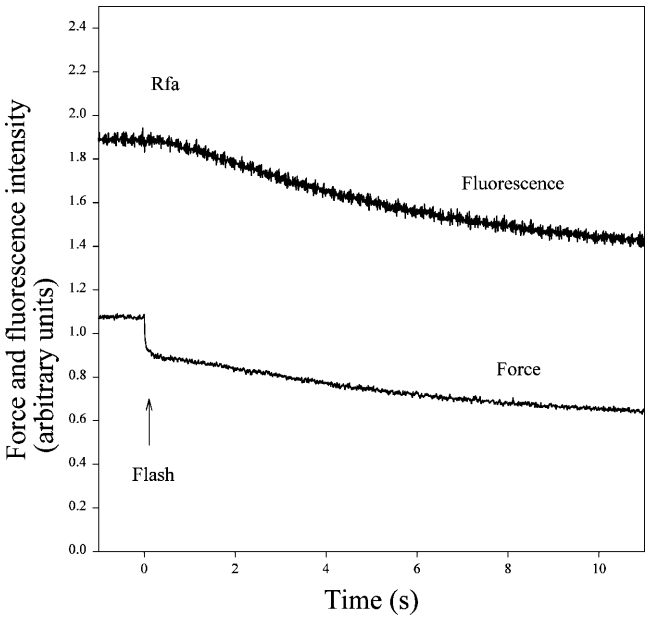

FIGURE 6 The time courses of force and fluorescence obtained simultaneously on the same smooth muscle (*Rfa*) labeled with 1 μM deac-edaADP, after photolysis of 5 mM caged ATP. Note the fluorescence decrease, which correlates with the slow phase of force decline.

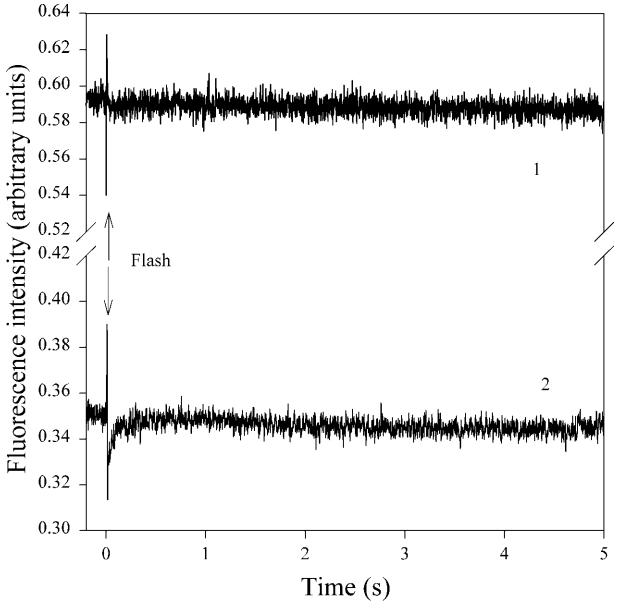

FIGURE 7 Control experiments. (*Trace 1*) Demonstrating that the decrease in fluorescence after photolysis of caged ADP in permeabilized smooth muscle (Rbl) is abolished when carried out in the presence of 1 mM preloaded nonfluorescent ADP. (*Trace 2*) Demonstrating that binding of deac-edaADP to the ecto-ATPase does not significantly contribute to the fluorescence changes observed in experiments in smooth muscle. Only a small decrease of fluorescence ($\sim$1%) was observed in intact smooth muscle (Rbl) loaded with 50 μM deac-edaADP after photolysis of 5 mM caged ADP.

characteristic exponential decrease in fluorescence amplitude of ~3% (Fig. 5 *A*). Therefore, under the conditions used, binding of deac-edaADP to ecto-ATPase site(s) did not significantly contribute to the fluorescence changes observed in experiments with smooth muscles.

DISCUSSION

The major findings of our study (Table 3) are 1), the rate of ADP release from rigor cross-bridges in smooth muscle is increased by thiophosphorylation of the myosin RLCs and decreased by positive strain; 2), the rates are higher in the phasic, bladder than in the tonic, femoral artery smooth muscle, and the effects of reduced strain and thiophosphorylation are additive: ADP release is fastest (1.4 s^{-1}) in unstrained phasic (bladder) smooth muscle in which the RLCs are thiophosphorylated (Table 3); and 3), the rates of ADP release from rigor bridges and of P_i release from cross-bridges during a maintained isometric contraction in a similarly phasic muscle, the portal vein (He et al., 1998) are similar.

Several controls verified that the fluorescent nucleotides used to report ADP release occupied the nucleotide-binding pocket of smooth muscle myosin cross-bridges. Namely, deac-edaATP is equivalent to MgATP as a substrate for striated muscle myosin (Webb and Corrie, 2001), supported the same maximal shortening velocity of smooth muscle as MgATP, and localized to the A-bands of rabbit psoas fibers (Fig. 4). The fluorescent signals were not affected by ecto-ATPases (Fig. 7) and the time course of fluorescence decay reporting ADP release (Fig. 6) was similar to the slow phase of cross-bridge detachment thought to be rate limited by ADP release (Somlyo et al., 1988).

The change in fluorescence that accompanied deac-edaADP release from smooth muscle was small and negative (Fig. 5 *A*), whereas the signal from skeletal muscle was large and positive (Figs. 2 and 3), consistent with the opposite polarities of the signals reporting deac-edaADP binding to isolated smooth muscle myosin (this study), skeletal HMM (this study) and skeletal myosin S1 in solution (Webb and Corrie, 2001). The differences in fluorescence quenching between the proteins and the medium presumably reflect the different atomic structures of the respective nucleotide-binding pockets and/or the neighboring loop 1 (reviewed in Sweeney, 1998) of the two myosins.

Removal of unregulated myosin (dead heads) is important for quantifying ATPase activity of isolated smooth muscle myosin (Sellers, 1985), and in the presence of unregulated myosin and/or contamination with actin, before dead head removal, ATPase activity of smooth muscle myosin was more than an order of magnitude higher (Table 2). After dead head removal, ADP release was slower and the ATPase activity was also decreased suggesting that the fast rates were largely due to contamination with actin. The rates of ADP release from isolated skeletal muscle myosin S1 subfragment (1.3 s^{-1}; Webb and Corrie, 2001), skeletal muscle in rigor (31.1 s^{-1}; Millar et al., 1999), gizzard myosin S1 (0.9 s^{-1}; Marston, 1982), permeabilized rabbit portal vein smooth muscle (0.2 s^{-1} in thiophosphorylated portal vein averaged over 10 s; Butler and Siegman, 1998; Vyas et al., 1994), and smooth muscle acto-S1 (22 s^{-1}; Cremo and Geeves, 1998; 15 s^{-1}; Marston and Taylor, 1980) are in agreement with the results presented here from skeletal HMM and smooth muscle myosin (Table 2).

Positive strain decreased the rate of product release, which in turn reflects the rate of cross-bridge detachment, consistent with the original proposal of A. F. Huxley (Huxley, 1957) about the relationship between strain and detachment kinetics. The (twofold) slowing of ADP release by positive strain is likely to be an underestimate because of the rapid decrease in stress that follows a rapid stretch of smooth muscle (stress relaxation). The strain absorbed by series-elastic elements results in a lower strain on the cross-bridges themselves than imposed during the initial stretch. Nevertheless, qualitatively, the result indicates that positive strain slows ADP release, prolonging the strong binding, AM.ADP state, and force maintenance (Khromov et al., 1996, 1998, 2001; Nishiye et al., 1993). The opposite effects of positive and negative cross-bridge strain on ADP off-rate are also implied by the 10-fold increase in ADP turnover rate with transition from isometric to isotonic (decreased strain) conditions (Butler et al., 1995).

The fast component of ADP release from isolated smooth muscle myosin, determined without removal of contaminating actin, was significantly faster than even the highest rates determined in smooth muscle (compare Tables 2 and 3), suggesting that in muscle, unlike in solution, limitations on the motion of the catalytic domain of myosin bound to actin slow the rate of ADP release. Thus, we attribute the much faster ADP release in solution (Marston and Taylor, 1980; Rosenfeld and Taylor, 1984; Rosenfeld et al., 1998; Siemankowski et al., 1985; this study) and laser traps (Baker et al., 2003) to the greater mobility of unstrained catalytic domains. It may also reflect a heterogenous population of AM.ADP states (Sleep and Hutton, 1980) on strained rigor cross-bridges that completed the powerstroke or unstrained actomyosin that bound the ADP in solution. Others (Gollub et al., 1999) also found differences in ADP release between myosin in solution and smooth muscle: phosphorylation of RLCs increased by sevenfold the apparent dissociation constant for ADP-induced rotations of the smooth muscle light chain domain compared with the nonphosphorylated state, whereas phosphorylation of myosin in solution was without effect. These authors also implicated strain in the differences.

The rate of P_i release was 0.3 s^{-1} during the plateau of isometric contraction at 21°C in the phasic, thiophosphorylated rabbit portal vein smooth muscle (He et al., 1998). This rate of P_i release that corresponds to the steady-state ATPase

rate of actomyosin in a strained state is slightly less than the value found here for the transient rate constant of ADP release in thiophosphorylated, strained Rfa and half the value found for the thiophosphorylated, strained phasic Rbl (Table 3; 20°C). The ADP-release rates measured here were comparable, within experimental error, to the steady-state rate of P_i release (He et al., 1998). This indicates that under the conditions of the experiments the ADP-release step or an isomerization preceding it may be limiting the ATPase rate or at least contributes to it.

The rate of P_i release during force development (He et al., 1998), while the muscle is less strained, was six times faster than during the steady plateau of contraction, whereas here, in unstrained muscle, the transient rate of ADP release was only three times faster than in the strained muscle. This difference may well be accounted for by the fact that in our experiments the strained muscles were less strained (only 30% P_{max}) than is achieved during steady isometric contraction. However, it is possible that a different step, one that precedes ADP release, is rate limiting in the unstrained muscle, a possibility that is compatible with the strain dependence of the ADP-release step observed here. In this case the step in the reaction mechanism that controls the rate of ATP hydrolysis may be the P_i release step, or a step preceding it, such as an isomerization. Thiophosphorylation of the RLCs increased the ADP off-rate (this study) consistent with its (albeit small) effect on K_D (Nishiye et al., 1993), suggesting that, unlike its lack of effect on myosin in solution, phosphorylation of RLC can modulate this transition in (actomyosin) cross-bridge kinetics.

The effect of RLC thiophosphorylation on ADP release from noncycling (rigor) cross-bridges implies that this modification of the RLC has a structural effect on the myosin head, independent of dissociation of myosin from actin and catalytic activity. This is consistent with thiophosphorylation increasing the separation of the two heads of a single myosin molecule (Sheng et al., 2003; Zhang et al., 1997) and the stiffness of smooth muscle in rigor (Khromov et al., 1998).

The K_Ds (0.6 μM and 0.3 μM) of both phasic and tonic smooth muscle myosins calculated from kinetic measurements (ADP association rates of both myosins in situ of 1×10^6 M^{-1} s^{-1}; Khromov et al., 2001; ADP-release rates 0.6 s^{-1} or 0.3 s^{-1}; this study) were lower than the K_Ds (1 μM and 5 μM) estimated from the ADP concentration dependence of the amplitude of the fast phase of detachment of rigor bridges by ATP (Fuglsang et al., 1993; Nishiye et al., 1993) or myosin in solution (Cremo and Geeves, 1998). This difference could reflect the existence of an intermediate, probably strain-dependent AM′.ADP state not contributing to steady-state measurements (Gollub et al., 1999; Nishiye et al., 1993) or a kinetic constant not directly related to affinity (Cremo and Geeves, 1998).

It is likely that the very high affinity ($K_D = 0.3$–0.6 μM), of smooth muscle myosin for MgADP reflects the state(s) present in contracting muscle that is a major, if not the sole, contributor to the ability of tonic smooth muscle to maintain force at low levels of phosphorylation (Khromov et al., 1995, 1998). This high affinity also supports the conclusion that, although addition of MgADP to permeabilized smooth muscle has both functional (Khromov et al., 2001) and structural (Gollub et al., 1999) effects consistent with axial rotation in the direction observed in cryoelectron micrographs (Whittaker et al., 1995), on thermodynamic grounds it is highly unlikely for ADP release to be a force-generating step (Dantzig et al., 1999).

The different properties of, respectively, phasic and tonic smooth muscle myosins were also apparent in this, as in previous, related studies. ADP release from bladder was faster than from the femoral artery (Table 3), and the faster rate of force development by phasic than by tonic smooth muscles each containing thiophosphorylated RLCs indicated that these differences were inherent to their myosin isoforms (Horiuti et al., 1989), independent of the rates of RLC phosphorylation. Such differences in cross-bridge kinetics could be related to different LC_{17} light chain isoforms (Malmqvist and Arner, 1991; Matthew et al., 1998), as well as to the presence or absence of an insert in myosin heavy chain isoforms (Karagiannis et al., 2003; Kelley et al., 1993; Lauzon et al., 1998; White et al., 1993) or, most likely, the variable combination of the two (reviewed in Somlyo, 1993).

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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