Time-Resolved Measurements of Phosphate Release by Cycling Cross-Bridges in Portal Vein Smooth Muscle

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ABSTRACT The rate of release of inorganic phosphate (P_i) from cycling cross-bridges in rabbit portal-anterior mesenteric vein smooth muscle was determined by following the fluorescence of the P_i-reporter, MDCC-PBP (Brune, M., J. L. Hunter, S. A. Howell, S. R. Martin, T. L. Hazlett, J. E. T. Corrie, and M. R. Webb. 1998. *Biochemistry*. 37:10370–10380). Cross-bridge cycling was initiated by photolytic release of ATP from caged-ATP in Triton-permeabilized smooth muscles in rigor. When the regulatory myosin light chains (MLC_{20}) had been thiophosphorylated, the rate of P_i release was biphasic with an initial rate of 80 $\mu M\ s^{-1}$ and amplitude 108 μM, decreasing to 13.7 $\mu M\ s^{-1}$. These rates correspond to fast and slow turnovers of 1.8 s^{-1} and 0.3 s^{-1}, assuming 84% thiophosphorylation of 52 μM myosin heads. Activation by Ca^{2+}-dependent phosphorylation subsequent to ATP release resulted in slower P_i release, paralleling the rate of contraction that was also slower than after thiophosphorylation, and was also biphasic: 51 $\mu M\ s^{-1}$ and 13.2 $\mu M\ s^{-1}$. These rates suggest that the activity of myosin light chain kinase and phosphatase ("pseudo-ATPase") contributes <20% of the ATP usage during cross-bridge cycling. The extracellular "ecto-nucleotidase" activity was reduced eightfold by permeabilization, conditions in which the ecto-ADPase was 17% of the ecto-ATPase. Nevertheless, the remaining ecto-ATPase activity reduced the precision of the estimate of cross-bridge ATPase. We conclude that the transition from fast to slow ATPase rates reflects the properties and forces directly acting on cross-bridges, rather than the result of a time-dependent decrease in activation (MLC_{20} phosphorylation) occurring in intact smooth muscle. The mechanisms of slowing may include the effect of positive strain on cross-bridges, inhibition of the cycling rate by high affinity Mg-ADP binding, and associated state hydrolysis.

INTRODUCTION

The basic mechanisms of cross-bridge cycling in smooth and striated muscles are similar, although subject to different regulatory mechanisms and kinetics (Somlyo et al., 1988; Somlyo and Somlyo, 1994; Hartshorne, 1987). Consequently, they both pose the challenge of determining the relationship between the biochemical actomyosin ATPase cycle and the mechanical events of contraction. A particular problem has been to identify the rate-limiting step(s) of the mechanical and biochemical processes, commonly thought to be associated with release of the actomyosin ATPase products: ADP and inorganic phosphate (P_i) (Moss, 1997). Of these, P_i release from the nucleotide binding sites of cross-bridges or a preceding rapid isomerization is thought to determine the rate of the force-generating transition of the cross-bridge. However, activation of smooth muscle myosin is through phosphorylation of its regulatory light chain (MLC_{20}) by myosin light chain kinase (MLCK). Therefore, P_i is released not only from the heavy chain of myosin (nucleotide-binding domain), but also from phosphorylated MLC_{20}, by smooth muscle myosin phosphatase (SMPP-1M). In activated intact smooth muscles, therefore, a pseudo-ATPase resulting from MLCK and smooth muscle myosin phosphatase activity contributes a variable fraction of the energy metabolism associated with contraction. An additional complication of evaluating the various sources of ATP breakdown in permeabilized smooth muscles exposed to exogenous ATP is the significant contribution of the extracellular ATPase (ecto-ATPase) to total ATP breakdown (Trinkle-Mulcahy et al., 1994). The purpose of the present study was to time-resolve the rate of total P_i release and partition, to the extent possible, its components, actomyosin ATPase, pseudo-ATPase, and ecto-ATPase, and to relate the rate of P_i release to force generation.

MDCC-PBP is a P_i-sensitive probe, a fluorophore attached to a bacterial P_i-binding protein that increases its fluorescence upon binding P_i (Brune et al., 1994) and can follow the kinetics of P_i release with micromolar sensitivity and millisecond time resolution ($K_d = 0.1\ \mu M$ and association rate constant $= 1 \times 10^8\ M^{-1}\ s^{-1}$), and its use allowed us to determine the rate of total P_i release at millisecond time resolution. An estimate of the contribution of the pseudo-ATPase contributed by MLCK/SMPP-1M activity could be obtained by comparing the rates of P_i release when MLCK phosphorylation was rate-limiting contraction (Horiuti et al., 1989; Zimmermann et al., 1995) and contributed to P_i release, with the rate when MLC_{20} had been previously thiophosphorylated and the rate of P_i release was determined primarily by the activity of actomyosin ATPase. Finally, an estimate of the contribution of ecto-ATPase activity was obtained by monitoring, in permeabilized

Received for publication 30 June 1998 and in final form 15 September 1998.

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0006-3495/98/12/3031/10 $2.00

smooth muscle, the rate of P_i release after photolysis in the absence of Ca^{2+}. Similar measurements on intact smooth muscle provided us with information about the extent to which the activity of the ecto-ATPase, a glycoprotein of unknown physiological function, could be reduced by permeabilization with Triton X-100 (Trinkle-Mulcahy et al., 1994). Preliminary reports of these findings have been presented to the Biophysical Society (He et al., 1997).

MATERIALS AND METHODS

Muscles

Portal-anterior mesenteric veins were removed from ~5-kg Dutch belted rabbits killed by a rising concentration of CO_2 in accordance with institutional policies. After dissection and cleaning of connective tissue, longitudinal strips, 200–250 μm wide, 2.3 mm long, and ~50 μm thick, were cut with razor knives and the ends attached to aluminum T-shaped clips (Goldman and Simmons, 1984) for mounting in the apparatus. Densitometry of PAGE gels of the preparations showed that the total myosin head concentration was 52 μM (see below). It is noteworthy that there is greater extracellular surface area and volume in smooth muscle preparations compared to skeletal muscle preparations, potentially contributing to the higher "ecto-nucleotidase" activity in the former (see below). Muscle strips in Hepes-buffered physiological salt solution at 21°C were mounted in the apparatus with the adventitial surface of the muscle strip facing the laser and stretched 30% of slack length. The viability of the intact muscle strips was checked by exposure to a depolarizing solution containing 140 mM K^+.

The apparatus

The apparatus was the same as used for experiments on skeletal muscle fibers using the P_i-sensitive probe (He et al., 1997). Muscle strips were mounted in one of a set of six small stainless steel troughs, each 30 μl in volume, built on the temperature-controlled stage of the Zeiss ACM microscope. The microscope was equipped with Zeiss 10× eyepieces and an epifluorescence attachment, which used a tungsten lamp and an excitation filter with 52% peak transmission at 420 nm and full bandwidth at half-maximum transmission (FWHM) of 7.5 nm (Ealing 35-3234). A Zeiss 40× water-immersion objective (0.75 NA) was used to illuminate the fibers and to collect the fluorescence emission through a dichroic mirror (Zeiss FT425) and an interference filter with 54% peak transmission at 470 nm, FWHM = 8 nm (Ealing 35-3425). Emitted light was measured by means of a photomultiplier tube (EMI 9524 QB) operating at 500 V cathode voltage mounted above the muscle on the microscope head. The troughs in which the muscles were bathed, typically in 30 μl of solution, could be changed within 3 s by vertical displacement and rotation of the stage below the muscle. One of the troughs was constructed with a quartz window to allow illumination of the muscle and its bathing solution with light pulses to effect photolysis. The pulses were produced during the early part of this study by a xenon arc flash lamp (Rapp and Güth, 1988) and later by a Q-switched frequency-doubled ruby laser (Type QSR 2, Lumonics Ltd., Rugby, UK) focused with a cylindrical lens. In practice, tension and fluorescence records were the same whether flash lamp or laser pulse was used, so the photolysis light source is not distinguished in the Results. The illumination trough was fitted with a 2 μl chamber at each end to contain water. The central section could then be filled with silicone oil (Dow Corning, 10 centistokes) without the oil running out of the end slits. The surface tension of the water in the end chambers served to contain the oil. The use of silicone oil reduced background fluorescence in the bathing medium, as only the muscle volume contained the fluorophore. ATP (1.0–1.5 mM) was released into the rigor muscle with a single flash (He et al., 1997) to initiate (acto)myosin ATPase and relaxation/contraction. The T-clips crimping the muscle ends were held to the apparatus by hooks made from 100 μm diameter stainless steel wire passing through slits in the ends of the troughs. One end of the muscle was attached to a strain gauge for tension measurements (AE801, AME, Horten, Norway). The other end was fixed. The tension and fluorescence signals were recorded on a chart recorder (Gould 2400S), on a digital oscilloscope (Tektronix 5223), and on a personal computer using an analog-to-digital converter (Amplicon Liveline PC30D, Brighton, UK or R.C. Electronics EGAA Computerscope, Goletta, CA). Typically, data points were collected at 50-ms intervals over 50 s. In some instances, such as for studies of the lag phase in the response, data were collected at 1 ms intervals. The data were stored and analyzed on a personal computer. The time resolution and degree of filtering in records of tension and fluorescence depended on the particular experimental trial and are indicated in the figure legends. The tungsten light of the epifluorescence attachment caused slow photolysis of the P^3-1-(2-nitrophenyl)-ethyl ester of ATP (caged ATP); to minimize this, a shutter was used to limit exposure to a few seconds before and after the flash from the photolysis lamp, effectively eliminating ATP generation from other than the light flash. Photobleaching of the P_i probe fluorescence (0.15% min^{-1}) was slow compared to the time scale of the experiments and, therefore, could be neglected.

MDCC-PBP and P_i contamination

MDCC-PBP, the A197C mutant of the *Escherichia coli* phosphate-binding protein labeled with *N*-(2-[1-maleimidyl]ethyl)-7-diethylaminocoumarin-3-carboxamide, was prepared as described previously (Brune et al., 1998; Corrie, 1994). As P_i binding to MDCC-PBP is tight (K_d = 0.1 μM), the amount of MDCC-PBP in the fiber determines the amount of P_i which can be detected during ATPase activity (He et al., 1997). The linear relationship between MDCC-PBP fluorescence and P_i concentration has been demonstrated in skeletal muscle fibers (Ferenczi et al., 1995; He et al., 1997).

All solutions used for experiments with MDCC-PBP were handled to minimize contamination by P_i, which would increase background fluorescence and reduce the accuracy of the calibration of P_i concentration. P_i contamination was reduced to micromolar levels by treating, for 10 min at 20°C, the rigor solutions and the muscles with an enzymatic "P_i-mop" (Brune et al., 1994) consisting of 2.5 mM MEG (7-methylguanosine) and 2 units/ml^{-1} PNPase (purine nucleoside phosphorylase) that catalyzes the formation of ribose 1-phosphate from P_i. This was discontinued in later experiments, as it did not significantly change background fluorescence.

Experimental solutions and protocol for the P_i release assay

Muscle strips were permeabilized by incubation for 8–15 min at 22°C in fresh 0.1% Triton X-100 (Boehringer-Mannheim, Indianapolis, IN) dissolved in relaxing solution (G1) consisting of (mM) 30 PIPES, 6.6 magnesium methanesulfonate ($MgMs_2$), 1 Mg^{2+}, 1.0 K_2 EGTA, 103 potassium methanesulfonate (KMs), 5.2 Na_2 ATP, pH 7.1, ionic strength 0.2 M, plus protease inhibitors leupeptin (10 μM) and AEBSF (4-(2-aminoethyl)benzene sulfonylfluoride · HCl) (1 mM) and the mitochondrial blockers FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (1 μM) and oligomycin (2 μg/ml). After Triton permeabilization, muscles were incubated in Ca^{2+}-free rigor solution exchanged twice over a 6-min period containing (mM) 20 PIPES, 127 KMs, 10 K_2 EGTA, 1.9 $MgMs_2$, pH 7.1, plus leupeptin, AEBSF, and FCCP, as indicated above. For experiments with caged ATP in the presence of calcium, muscles were next incubated in a calcium-containing solution, pCa 4.5 (same as the Ca^{2+}-free rigor solution, but with Ca^{2+} added). For experiments with caged ATP following thiophosphorylation of the 20-kDa myosin light chains, muscles were incubated with 2 mM ATPγS (Boehringer-Mannheim) and 5 μM calmodulin in Ca^{2+}-rigor solution for 10 min, followed by a 3-min wash in calcium-free rigor solution.

The above muscles were incubated before photolysis for 5 min in rigor solution containing 0.8 mM MDCC-PBP plus 5 mM caged ATP. The rigor solution used for photolysis of caged ATP in the presence of calcium

contained (mM) 60 TES pH 7.1, 1.36 EGTA, 18.6 Ca-EGTA (pCa 4.5), 2.2 $MgCl_2$ (resulting in 1.5 mM free Mg^{2+} after release of 1 mM ATP), 72 potassium propionate, 40 glutathione, and 5 μM calmodulin, ionic strength 0.2 M. In experiments designed to investigate the kinetics of P_i release after the 20-kDa myosin light chains had been previously thiophosphorylated, as well as in control experiments designed to measure the background P_i release in the absence of cross-bridge cycling, the loading solution contained 20 mM EGTA and no added calcium or calmodulin. All loading solutions contained leupeptin, AEBSF, and FCCP as above, as well as 2 μM thapsigargin and 2 μg/ml oligomycin to inhibit the sarcoplasmic reticulum and mitochondrial ATPases, and 1 mM ouabain to inhibit the Na/K ATPase.

For experiments in which caged calcium (NP-EGTA; Ellis-Davies and Kaplan, 1994) and caged ATP were photolysed simultaneously in the presence of MDCC-PBP, exposure of the muscles to high concentrations of EGTA was avoided (Zimmermann et al., 1995). After Triton permeabilization in G1 solution (as above), the muscles were washed for 5 min in calcium-free rigor solution containing 0.5 mM EGTA, followed by a 2-min wash in EGTA-free, Ca^{2+}-free rigor solution. The loading solution contained 2 mM NP-EGTA, 1.25 mM added Ca^{2+}, 800 μM MDCC-PBP, 5 mM caged ATP, 25 μM calmodulin, 25 mM glutathione, 1 mM $MgMs_2$, 20 mM PIPES (pH 7.1), 137 mM KMs plus protease inhibitors, and mitochondrial and sarcoplasmic reticulum ATPase blockers as indicated above. Under these conditions, [Ca^{2+}] is pCa 7.0 before and pCa 5.5 after photolysis (Zimmermann et al., 1995).

After incubation in the loading solution, the muscle bundles were transferred into silicone oil (Dow Corning, 10 centistokes) in the trough with the quartz window. After photolysis and collection of data, the muscle strips were transferred to a trough containing relaxing solution for an additional cycle. Because of "rundown" in the amplitude of force during repeated stimulation of Triton permeabilized smooth muscle, only two trials were run on a given strip or, alternatively, neighboring strips of identical size were cut from the muscle sheet and treated as pairs.

Ecto-ATPase, ecto-ADPase activity and its subsequent inhibition by Triton permeabilization (Trinkle-Mulcahy et al., 1994) were characterized first in intact and subsequently in Triton-permeabilized fibers using the Ca^{2+}-free or Ca^{2+}-containing loading solutions and protocols described above, followed by photolysis of 5 mM caged ATP or 5 mM caged ADP. To assess whether ATPγS affects the ecto-ATPase activity, intact strips were pretreated with ATPγS as described above for permeabilized preparations and the rate of P_i released upon photolysis of 5 mM caged ATP was measured. The Triton permeabilization protocol used in these experiments inhibited approximately eightfold, but did not eliminate, the ecto-ATPase, ecto-ADPase activity. Triton concentrations and time of exposure were varied to optimize the inhibition of the ecto-ATPase and to minimize rundown of contractile responses.

When appropriate, P_i release rates were normalized to the concentration of myosin heads in rabbit portal vein smooth muscle (52 μM), determined as indicated below. Experiments were conducted at 21°C.

Myosin head concentration

Intact and Triton-permeabilized muscle strips collected at the completion of experiments were frozen in liquid nitrogen and freeze-substituted in acetone plus 10% trichloroacetic acid, subsequently homogenized, and the MLC_{20} was separated on 15% gels by SDS-PAGE (Kitazawa et al., 1991). Serial dilutions of known concentrations of recombinant chicken gizzard MLC_{20} kindly provided by Dr. Kendrick-Jones LMB, Cambridge, were run on each sample gel. SDS gels were stained with AllPro (Promega, Madison, WI) and quantitated with a fluorescence imager (FluoroImager TM575, Molecular Dynamics using Image Quant, Sunnyvale, CA). Western blots using MLC_{20} antibodies were used to confirm the position of the MLC_{20} bands. The micromolar concentration of myosin heads was estimated by comparison of the fluorescence intensity of the AllPro-stained MLC_{20} bands in gels of tissue homogenates with serial dilutions of recombinant MLC_{20} taking into account the volume of the muscle strips used. The rates of P_i release are calculated on the basis of [myosin head]/total strip volume. The [myosin head]/cell volume can be estimated by taking into account that the smooth muscle cell volume determined by morphometric analysis of electron microscopic images is 56% of the strip volume. The density of the tissue homogenate bands fell within the linear range of the serial dilutions of the recombinant MLC_{20} bands.

Reagents

Caged ATP and caged ADP were synthesized by the method of Walker et al. (1989) and purified by the method of Corrie and Reid (1994). An S139C mutant of the PBP that is insensitive to P_i was prepared and labeled with MDCC as a control for potential mechanical or laser-pulse artifacts (Brune et al., 1994). MLC_{20} antibody was a gift from Dr. Chris Kamm, University of Texas, Southwestern, Dallas, TX; 4-(2-nitrophenyl)-EGTA (NP-EGTA) was obtained from Molecular Probes (Eugene, OR). Reagents not otherwise specified were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Characterization of ATPases in intact and Triton-permeabilized muscles

The rate of ecto-nucleotidase activity was quantitated by the appearance of P_i that followed photolytic release of ~1.2 mM ATP from 5 mM caged ATP (He et al., 1997) in the presence of MDCC-PBP in intact portal vein (Fig. 1). The rate of P_i release during the first second after photolysis of caged ATP was 320 μM s^{-1} ± 26 mean ± SE (n = 4) in the presence of 32 μM Ca^{2+}, and not significantly different, 300 μM s^{-1} ± 14 mean ± SE (n = 4), in the absence of Ca^{2+}. In two experiments, pretreatment of the intact muscle with ATPγS before photolysis of caged ATP using a protocol similar to that used for permeabilized muscles had no effect on the ecto-ATPase activity, whereas permeabilization with Triton (0.1%) for 8–15 min led to an eightfold inhibition to 37 μM s^{-1} derived from the average of 12 traces during the first second after photolysis of caged ATP in the absence of Ca^{2+}. After Triton treatment, the ecto-nucleotidase is predominantly an ATPase, as release of ~1.2 mM ADP from 5 mM caged ADP in rigor solution resulted in a slow linear increase in fluorescence with a rate of 6.2 μM s^{-1} (n = 2). We were unable to abolish the remaining ecto-nucleotidase activity. During the first 5 s the average rate of P_i release in the absence of calcium, after Triton treatment, was 42 μM s^{-1} ± 4.0 mean ± SE (n = 16). Therefore, a trace collected in the absence of Ca^{2+} and representing the background nucleotidase activity was collected for each experiment and subtracted from the experimental trace obtained from the same strip, as illustrated in Fig. 1. The envelope of fluorescence between traces *A* and *B* reflects the P_i generated by actomyosin ATPase. In this case the MLC_{20} was previously thiophosphorylated and thus there is no P_i contribution from the "pseudo-ATPase" of MLCK/SMPP-1M activity.

Myosin content

The myosin head concentration measured from AllPro-stained gels of a pooled sample of four Triton-treated thio-

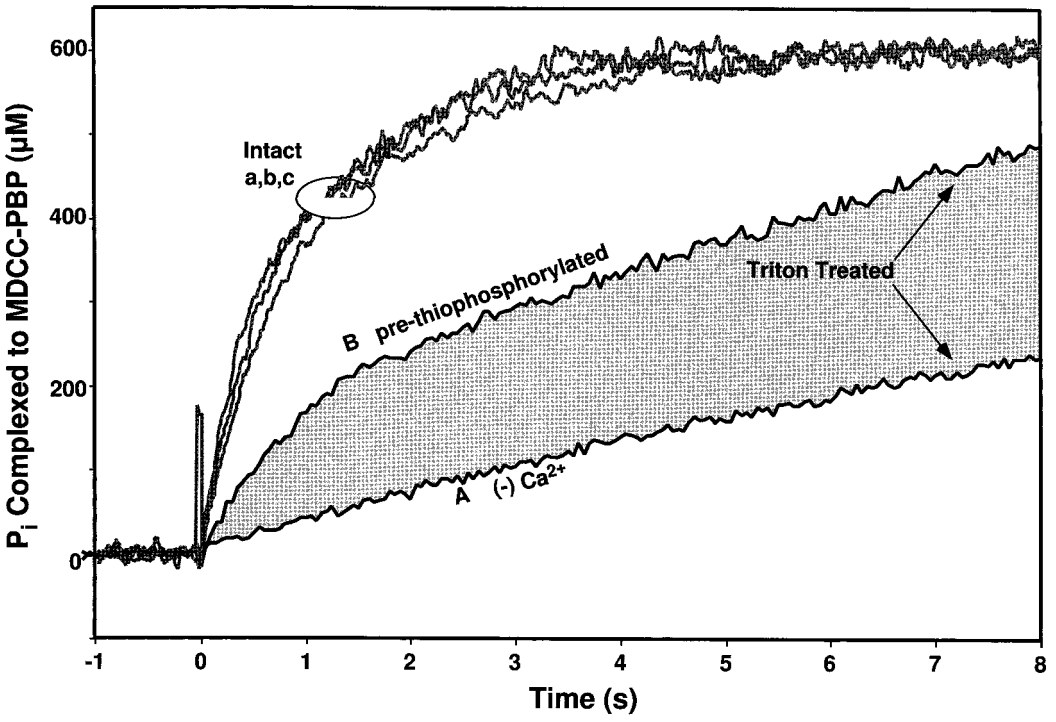

FIGURE 1 Time course of P_i release after photolysis of caged ATP in intact (*a, b, c*) and Triton-permeabilized (*A, B*) rabbit portal vein smooth muscle. Muscle strips were incubated in rigor solution in the absence of phosphate-containing compounds, followed by incubation in rigor solution containing 0.8 mM MDCC-PBP plus 5 mM caged ATP. Typically, the capacity of MDCC-PBP for P_i in the assay was 75% of the total protein concentration (Brune et al., 1994; 1998). Intact smooth muscle, unlike skeletal muscle, has a high ecto-ATPase activity (*traces a–c*) which was inhibited approximately eightfold after permeabilization with 0.1% Triton X-100 for 8–15 min at 22°C (*traces A* and *B*). The time course of P_i release in the intact muscle was similar in the presence (*a*) or absence (*b*) of 32 μM calcium or pretreatment with 2 mM ATPγS (*c*). Photolysis of caged ATP in Triton-permeabilized, thiophosphorylated muscles increased the fluorescence signal, typically with a fast early phase (*trace B*). The residual ecto-ATPase activity after Triton treatment was determined by photolysis of caged ATP in the absence of calcium (*trace A*) and was 37 μM s^{-1}. The kinetics and magnitude of P_i release not resulting from ecto-nucleotidase were determined from the paired experiments by subtracting *trace A* from *trace B*. The light flash occurs at time 0 on the abscissa.

phosphorylated strips collected after measurements of P_i release was 52 μM, based on standards of known concentrations of recombinant MLC_{20} and strip volume. The MLC_{20} content of three intact strips was 44, 35, and 52 μM. There was no detectable difference between the content of myosin heavy chains in intact and Triton-treated strips, indicating no major loss of myosin from the permeabilized muscle strips under the experimental paradigm used.

P_i release kinetics and force development in muscles in which MLC_{20} had been thiophosphorylated

After photolysis of caged ATP in muscles containing thiophosphorylated MLC_{20}, the time course of the fluorescence change tracks P_i complexed to MDCC-PBP. After subtraction of the contribution by the ecto-ATPase this fluorescence exhibited an initial fast phase with a rate of 80 μM s^{-1} ± 7.6 mean ± SE, followed by a 5.8-fold decrease to 13.7 μM s^{-1} ± 1.1 (Table 1 and Fig. 2). The amplitude of the fast phase, measured from the time of the light flash to the intercept of a line extrapolated from the slow phase, was 108 μM ± 14.8 mean ± SE. Tension had reached 81% ± 6 mean ± SE of maximum tension at the peak amplitude of the fast phase of P_i release (Table 1). Based on the average 52 μM myosin head content in these preparations and assuming that all heads were active upon photolysis of caged ATP, the rate of P_i release was 1.5 s^{-1} during the first and second turnovers and 0.26 s^{-1} during the third turnover. If only 84% of heads were thiophosphorylated as determined in a previous study using the same thiophosphorylation protocol on the same muscle type (Zimmermann et al., 1995), then the rates were 1.8 s^{-1} and 0.3 s^{-1}, respectively. Average measurements of fluorescence and tension changes of nine strips indicated that 70% of maximum force increment (measured from the rigor force before the light flash) was achieved by the end of the first turnover (for 100% of the heads thiophosphorylated; Fig. 2); this becomes 60% of maximum force increment based on 84% of heads thiophosphorylated. Measurement of the time between the light pulse and the beginning of the change in force and in fluorescence gave values of 40 ms ± 4 and 54 ms ± 20 mean ± SE for the force and fluorescence lags, respectively, for seven traces of previously thiophosphorylated muscles collected at high time resolution (1 kHz sampling). This measurement has considerable scatter, probably be-

TABLE 1 Rates of P_i release and tension generation in isometrically contracting portal vein smooth muscle

Condition	P_i Release					Tension		
	Fast Phase			Slow Phase				
	Rate (μM s^{-1})	k* (s^{-1})	Amplitude (μM)	Rate (μM s^{-1})	k* (s^{-1})	Initial Rate (s^{-1})	% T_{max} at End of Fast Phase#	n
Photolysis of caged ATP following thiophosphorylation	80 ± 7.6	1.5 ± 0.1 (1.8)	108 ± 15	13.7 ± 1.1	0.27 ± 0.02 (0.3)	4.4 ± 1.0	81 ± 6	13
Photolysis of caged ATP in presence of Ca^{2+}	51 ± 7.0	1.0 ± 0.1	81 ± 13	13.2 ± 2.3	0.26 ± 0.05	0.8 ± 0.1	61 ± 4.8	9
Simultaneous photolysis of NP-EGTA and caged ATP	34 ± 5.6	0.7 ± 0.1	94 ± 10	8.0 ± 3.3	0.16 ± 0.08	0.3 ± 0.1	53 ± 5.2	3

All measurements shown are values following subtraction of ecto-ATPase activity.

*Based on 52 μM myosin heads (mean ± SE). Rates based on 84% thiophosphorylated myosin heads (Zimmermann et al., 1995) are shown in parentheses.

#Percent of T_{max} at the end of the fast phase is the percent of maximal tension measured from the relaxed state reached at the intercept of the slopes of the fast and slow phases of the P_i release trace. The initial rate of tension development was obtained by fitting the trace to two exponentials.

cause of the necessity of subtracting the paired trace (minus Ca^{2+}) for the residual ecto-ATPase activity.

P_i release kinetics and force development in nonthiophosphorylated muscles in the presence of Ca^{2+}

The rate of rise of the initial fast phase of P_i fluorescence (and of force) after photolysis of caged ATP in rigor muscles in which MLC_{20} had not been thiophosphorylated was significantly slower ($P \leq 0.017$) than in previously thiophosphorylated muscles (Table 1 and Fig. 3): 51 μM s^{-1} ± 7.0 mean ± SE vs. 80 μM s^{-1} ± 7.6 mean ± SE, respectively. The slow (steady-state) phase of P_i release was 3.9-fold slower than the initial phase, and not significantly different from the slow phase in thiophosphorylated muscles: 13.2 μM s^{-1} ± 1.1 mean ± SE vs. 13.7 μM s^{-1} ± 2.3 mean ± SE. The intercept of the fast and slow phases, at 2.2 s, was later than in the thiophosphorylated muscles (at 1.7 s). Starting from rigor, tension reached 61% ± 4.8 mean ± SE of maximum tension at the end of the fast phase of P_i release. After a lag phase (typically 100 ms, as in Fig. 3), the rate of force development was 5.5-fold slower than in thiophosphorylated muscles.

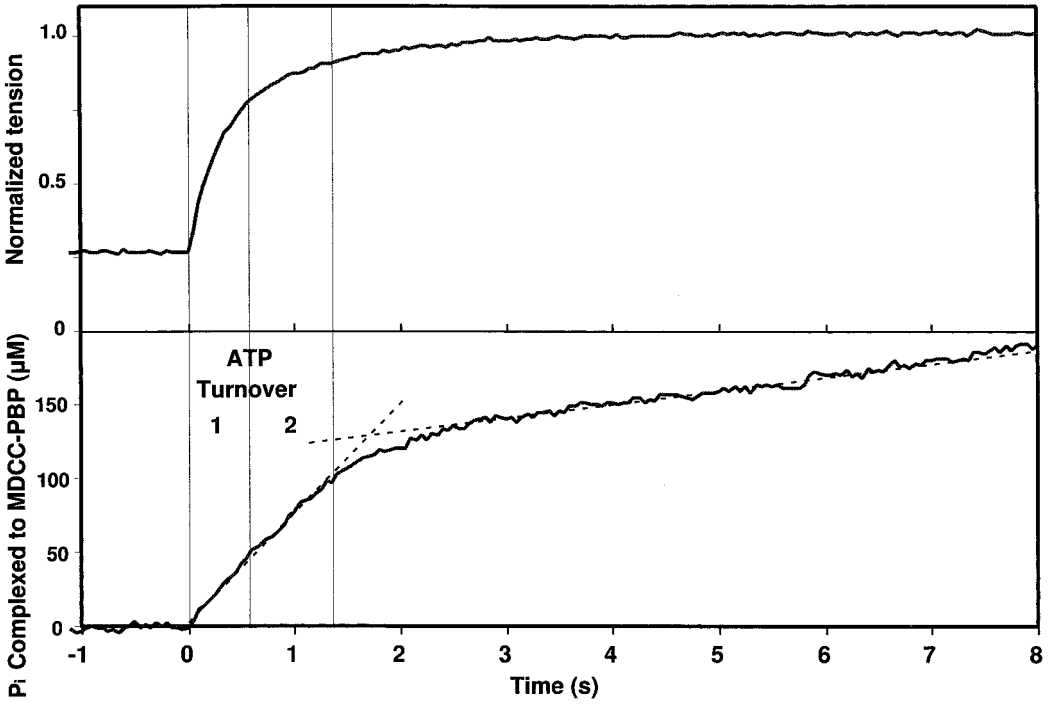

FIGURE 2 Average time course of P_i release and tension development after photolysis of caged ATP in Triton-permeabilized muscles previously thiophosphorylated (MLC_{20} phosphorylation > 80%; n = 7). Fluorescence increased with an initial fast phase of 80 μM s^{-1}, followed by a slow phase of 13.7 μM s^{-1}. Based on 52 μM myosin heads measured in this type of smooth muscle, tension had reached 72% of maximal tension by the end of the first cross-bridge turnover. The fast phase of P_i release encompassed about two turnovers of the myosin heads. No phosphate burst was detectable before force development. *The contribution of the calcium-independent ATP breakdown has been subtracted from the traces.*

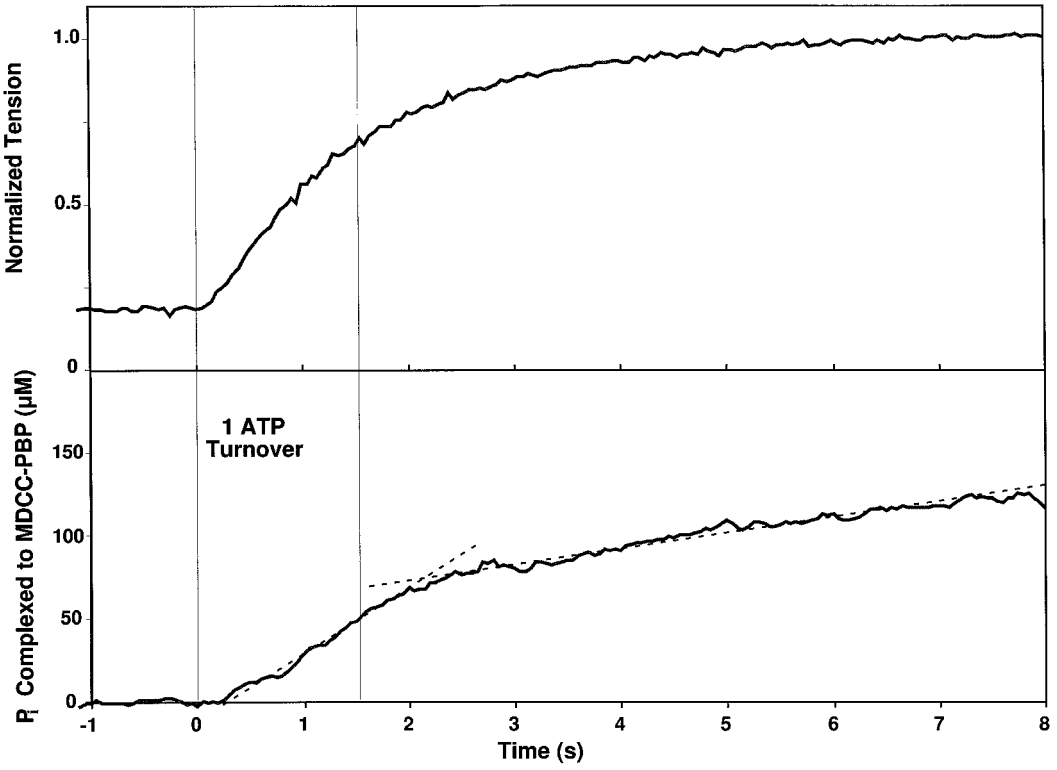

FIGURE 3 Average time course of P_i release and tension development after photolysis of caged ATP in Triton-permeabilized muscles in the presence of 32 μM Ca^{2+} and 5 μM calmodulin. Fluorescence increased with an initial fast phase of 51 μM s^{-1}, followed by a slow phase of 13.2 μM s^{-1}. Tension had reached 54% of maximal tension by the end of the first cross-bridge turnover, based on 52 μM myosin heads and reached 61% by the intercept of the time course of the fast and the slow phase of the fluorescence trace. No phosphate burst was detectable before force development. *The contribution of the calcium-independent ATP breakdown has been subtracted from the traces.*

P_i release kinetics and force development upon simultaneous photolysis of caged ATP and NP-EGTA

The contractile response to simultaneous release of Ca^{2+} and ATP in Ca^{2+}-free rigor muscles (Ashley et al., 1988; Zimmermann et al., 1995) containing 25 μM calmodulin was determined in order to evaluate the contribution of activation steps that precede myosin light chain phosphorylation (Zimmermann et al., 1995) to the kinetics of ATP hydrolysis and force development. The initial rate of P_i release, 34 μM s^{-1} $\pm$ 6 mean $\pm$ SE, was significantly slower than the fast phase of P_i release in thiophosphorylated muscles, $P \leq 0.02$; it was also slightly slower than the 51 μM s^{-1} $\pm$ 7 mean $\pm$ SE measured after photolysis of caged ATP in the presence of Ca^{2+} (Table 1), but this difference was not statistically significant ($P \leq 0.29$). The intercept of the fast and slow phases of P_i release occurred at later times, and the initial rates of force development after simultaneous photolysis of the two caged compounds were also slower than in thiophosphorylated muscles and muscles activated with ATP in the presence of Ca^{2+} (Table 1), similar to previous results obtained with force measurements (Zimmermann et al., 1995).

DISCUSSION

Our results, relating the kinetics of P_i release to those of force development, show that in maximally activated smooth muscle there is a clear transition of the ATPase activity, on the average after about two turnovers, from a fast to a slow rate. The initial rate of cross-bridge cycling (1.5–1.8 s^{-1}) in portal vein smooth muscle is comparable to the maximal (gizzard) acto-HMM ATPase activity in solution (1.9 s^{-1}; Sellers, 1985), and the pseudo-ATPase (resulting from MLCK and phosphatase activity) contributes little to energy consumption during force maintenance under our experimental conditions. These issues, and a brief discussion of the present method and its limitations, will follow.

The initial rate of P_i release (present study), as well as the rate of force development (Horiuti et al., 1989; Zimmermann et al., 1995; present study) are significantly faster when contraction is initiated by photolytic release of ATP into muscle in which MLC_{20} had already been thiophosphorylated than when phosphorylation of MLC_{20} by Ca-calmodulin-activated MLCK is rate-limiting. This is consistent with the conclusion that under physiological conditions (not thiophosphorylation), the rate of force de-

velopment (Horiuti et al., 1989) and actomyosin ATPase activity (present study) are rate-limited by the rate and extent of MLC_{20} phosphorylation, respectively. The initial rate of calculated P_i release would be even higher, perhaps implausibly so following phosphorylation, if only a fraction of the thiophosphorylated heads had been available for activation by actin (Butler and Siegman, 1998). Furthermore, our measurements were made at the optimum of the length-tension curve, where the maximum number of actin targets are expected to be available. The biphasic time course of P_i release and its relation to force development are strikingly similar to what is observed in permeabilized skeletal muscle fibers of the frog, but on a different time scale (Fig. 9 in He et al., 1997). An important conclusion derived from our results is that the transition from fast to slow rate is not because of a decline in MLC_{20} phosphorylation, but to other factors acting directly on cross-bridges, as in skeletal muscle (He et al., 1997).

P_i release occurs over a rapid ($1.8 \pm 0.1\ s^{-1}$) and a slow ($0.3 \pm 0.02\ s^{-1}$) phase when muscles are activated by photolytic release of ATP from the (MLC_{20}) thiophosphorylated state (Fig. 2; Table 1). The rapid P_i release rate is considerably greater than the rate of ADP release ($0.2\ s^{-1}$) measured over the first 10 s, also in rabbit portal vein at comparable temperature (20°C; Vyas et al., 1994). Based on a concentration of myosin heads of 52 μM (84% of which are thiophosphorylated) and the initial rate of P_i release ($1.8\ s^{-1}$), the heads completed, on the average, two cycles before the transition from fast to slow force development. Completion of two cycles with an initial rate of P_i release of $1.8\ s^{-1}$, however, is inconsistent with the rate-limiting step of $0.2\ s^{-1}$ ADP release during completion of the first cycle. We therefore consider it likely that the rate of ADP release during the first turnover is also faster than that obtained by steady-state measurements. Time-dependent transitions in energy consumption from a fast phase during force development to lower consumption during force maintenance occur in both striated and smooth muscle (e.g., He et al., 1997; Lionne et al., 1995; Siegman et al., 1980). In electrically stimulated intact (rabbit taenia coli) smooth muscle energy consumption decreases by fourfold between the initial 25 and the subsequent 35 s of contraction (Siegman et al., 1980). However, in intact smooth muscle the rate of contractile activation is limited by the rate of rise in Ca^{2+} and MLCK activity (Kamm and Stull, 1986; Zimmermann et al., 1995; reviewed in Hartshorne, 1987), and MLC_{20} phosphorylation reaches lower values than attainable after thiophosphorylation of permeabilized smooth muscle (Cassidy et al., 1979; Kenney et al., 1990; Zimmermann et al., 1995). Furthermore, in smooth muscles in which MLC_{20} is phosphorylated, rather than thiophosphorylated, energy metabolism could decrease as the result of time-dependent dephosphorylation of MLC_{20}. Nevertheless, the approximately three to fivefold difference between the fast and slow rates of P_i release in both nonthiophosphorylated and thiophosphorylated permeabilized muscles (present study) is similar to the change in rate in energy consumption found in intact smooth muscle. In striated muscle the slowing of P_i release has been suggested to be the result of inhibition by positive strain on cross-bridges, and it is likely that the same mechanism, transition from shortening of the series elastic into the isometric state, also operates in smooth muscle. In view of the high affinity of smooth muscle myosin for MgADP (Fuglsang et al., 1993; Nishiye et al., 1993; Khromov et al., 1995), inhibition by MgADP ($\sim$50 μM released on the first turnover by cycling cross-bridges plus the amount contributed by ecto-ATPase activity) may also contribute to the slowing of the cycle. This mechanism would be less prominent in phasic, than in tonic, smooth muscles (Khromov et al., 1995). Given the very high ratio of actin to myosin filaments in smooth muscle (Ashton et al., 1975), we would also entertain the possibility that following the initial attachment of cross-bridges, at least some of the ATP breakdown occurs through the slower, associated state hydrolysis pathway (White et al., 1997) and/or cooperative cycling of nonphosphorylated myosin (Somlyo et al., 1988; Himpens et al., 1988; Vyas et al., 1992; Butler and Siegman, 1998).

In smooth, as in striated muscle (He et al., 1997), there is no evidence of a burst of P_i release into the surrounding solution preceding force development, showing that P_i generated by the first turnover remains bound to the catalytic domain of myosin. This means that initially the rate-determining step precedes or includes AM · ADP formation so that biochemical states such as AM · ATP or AM · ADP · P_i predominate. However, there is evidence that when the plateau of isometric contraction is reached AM · ADP contributes to the steady-state complex (e.g., Österman and Arner, 1995; Nishiye et al., 1993). Thus the time course of P_i formation in Figs. 2 and 3 is accompanied by redistribution of biochemical states (AM · ATP, AM · ADP · P_i, AM · ADP, etc.). It follows that defining the biochemical states bearing force is not possible without further information. The shorter lag phase in force development compared to P_i release by contrast is direct evidence for states such as AM · ADP · P_i contributing to force, though as noted earlier, the difference in lag times is less than the mean $\pm$ SE of the data.

The rate of P_i release during the plateau of isometric contraction was not significantly different in the thiophosphorylated ($13.7\ s^{-1}$) from that in the nonthiophosphorylated ($13.2\ s^{-1}$) muscles. At first glance, this suggests that the contribution of the pseudo-ATPase (MLCK/SMPP-1M activity) to total energy consumption during the force plateau is negligible. However, in making the comparison we have to consider the higher levels of MLC_{20} thiophosphorylation than phosphorylation reached with the protocols used in these experiments (84% vs. 57%; Zimmermann et al., 1995). Considering the near-linear relationship among MLC_{20} phosphorylation, ATPase, and force (Kenney et al., 1990; Kitazawa et al., 1991) and, therefore, the $\sim$20% greater force developed when MLC_{20} is near maximally thiophosphorylated, we could estimate that the pseudo-ATPase may account for up to 20% of the slow phase of P_i release. This is probably an upper limit that assumes thiophosphorylation and phosphorylation are equivalent and is somewhat higher than an earlier estimate (12%) based on

studies of thiophosphorylated muscles that did not take into account different levels of MLC_{20} phospho and thiophosphorylation (Zhang and Moreland, 1994). Furthermore, the uncertainty of the difference (± 4.4 $\mu M\ s^{-1}$; see Results) in P_i release rates in, respectively, thiophosphorylated and phosphorylated preparations is sufficiently large (>20% of the respective rates) to preclude a precise estimate of pseudo-ATPase activity. An estimate of this activity can also be obtained by considering that it cannot exceed the dephosphorylating activity of SMPP-1M. Then, for a phosphatase activity of $\sim 0.07\ s^{-1}$ (Khromov et al., 1995), when 57% of the heads are phosphorylated during a peak contraction (Zimmermann et al., 1995), the pseudo-ATPase activity would account for ATP breakdown of 2 $\mu M\ s^{-1}$, or ~4% of the total P_i release rate (51 $\mu M\ s^{-1}$). Similar calculations in intact smooth muscle led to the conclusion that the phosphatase reaction rate was no more, and probably less than, 15% of the actomyosin ATPase rate (Butler et al., 1986; Driska et al., 1989; Paul, 1989). Our conclusions would not be greatly affected by considering the approximately two-fold higher MLC_{20} phosphatase activity of phasic smooth muscles (Gong et al., 1992), and our results remove the uncertainty (Driska et al., 1989) concerning the maximum and steady-state activated actomyosin ATPase rate of myosin organized in filaments in smooth muscle (Table 1). Therefore, we conclude that during both force development and force maintenance the pseudo-ATPase activity due to SMPP-1M is only a small fraction of the actomyosin ATPase activity.

The major factor complicating the evaluation of our results was the very significant ecto-ATPase activity of smooth muscle (Trinkle-Mulcahy et al., 1994). Fortunately, although relatively high even after Triton treatment (42 $\mu M\ s^{-1}$), this activity was sufficiently reproducible to be subtracted from the experimental records and was neither Ca^{2+}-sensitive nor affected by ATPγS (present study). The major difficulty due to the ecto-ATPase was that the ATP-breakdown measured in the absence of Ca^{2+} includes P_i liberated by both the ecto-ATPase and (resting) myosin ATPase. Therefore, an accurate value of basal myosin ATPase activity and of the extent of regulation by MLC_{20} phosphorylation is not feasible without resorting to comparison with published values obtained from intact smooth muscle in the absence of extracellular ATP. The basal energy consumption (2.3 $\mu M\ s^{-1}$) of intact resting smooth muscle (Siegman et al., 1980) is $\sim 0.05\ s^{-1}$ (based on 52 μM heads), and more recent measurements range from ~7 $\mu M\ s^{-1}$ (equivalent to $0.15\ s^{-1}$) at 37°C in swine carotid artery (Wingard et al., 1997), and 9.7 $\mu M\ s^{-1}$ in rat portal vein at 22°C (Arner and Hellstrand, 1983) to even higher values (reviewed in Paul, 1989) and would yield lower estimates of the range of activation. Using a basal rate of $0.05\ s^{-1}$ as representing resting myosin ATPase activity and the maximal rate of P_i release (1.5–1.8 s^{-1}; present study) as the active level, we obtain ~37-fold as the lower limit of activation. The true value is probably somewhat higher, since the total energy consumption in resting smooth muscle includes contributions, perhaps as much as 50%, from the sodium-pump and other nonmyosin ATPases. The reported range of actomyosin ATPase activation by myosin light chain phosphorylation is between 30- and 1,000-fold (Trybus, 1989; Dash and Hackney, 1991; Sellers, 1985; Vyas et al., 1994), with the highest estimate assuming that the basal actin-activated activity of isolated, nonphosphorylated HMM was due to denatured, nonregulated heads (Sellers, 1985). In contrast, the increase in energy consumption by intact rabbit taenia coli, from basal rate, during the initial 25 s of a tetanus is only ~10-fold (Siegman et al., 1980) and heat production during force development (10 s) by rabbit bladder increases approximately threefold (Wendt and Gibbs, 1987). Phosphorylation of MLC_{20} of whole myosin (rather than HMM) increases its actin-activated ATPase activity (compared to ATPase activity of nonphosphorylated whole myosin) by ~100-fold (Dash and Hackney, 1991); this and the present study suggest that the higher range of activation of acto-HMM in solution compared to that found in muscle reflects a higher basal (nonphosphorylated) actomyosin ATPase activity of the organized filamentous system. Single ADP-turnover experiments on Triton-permeabilized portal vein smooth muscle yielded a resting (myosin) ATPase rate of $0.004\ s^{-1}$. Comparison of these with our results is complicated by the order of magnitude difference in ecto-ATPase activities: 37 $\mu M\ s^{-1}$ (present study) and 3.5 $\mu M\ s^{-1}$ (Vyas et al., 1994). This difference may be because of the more extensive permeabilization that may have also affected the myosin ATPase activity in the experiments of Vyas et al. (1994). In any case, the uncertainty of estimating the effect of MLC_{20} phosphorylation on the range of actomyosin ATPase activation is largely a result of the uncertainty of estimating basal (myosin) ATPase activity.

The ecto-ATPase activity after permeabilization with 0.1% Triton X-100 for 8–15 min at 22°C contributed ~32% of maximal ATPase activity measured as P_i release. More extensive treatment with Triton X-100 could reduce this activity further (Trinkle-Mulcahy et al., 1994, see above), but at the expense of reduced force-bearing capacity of the smooth muscle associated with loss of myosin (data not shown). Mitochondrial and ion-transporting ATPase activities are below detection levels in permeabilized smooth muscle (Lönnbro and Hellstrand, 1991), but, unfortunately, regardless of the method used, whether coupled NADH assay, HPLC of nucleotides, or heat measurements, accurate estimates of basal myosin ATPase activity in permeabilized smooth muscle are limited by the ecto-ATPase (Trinkle-Mulcahy et al., 1994; present study).

In conclusion, P_i release after activation of smooth muscle occurs in two phases, and transition from rapid to slow cycling occurs independently of any change in the level of MLC_{20} phosphorylation. The range of activation by MLC_{20} phosphorylation, considering a maximum ATPase activity of 1.5–1.8 s^{-1} and the published values of basal energy metabolism of smooth muscle, is unlikely to reach 1000-fold in situ, and ATP breakdown attributable to MLCK/

SMPP-1M activity is relatively low. A precise evaluation of the basal (acto) myosin activity in situ will require the development of specific ecto-ATPase inhibitors.

We are grateful to Jackie L. Hunter (National Institute for Medical Research, London) for preparation of phosphate binding protein, to Jama Coartney for preparation of figures, and to Barbara Nordin for help in preparation of the manuscript.

This research was funded by National Institutes of Health Grant HL19242 and by the Medical Research Council.

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