Effects of magnesium pyrophosphate on mechanical properties of skinned smooth muscle from the guinea pig *Taenia coli*

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ABSTRACT Effects of the non-hydrolyzable nucleotide analogue magnesium pyrophosphate ($MgPP_i$) on cross-bridge properties were investigated in skinned smooth muscle of the guinea pig *Taenia coli*. A "high" rigor state was obtained by removing MgATP at the plateau of an active contraction. Rigor force decayed slowly towards an apparent plateau of ~25–35% of maximal active force. $MgPP_i$ markedly increased the rate of force decay. The initial rate of the force decay depended on [$MgPP_i$] and could be described by the Michaelis-Menten equation with a dissociation constant of 1.6 mM. The decay was irreversible amounting to ~50% of the rigor force. Stiffness decreased by 20%, suggesting that the major part of the cross-bridges were still attached. The results can be interpreted as "slippage" of PP_i-cross-bridges to positions of lower strain. The initial rate of $MgPP_i$-induced force decay decreased with decreasing ionic strength in the range 45–150 mM and was ~25% lower in thiophosphorylated fibers. MgADP inhibited the $MgPP_i$-induced force decay with an apparent K_i of 2 μM. The apparent K_m of MgATP for the maximal shortening velocity in thiophosphorylated fibers was 32 μM. This low K_m of MgATP suggests that steps other than MgATP-induced detachment are responsible for the low shortening velocity in smooth muscle. No effects were observed of 4 mM $MgPP_i$ on the force–velocity relation, suggesting that cross-bridges with bound $MgPP_i$ do not constitute an internal load or that binding of $MgPP_i$ is weaker in negatively strained cross-bridges during shortening.

INTRODUCTION

During actomyosin ATPase activity, myosin is considered to exist in a series of states with different affinity for actin (cf. Eisenberg and Hill, 1985). On the basis of biochemical data, myosin alone (M) and the myosin-ADP complex (M-D) have been characterized as "strong" actin binding states, and myosin-ATP (M-T) and myosin-ADP-P_i (M-D-P) as "weak" binding states (Stein et al., 1979). In the presence of actin, the weakly binding states have been proposed to exist in a rapid equilibrium between bound and dissociated forms (Stein et al., 1979). Evidence has been presented that weakly bound cross-bridges can exist in relaxed striated muscle fibers (Brenner et al., 1982). The transition from weakly to strongly bound states is considered to be the rate-limiting step for force generation (cf. Eisenberg and Hill, 1985). The strongly bound states have a higher affinity for actin and dissociate more slowly both in solution and in muscle fibers (cf. Brenner, 1987).

Non-hydrolyzable ATP analogues, e.g., pyrophosphate (PP_i) or adenylyl imidodiphosphate (AMP-PNP; Yount et al., 1971) have been used to study the actin–myosin interaction in solution. These substances bind to the nucleotide site on myosin without being hydrolyzed and defined biochemical states are produced. PP_i and AMP-PNP have pronounced weakening effects on the myosin subfragment-1 (S-1) binding to actin (Greene and Eisenberg, 1980), although the binding is still stronger than in the presence of ATP. The addition of PP_i to striated insect flight muscle fibers in rigor caused a reduction in force with little reduction in stiffness (White, 1970), which was interpreted as cross-bridges being dissociated by PP_i but remaining in close proximity to the thin filaments. The high stiffness at high stretch rate and the low force responses at low stretch rate ("slip") was explained by a strong viscous interaction between the filaments. In a study by Schoenberg and Eisenberg (1985), the effects of $MgPP_i$ on cross-bridge behavior in skinned rabbit psoas fibers were investigated. According to an equilibrium model for cross-bridge behavior (Schoenberg, 1985), the force after a stretch would decay to zero according to rate constants reflecting cross-bridge dissociation rates. Whereas the force after a 2 nm/half sarcomere stretch in rigor showed very little decay, the force in $MgPP_i$ rigor decayed rapidly to zero following a multiexponential process. An interpretation of these results has been that binding of $MgPP_i$ increases the rate of myosin detachment from actin, and that either the binding of $MgPP_i$ or the detachment rate is strain dependent (Schoenberg and Eisenberg, 1985; Schoenberg, 1989). In active contractions, $MgPP_i$ decreased force and acted as a pure competitive inhibitor with regard to the ATP-depen-

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dence of maximal velocity of shortening, V_{max} (Pate and Cooke, 1985).

The contraction of smooth muscle is considered to be the result of a cyclic interaction of cross-bridges between thick and thin filaments, in a similar manner as in striated muscle. However, compared with striated muscle fibers, smooth muscle has low V_{max}. The guinea pig *Taenia coli* (V_{max} ~0.2 lengths/s at 22°C) is more than 20 times slower than the rabbit psoas muscle (~2 lengths/s at 10°C; Cooke and Bialek, 1979), assuming a Q_{10} value of 2. The smooth muscle S-1 and acto S-1 ATPase in solution, appear to follow essentially the same sequence of steps as in striated muscle (Marston and Taylor, 1980; Marston, 1980). The myosin S-1 ATPase is similar in striated and smooth muscles, whereas the maximal rate of the acto S-1 ATPase in smooth muscle is ~40 times lower than that of the fast skeletal acto S-1. Several of the reaction steps in the smooth actomyosin ATPase are slower (Marston, 1980), but the specific properties of the smooth muscle contraction (low V_{max} and low tension cost) have at present not been linked to properties of specific states or biochemical transitions in the actin–myosin interaction. The organization of the contractile proteins in the filamentous system in the smooth muscle cell may impose structural constraints and alter several of these cross-bridge reactions compared with the situation in vitro.

The aim of this study was to obtain information regarding the strongly bound cross-bridge states in smooth muscle fibers. Chemically skinned preparations from the guinea pig *Taenia coli* were used. The mechanical effect of $MgPP_i$ on rigor force was investigated. The results show that $MgPP_i$ binds to the smooth muscle fiber under these conditions and causes a reduction of force. The influence of ionic strength and myosin light chain phosphorylation was characterized. The effect of a rapid increase in PP_i in the muscle fiber was investigated by photolytic release of PP_i from caged PP_i. ADP did not alter the rigor force but inhibited the $MgPP_i$-induced force decay. By competition experiments the dissociation constant for ADP was estimated in nonphosphorylated and phosphorylated muscle fibers. The ATP dependence of force and V_{max} in active contractions was investigated in the absence and in the presence of $MgPP_i$.

Some of the results have been presented in preliminary form (Arner and Arheden, 1990).

MATERIALS AND METHODS

Preparation and solutions

Guinea pig *Taenia coli* preparations were skinned with Triton X-100, as described previously (Arner and Hellstrand, 1985), and stored in a 50% glycerol solution at −15°C. In isometric experiments and in isotonic quick release experiments (see below) fiber length was in the range 3–5 mm and diameter 0.2–0.3 mm. In stiffness experiments, length was 1.5–2.5 mm with a diameter ~0.1 mm. Glycerinated rabbit psoas fibers were prepared as described by Goldman et al. (1984) and mounted at a sarcomere length, determined by laser diffraction, of ~2.2 μm.

The experimental solutions contained: 30 mM TES (*N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid), 0.5 mM sodium azide, and 2 mM free-Mg^{2+}. Solutions with pCa(-log[Ca^{2+}]) 9 and pCa 4.5 were obtained by adding appropriate amounts of K_2EGTA (ethyleneglycol-bis(amino-ethylether)-*N,N'*-tetraacetic acid) and K_2CaEGTA, while keeping total EGTA at 4 mM. Ionic strength was adjusted with KCl to 150 mM, unless stated otherwise, and pH with KOH to 6.9. All ATP-free rigor solutions contained 10 mM glucose and 20 U/ml hexokinase. Relaxing and activating solutions contained 3.2 mM MgATP, 12 mM phosphocreatine (PCr), and 0.5 μM calmodulin if not stated otherwise. In the isotonic quick release experiments, performed to determine the force–velocity relations at varied MgATP in the presence of 0 and 4 mM $MgPP_i$, 75 U/ml creatine phosphokinase (CK) was added. In solutions with MgADP, 0.2 mM of the myokinase inhibitor $Ap_5A(p^1,p^5$-di(adenosine-5')pentaphosphate; Feldhaus et al., 1975) was added in addition to the glucose/hexokinase system. Solutions containing $MgPP_i$ were prepared by mixing all constituents except PP_i and $MgCl_2$, and thereafter from stock solutions (100 mM) adding first PP_i, then slowly and during stirring adding $MgCl_2$, and finally adjusting pH. For each mM of $MgPP_i$ in the solution, the amount of KCl was reduced by 5 mM. Solution compositions were computed using a program similar to that described by Fabiato and Fabiato (1979). Stability constants were obtained from Fabiato (1981). Experiments were performed at room temperature (22°C). Hexokinase was obtained from Boehringer (Mannheim, FRG), and all other chemicals from Sigma Chemical Co. (St. Louis, MO). Calmodulin was a gift from Dr. Eva Thulin (Division of Physical Chemistry 2, Chemical Centre, University of Lund).

Isometric experiments

Muscle fiber preparations were cut out and attached with glue (celluloid acetate dissolved in acetone) horizontally between two carbon fiber pins. One of the pins was fixed to a micrometer screw and the other was attached to a force transducer (AE 801; SensoNor a.s., Horten, Norway). The muscles were immersed in the experimental solutions contained in plastic troughs (0.5 ml). The solutions could be changed in ~5 s by lowering the trough stand and exchanging the troughs. Force was recorded on paper using a W + W 340 chart recorder (Kontron Electronics, Basel, Switzerland). For curve fitting, the force records were digitized using a HiPad Plus digitizer (Houston Instrument Co., Austin, Texas).

The effect of $MgPP_i$ on rigor force was investigated in unphosphorylated and thiophosphorylated fibers. A "high rigor" state (cf. Kawai and Brandt, 1976) was obtained in the smooth muscle fibers by removing substrate during an active contraction (Arner and Rüegg, 1985). To obtain fibers in an unphosphorylated high rigor state the preparations were activated by Ca^{2+} (pCa 4.5) in the presence of MgATP. At the plateau of the active contraction the fibers were transferred to a rigor solution at pCa 4.5, containing hexokinase and glucose. After 10 min the solution was changed to a rigor solution at pCa 9. After exposure to $MgPP_i$ the muscle was again activated with Ca^{2+} and MgATP. Repeated contraction–rigor $MgPP_i$ cycles were performed on each fiber. Thiophosphorylation was achieved by initially exposing the muscle strips to rigor solution, without hexokinase and glucose, at pCa 4.5 with 2 mM ATP-γ-S and 0.5 μM calmodulin for 15 min. Contraction was then initiated by transfer to MgATP containing solution at pCa 9. At the plateau of contraction, high rigor was

induced by transfer to pCa 9 rigor solution. After exposure to $MgPP_i$ the muscle was again thiophosphorylated for 5 min before the next contraction–rigor $MgPP_i$ cycle. In these experiments $MgPP_i$ was introduced by change from rigor to $MgPP_i$ solution and allowing the $MgPP_i$ to diffuse into the fiber.

Experiments with caged pyrophosphate

In order to investigate tension transients in response to rapid increase in $MgPP_i$, photolysis of caged PP_i (2-nitro-phenylethyl pyrophosphate) was used. Fibers were mounted as described above and high rigor was induced. When rigor force had decayed to a steady level, usually ~30–35% of maximal active force, the muscle preparation was immersed for 3 min in 50 μl of rigor solution with 5 mM caged PP_i. Photolysis was performed using a xenon flash lamp as previously described for caged ATP (Arner et al., 1987a). Isotachophoresis (cf. Gower and Woledge, 1977) performed with an LKB 2127 Tachophor (LKB, Bromma, Sweden) was used to determine the concentration of released PP_i. Force was recorded on magnetic tape (Store 4 recorder; Racal-Thermionic Ltd., Hythe Southhampton, England), and digitized (0.5 kHz; using an IBM-compatible personal computer (Danbury, CT) equipped with an Analog Devices RTI-800F analogue-to-digital board; Norwood, MA) for subsequent analysis.

Isotonic quick release experiments

Isotonic quick release experiments for determination of force–velocity relations were performed using a lever system as described earlier (Hellstrand and Johansson, 1979). The force transducer was an AE 801 extended by 2.4 mm by a carbon fiber pin, which gave a resonance frequency in air of 5.8 kHz. The total compliance of the lever and force transducer was 5 μm/mN. The fibers were attached to the lever and force transducer by means of aluminum clips wrapped around the ends of the fiber preparation. Force–velocity relations were determined at varied concentrations of MgATP in the presence of 0 and 4 mM $MgPP_i$, using maximally thiophosphorylated preparations as described by Arheden et al. (1988). The muscles were thiophosphorylated for 15 min in pCa 4.5 rigor solution as described above and contracted by introducing MgATP at pCa 9. A series of releases (8–12) to different afterloads was applied at the plateau of the contraction. Before each new test condition (different MgATP at 0 or 4 mM $MgPP_i$) the muscle preparation was thiophosphorylated for another 2 min. Force and length data were digitized at 1 kHz using an A/D board in a personal computer (see above). Since the shortening velocity decays with time after the release, velocities were determined at different points in time after the release (cf. Arner and Hellstrand, 1985). Force and velocity data were fitted by the Hill (1938) equation in the form: $V = b(1 - P/P_o)/(P/P_o + a/P_o)$, where all force values (P) were normalized to isometric force before release (P_o). V is shortening velocity and a and b are constants. V_{max} was calculated as bP_o/a.

Stiffness experiments

Stiffness was determined consecutively at the plateau of a Ca^{2+}-activated contraction, after 30 min in high rigor, and after 20 min after the addition of 4 mM $MgPP_i$. The experiments were performed using the experimental setup described by Arheden and Hellstrand (1991). The fibers were mounted between an AE 801 force transducer (shortened to give a resonance frequency of 50 kHz) and a length step generator. Length steps of different amplitude, complete within 0.3 ms, were imposed on the fiber. T_1 curves (Ford et al., 1977) were constructed by plotting the extreme force reached during the length change, normalized to the initial force P_o, against the amplitude of the imposed length step. Stiffness was determined as the slope of the linear relation between force and length for positive stretches of up to 0.02 muscle lengths.

Statistics

Values are given as mean ± SEM, with the number of observations indicated. Curve fitting was done using a nonlinear least squares method (Fletcher and Powell, 1963).

RESULTS

Effects of $MgPP_i$ on rigor force

The high rigor state was obtained as illustrated in Fig. 1. When MgATP was removed at the plateau of the active contraction, force decayed to 30–35% of the maximal active isometric value after 30 min. The further decay was very slow and after 4 h force was at 20–25% of the maximal isometric value. We chose to investigate the

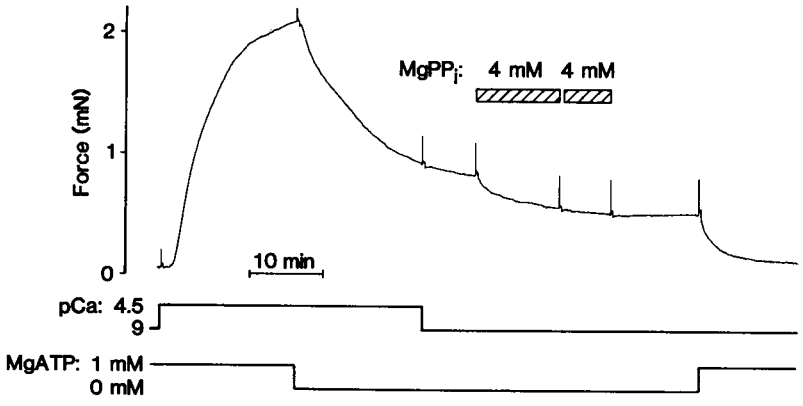


FIGURE 1 Original recording of force from a skinned guinea pig *Taenia coli* preparation. Contraction is initiated by a rise in free Ca^{2+}. Rigor is induced by removal of MgATP. Addition of 4 mM $MgPP_i$ results in an incomplete relaxation. Introduction of fresh $MgPP_i$ solution does not alter the time course of the $MgPP_i$-induced force decay. Addition of MgATP causes relaxation.

effects of $MgPP_i$ on rigor force by introducing the test solutions at 30 min after induction of high rigor, when force had approached an apparent plateau. The subsequent force values in the experiments were normalized relative to this value. When a solution containing $MgPP_i$ was introduced at this stage, a marked increase in the rate of force decay was observed (Fig. 1).

The decay of rigor force induced by $MgPP_i$ solutions was not observed in rigor solutions containing 6 mM $MgCl_2$ without PP_i or 4 mM PP_i without $MgCl_2$, which suggests that the force decay is caused by $MgPP_i$. $MgPP_i$ may precipitate at higher concentration. For the concentrations used (0.5–4 mM) no visible precipitation occurred within 45 min. In order to investigate if the relaxing effects of the $MgPP_i$ solutions changed with time, we introduced fresh $MgPP_i$ solution, 10 min (see Fig. 1) and 4 h after the first introduction. In either case, this did not have any additional effects and did not alter the time course or amplitude of the force decay.

The $MgPP_i$-induced force decay was irreversible, because removal of $MgPP_i$ inhibited the slow force decay but did not cause an increase in force (Fig. 1). The force in the presence of $MgPP_i$ approached a lower, but nonzero, force level. After 20 min, force in 4 mM $MgPP_i$ had decreased by ~40% from the starting point in rigor. In separate experiments it was found that the rigor force, in the absence of $MgPP_i$, decayed by ~5% during the same time and did not approach the force value in the presence of $MgPP_i$ within 4 h. The addition of $MgPP_i$ to rigor at this stage (4 h in rigor) caused an additional force decay similar to that observed when added after 30 min in rigor. These results suggest that the two conditions, rigor and $MgPP_i$ states, are associated with different force levels.

Caged PP_i and the amount of photolytically released PP_i was determined with the isotachophoresis system. Since PP_i is not UV absorbing, the amount of this substance was determined from the conductivity detector signal. In test experiments, solutions were illuminated with the flash lamp system (120 mJ in the wave length range 300–400 nm; Arner et al., 1987a) using an UV-transmitting filter (UG-11). Increasing the concentration of caged PP_i up to 5 mM gave increased concentrations of released PP_i. At 5 mM ~1.8 mM PP_i was released. This concentration was used because 10 mM caged PP_i did not further increase the amount of released PP_i.

Illumination of the fiber in rigor solution without caged PP_i did not influence the force level, and immersion of the fiber in rigor solution with 5 mM caged PP_i did not affect the rigor force. When the fiber was illuminated in the presence of caged PP_i (Fig. 2), force decayed in a similar manner as when 2 mM $MgPP_i$ was added by diffusion. This shows that the rate of relaxation is not limited by the rate of $MgPP_i$ diffusion in the isometric experiments above.

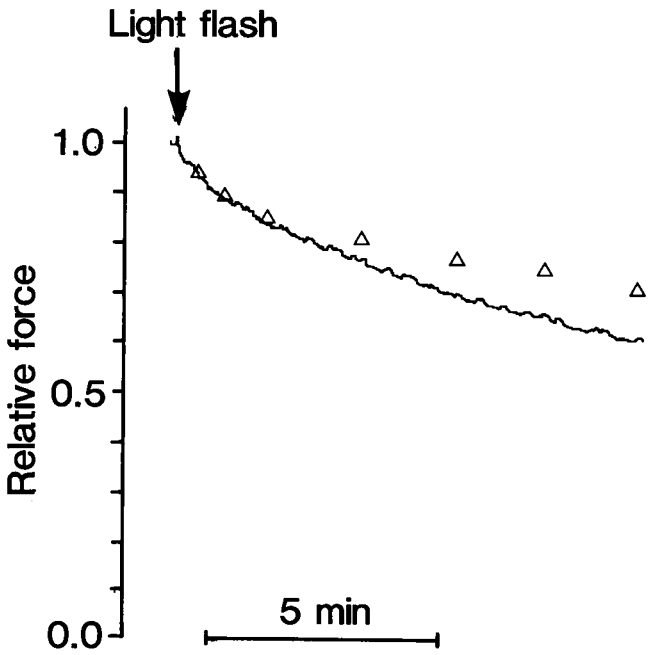


FIGURE 2 Original recording of force after photolytic release of PP_i. A rigor state was induced as shown in Fig. 1. At the plateau of the rigor force the preparation was transferred to a rigor solution with 5 mM caged PP_i. After 3 min the muscle was illuminated with a light flash which released ~1.8 mM PP_i. Force is normalized to the rigor force level before the light flash. For comparison, mean values of force obtained by diffusion of 2 mM $MgPP_i$ into the muscle fibers are shown (*open triangles* from Fig. 6).

Effects of $MgPP_i$ on stiffness

The extreme force reached during rapid length steps (complete within 0.3 ms) is plotted against the amplitude of the imposed length steps (T_1 curves) for three fiber preparations in Fig. 3. Length (L) is normalized to initial muscle length (L_i) and force (P) to the isometric value in the Ca^{2+} activated state for each muscle (P_o). For shortening steps the relation between length and force changes was concave upwards, and for stretches the relation was near linear. Linear regression (force change vs. length change) gave for the stretches in the activated muscle a slope (stiffness) of 100.4 (P_o/L_i), which corresponds to an intercept with the x-axis of $-0.010\ L_i$ ($r = 0.99$). In rigor, force was 0.30 P_o and the slope was 61.0 ($r = 0.99$). Thus, both force and stiffness are lower compared with the active contraction. However, relative stiffness (stiffness/force) is ~2-fold higher in the rigor state compared with the active contraction. In the $MgPP_i$ solution, force was 0.16 P_o and stiffness was

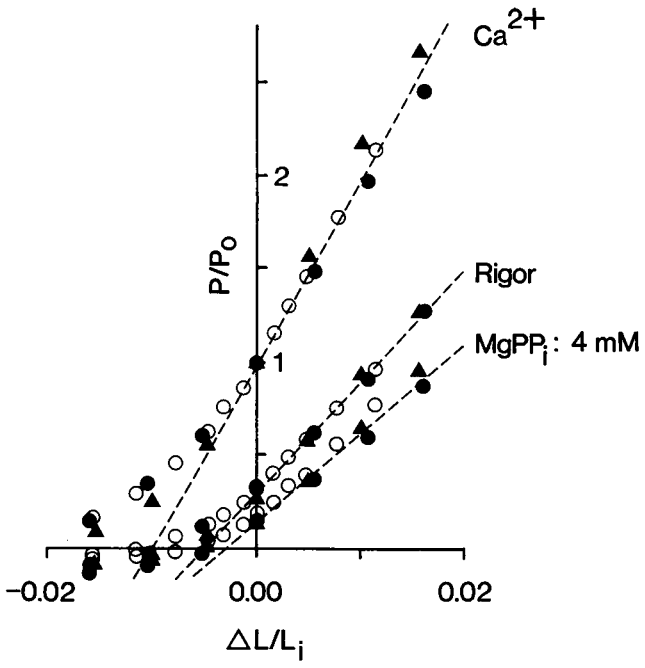


FIGURE 3 Stiffness in skinned guinea pig *Taenia coli* preparations during, consecutively, active Ca^{2+}-induced contraction, rigor, and rigor with 4 mM $MgPP_i$. Data from three different preparations are indicated with different symbols. The extreme force reached during the length change (P), normalized to the maximal isometric force during Ca^{2+}-induced active contraction (P_o), is plotted against the amplitude of the imposed length change (ΔL, normalized to initial muscle length (L_i). Regression lines ($r > 0.98$ for each group) for force change vs. length change for stretches (positive $\Delta L/L_i$ values) are shown in the figure and extrapolated across the intercept with the x-axis.

48.8 ($r = 0.98$). Thus, compared with the rigor state, the $MgPP_i$ state is associated witih a 48% decrease in force with a 20% decrease in stiffness, corresponding to an increase in the relative stiffness by a factor of 1.5. Removal of $MgPP_i$ did not alter the force (cf. Fig. 1) or stiffness (data not shown).

Quantitative analysis of $MgPP_i$-induced decay of rigor force

The experimental design shown in Fig. 1 was modified to include several contraction–rigor $MgPP_i$ cycles (Fig. 4, *left record*). Using this protocol we investigated the concentration dependence of the $MgPP_i$ responses, and the influence of MgADP, ionic strength, strain, and myosin light chain phosphorylation on the $MgPP_i$-induced force decay. When rigor force approached a plateau the fiber was exposed to $MgPP_i$. The resulting force decay was followed for 20 min before a new cycle began. Up to six cycles were repeated on each fiber preparation. For comparison, the effects of 4 mM $MgPP_i$ on rigor force in striated muscle fibers from the rabbit psoas was investigated using the same experimental conditions (identical solutions with ionic strength 150 mM, pH 6.9 at 22°C). Compared with the smooth muscle the $MgPP_i$-induced force decay in striated muscle was greater and considerably faster (Fig. 4, *right*).

In order to quantitate the $MgPP_i$-induced responses, the force decays were digitized (2–4 samples/min) and the data points fitted by exponential functions using a nonlinear least squares curve fitting routine (Fletcher and Powell, 1963). The force data were normalized to the force in rigor before the addition of $MgPP_i$. Force in

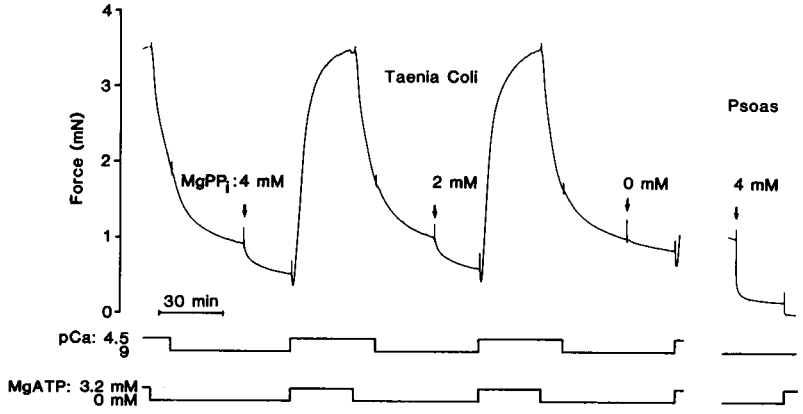


FIGURE 4 (*Left record*) Original recording of force from a skinned guinea pig *Taenia coli* preparation. Three contraction-rigor-$MgPP_i$ cycles are shown. (*Right record*) Recording of force from a bundle of skinned rabbit psoas fibers. Rigor force was induced by removing ATP in the relaxed state. Rigor force decays rapidly in the presence of 4 mM $MgPP_i$. Full relaxation is seen in the presence of MgATP.

rigor and in the presence of $MgPP_i$ did not decay to zero within the time of observation as described above. This suggests the presence of very slow processes or that force approaches a nonzero level under these conditions. This behavior was therefore approximated by including a constant term in the fitted functions. The decay of force (P) with time (t) in the presence of $MgPP_i$ was clearly multi-exponential but could be adequately fitted, for the 20-min period, by the sum of two exponentials (amplitudes A_1 and A_2, rates r_1 and r_2) and the constant (K). Force (P) was expressed relative to the rigor force (P_r) immediately before $MgPP_i$ was added ($t = 0$).

$$P/P_r = A_1e^{(-r_1t)} + A_2e^{(-r_2t)} + K \quad (1)$$

In rigor, the decay was very slow and one exponential function ($A_1 = 0$) was used to fit the data. Digitized force data at 0 and 4 mM $MgPP_i$, from the experiment in Fig. 4 (*left record*), and fitted functions are shown in Fig. 5. As seen in the figure, good fits to the data were obtained. Although the function adequately described the force decay, most of the experimental variables influenced several parameters in the function. Therefore we also present the initial rate of $MgPP_i$-induced force decay (in units of P_r/time) calculated as the time derivative of the fitted equation (1) P/P_r at $t = 0$.

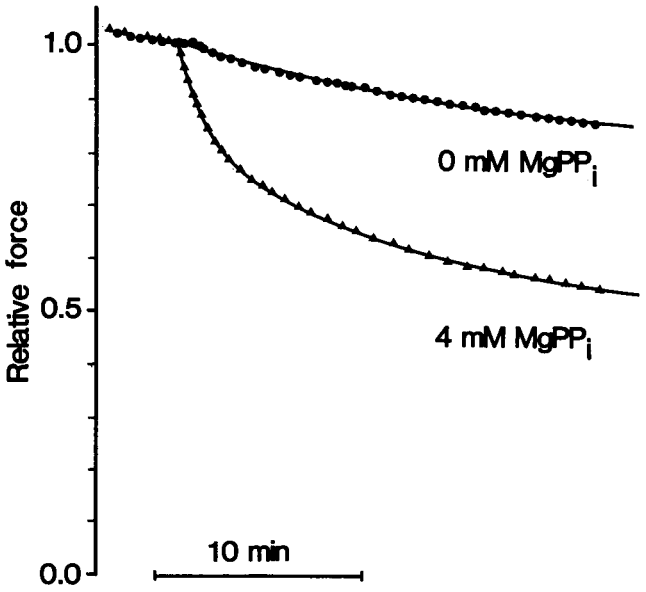


FIGURE 5 Digitized force data at 0 (*filled circles*) and 4 mM (*triangles*) $MgPP_i$ from the experiment shown in Fig. 4. Force is normalized to the value immediately before the addition of $MgPP_i$-test solutions. Eq. 1 was fitted to the data as described in the text and displayed in the diagram. Two exponential terms and a constant was used for 4 mM $MgPP_i$, and one exponential term and a constant for 0 $MgPP_i$.

Concentration-dependence of $MgPP_i$ effects

The rate of the $MgPP_i$-induced force decay was concentration dependent. Fig. 6 shows the parameters of Eq. 1 fitted to the force decay during 20 min after transfer to solutions with different $MgPP_i$ concentrations in nonphosphorylated rigor fibers. The rigor force at the time when the $MgPP_i$ was added (P_r), expressed as a fraction of the maximal active force, was 0.33 ± 0.01 ($n = 8$). The rate constants (r_1, r_2; *upper left*) for the two exponential decays in Eq. 1 were separated by about one order of magnitude.

Increasing [$MgPP_i$] was associated with increases in the amplitudes (A_1, A_2; *upper right*) and the rate constants r_1 and r_2 of both processes. The constant term, K, decreased with increasing $MgPP_i$ concentration. The calculated initial $MgPP_i$-induced relaxation rate (*lower*) showed a clear dependence on the concentration. The mean values for the initial rates (r_i) were fitted by the Michaelis-Menten equation:

$$r_i = r_{max}[MgPP_i]/(k_d + [MgPP_i]). \quad (2)$$

The apparent dissociation constant, k_d and the maximal rate, r_{max}, were found to be 1.6 mM and 0.28 P_r/min, respectively. Eq. 1 did not adequately fit the $MgPP_i$-induced force decay in the psoas preparation (*right*, Fig. 4). In that experiment, the fastest decay had an amplitude of 0.5 P_r and a rate constant of 3.5 min^{-1}. The calculated initial rate was ~1.9 P_r/min, which is ~10-fold higher than the highest rates observed in the smooth muscle. The rate of relaxation in the striated muscle might be underestimated because the experiments were performed by diffusing $MgPP_i$ into the fiber.

In the presence of $MgPP_i$ (> =0.5 mM) the amplitude of the slow exponential process (A_2) increased compared with rigor, and a second exponential term (A_1, r_1) was required to fit the force decay. A corresponding decrease in K was found. In order to investigate if the apparent steady-state force in the presence of $MgPP_i$ is dependent of the concentration of $MgPP_i$, one fiber preparation was maintained in 1 mM $MgPP_i$ for 3 h and then exposed to 4 mM $MgPP_i$. No further decay was observed when the $MgPP_i$ concentration was raised at this point in time. Fiber preparations maintained in rigor for 3 h still responded to 4 mM $MgPP_i$ with a force decay similar to that after 30 min in rigor, although the rate of relaxation was slightly lower. These results

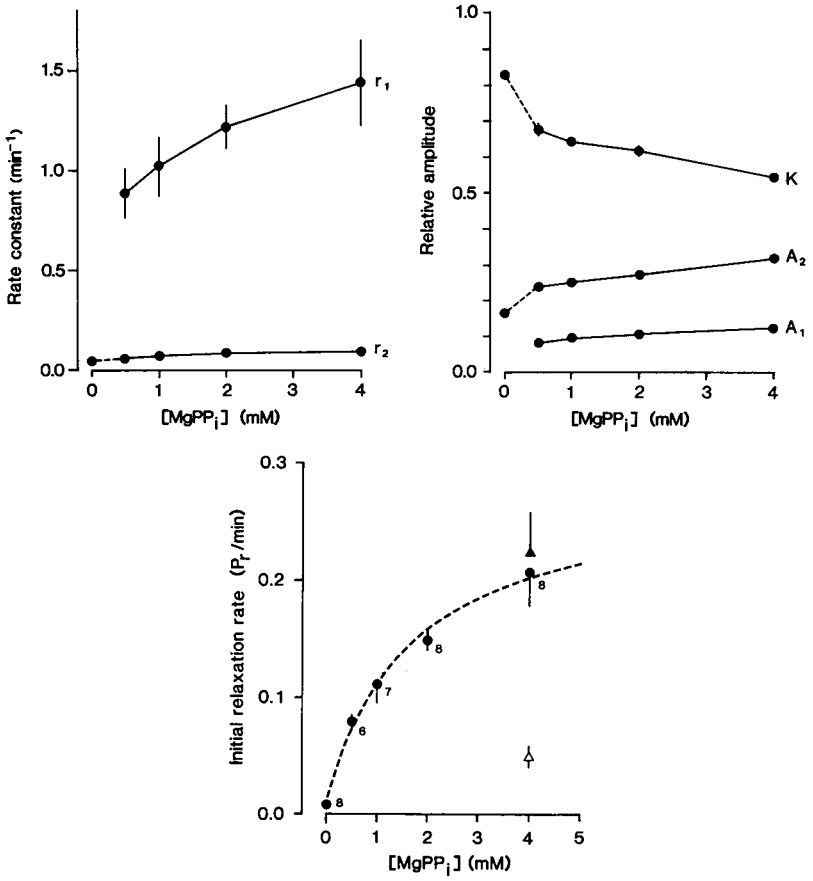


FIGURE 6 (*Upper*) Rate constants (r_1 and r_2), amplitudes (A_1 and A_2), and the constant term (K) at different $MgPP_i$ concentrations obtained by fitting Eq. 1 to the force decay. (*Lower*) The initial rate of relaxation calculated from the data in the upper panels. Broken line shows the fit of Michaelis-Menten equation to the mean values. Data are normalized to the rigor force immediately before the addition of $MgPP_i$ (P_r). P_r values were ~30% of the maximal active isometric force. The filled circles in the three panels refer to one series of experiments, where the number of observations are shown in the lower diagram. The triangles show data ($n = 4$) from a separate series of experiments where the effects of 4 mM $MgPP_i$ were investigated in unshortened fibers (*filled triangles*) and in fibers shortened to give P_r values of ~15% of the maximal active isometric force (*open triangles*). Error bars are not shown when smaller than size of symbols.

suggest that in the presence of $MgPP_i$, rigor force decays to a new lower steady-state level which is independent of the $MgPP_i$ concentration in the range 1 to 4 mM.

In order to investigate if the initial rate was dependent on force, a separate series of experiments was performed where fibers were shortened in rigor (by less than 1%) to give about half the rigor force (P_r values were ~15% of the maximal active force) before the addition of $MgPP_i$. The rate of relaxation in shortened fibers in rigor solution was very slow and almost zero. The data in the presence of 4 mM $MgPP_i$ are inserted in the lower panel of Fig. 6 (*open triangles, n = 4*). For comparison, recordings at 4 mM $MgPP_i$ were also performed in the same fiber preparations without shortening (*filled triangles*). The initial rate at 4 mM in the shortened fibers was reduced to 23 ± 5% of that in unshortened fibers, showing that the rate of the $MgPP_i$-induced force decay is dependent on the force level.

Dependence of $MgPP_i$-induced effects on ionic strength

Fig. 7 shows the rate constants (*upper left*), amplitudes, and the constant (*upper right*) obtained by fitting Eq. 1 to the force decay in response to 4 mM $MgPP_i$ at varied ionic strength. Ionic strength, adjusted with KCl, had a marked influence on the rate of the faster exponential decay (r_1), mainly in the range 45–90 mM, whereas the lower rate (r_2) and the amplitudes (A_1, A_2) and the constant (K) were little affected. The influence of ionic strength is also evident in the calculated initial rate (*lower*). The rate was decreased at decreasing ionic

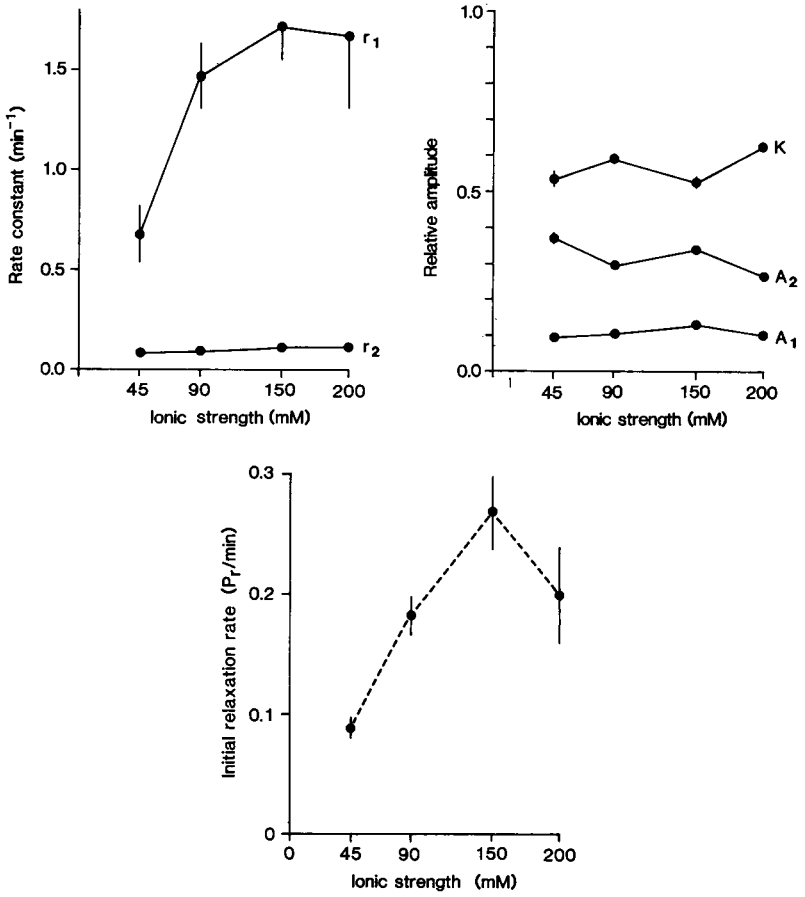


FIGURE 7 (*Upper*) Rate constants (r_1 and r_2), amplitudes (A_1 and A_2) and the constant term (K) at different ionic strength obtained by fitting Eq. 1 to the force decay in response to 4 mM $MgPP_i$. (*Lower*) The initial rate of relaxation calculated from the data in the upper panels ($n = 6$). Data are normalized to the rigor force immediately before the addition of $MgPP_i$ (P_r). Error bars are not shown when smaller than size of symbols.

strength from the maximum at ~150 mM. At increased ionic strength (200 mM) the rate decreased by ~25%.

Effects of MgADP on the force decay induced by $MgPP_i$ in non- and thiophosphorylated fibers

The effects of $MgPP_i$ on high rigor force in nonphosporylated and irreversibly thiophosphorylated fibers were compared. Experiments were performed at varied concentrations of MgADP. Before each contraction-rigor $MgPP_i$ cycle the thiophosphorylated fibers were exposed to ATP-γ-S for a 5-min period (see Methods). The active force levels decayed by less than 15% between the first and last of the repeated contractions in both thiophosphorylated and Ca^{2+}-activated fibers, which shows that the repeated contraction-rigor PP_i cycles do not markedly influence the active force generation. When MgATP solution in the absence of Ca^{2+} (pCa 9) was given to the fibers at the end of the rigor $MgPP_i$ period, the nonphosphorylated fibers relaxed completely, whereas the thiophosphorylated fibers contracted, giving ~85% of maximal active force. This shows that the thiophosphorylated fibers remain phosphorylated to a high degree during the rigor $MgPP_i$ period. Myosin light chain phosphorylation in nonphosphorylated fibers in the rigor state has previously been determined to be near zero (Arner et al., 1987a). The rigor force at the time when the $MgPP_i$ was added (P_r), expressed relative to the maximal active force, was 0.31 ± 0.01 ($n = 8$) for the nonphosphorylated (rigor state induced from a Ca^{2+}-activated contraction), and 0.35 ± 0.01 ($n = 8$) for the thiophosphorylated fibers (rigor state induced from a contraction after thiophosphorylation).

In the absence of MgADP the rate constant for the fast process (r_1) and the calculated initial rate (*lower*) were sigificantly lower in the thiophosphorylated com-

pared with nonphosphorylated fibers ($P < 0.05$, Student's t-test, unpaired data; Fig. 8). In two thiophosphorylated and two nonphosphorylated fibers, the ratio between the initial rates of relaxation at 0.5 and 4 mM $MgPP_i$ were compared and found to be similar (0.51 and 0.49, respectively), which suggests that the decreased rate of force decay in the thiophosphorylated compared with the nonphosphorylated fibers was not caused by a change in the concentation dependence of the $MgPP_i$ effects.

In both non- and thiophosphorylated fibers MgADP inhibited the $MgPP_i$-induced relaxation. No effect of MgADP alone on rigor force could be noted. For increasing MgADP, the amplitudes of the exponential decays (A_1, A_2) decreased while K increased. The calculated initial rate for $MgPP_i$-induced force decay decreased at increasing MgADP (*lower*). At 0.32 mM MgADP the inhibition was complete, with no observed effects on rigor force of 4 mM $MgPP_i$. Half-maximal effect of MgADP on the initial rate of the $MgPP_i$-induced force decay (estimated from the data in the *lower panel*) was observed at ~10 μM for both non- and thiophosphorylated fibers. In experiments where two fiber preparations were kept in 0.032 mM MgADP at 4 mM $MgPP_i$, the final force level reached after 3 h (0.49 and 0.48 P_r) was the same as that reached in 4 mM $MgPP_i$ without MgADP ($0.47 \pm 0.04 P_r$, $n = 4$) although the rate of the force decay was much lower. These results suggest that MgADP slows the $MgPP_i$-induced force decay in a similar manner as a reduction in the concentration of $MgPP_i$.

Effects of MgATP and $MgPP_i$ on the force velocity relation

The effects of varied MgATP, at 0 and 4 mM $MgPP_i$, on the force–velocity relation were investigated. The fibers were activated by thiophosphorylation in order to avoid

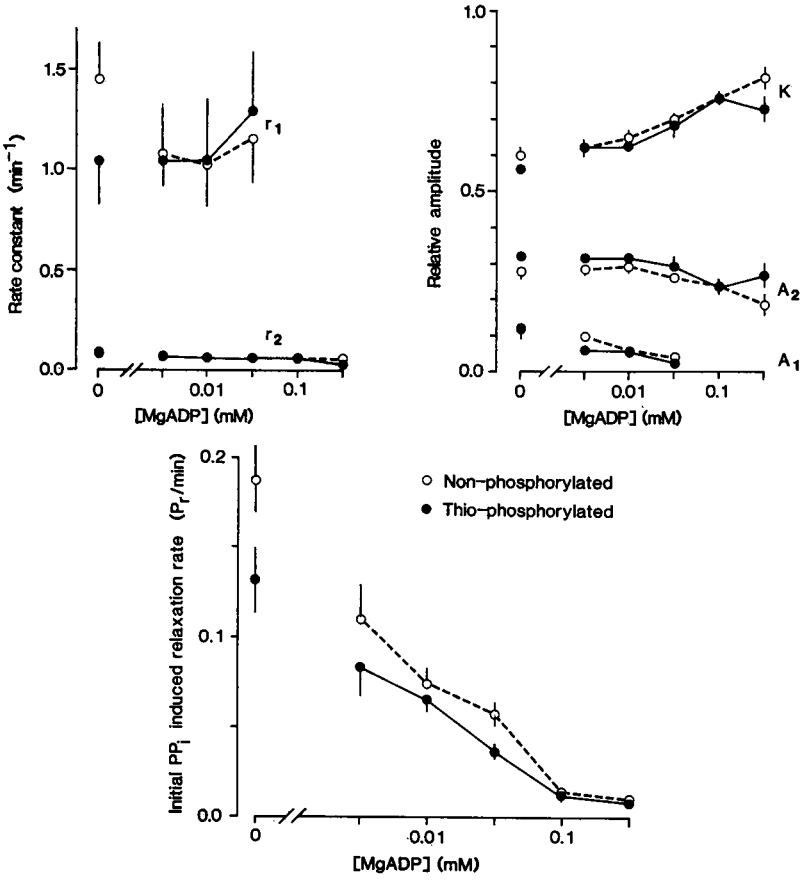


FIGURE 8 (*Upper*) Rate constants (r_1 and r_2), amplitudes (A_1 and A_2), and the constant term (K) at different MgADP concentrations obtained by fitting Eq. 1 to the force decay in response to 4 mM $MgPP_i$ for nonphosphorylated (*open circles*) and thiophosphorylated (*filled circles*) fibers. (*Lower*) The initial rate of relaxation calculated from the data in the upper panels ($n = 7$–8). Data are normalized to the rigor force immediately before the addition of $MgPP_i$ (P_r). Error bars are not shown when smaller than size of symbols.

possible influences on cross-bridge kinetics indirectly via effects on the phosphorylation–dephosphorylation reactions. In a previous study (Arheden et al., 1988), we found that 2 mM PCr (15 U/ml creatine phosphokinase, CK) was sufficient as an ATP regenerating system for maximal shortening velocity (V_{max}) and isometric force in thiophosphorylated fibers at 0.01 and 1 mM MgATP. Since $MgPP_i$ has been suggested to inhibit the creatine phosphokinase reaction (cf. Pate and Cooke, 1985) and the experiments were performed at low MgATP, high concentrations of PCr (12 mM) and CK (75 U/ml) were used. In two experiments the concentration of CK was further increased to 150 U/ml, which did not affect force or V_{max}, with or without PP_i, suggesting that the enzyme concentration used was adequate.

The shortening response to a quick release in smooth muscle is not linear with time but exhibits a gradually decreasing shortening velocity (cf. Arner and Hellstrand, 1985). Shortening velocities were therefore determined at different points in time after the release (100, 200, and 500 ms). Fig. 9 (upper) shows force–velocity relations (at 100 ms) from one fiber preparation at saturating (1 mM) and low (0.032 mM) MgATP in the absence (*open symbols*) and presence (*filled symbols*) of 4 mM $MgPP_i$. The data were fitted by the Hill (1938) equation and the maximal shortening velocity (V_{max}) was determined as the intercept of the fitted curve with the ordinate. At 0.032 mM MgATP, V_{max} decreased to ~50% of its maximal value. 4 mM $MgPP_i$ did not affect the force–velocity relation at either MgATP concentration. In all of the investigated solution conditions, releases to the same afterload, early and late during the series of releases, gave identical velocities. All points in a series of releases fell close to the hyperbolic equation as seen in the upper panel of Fig. 9. The force–velocity relations obtained in the different solution conditions were independent of the order at which these were applied to the muscle. These results exclude an influence of time-dependent phenomena on the determination of the force–velocity relation with this experimental design (cf. also Arheden et al., 1988). In Table 1 the fitted parameters of the Hill equation and the calculated

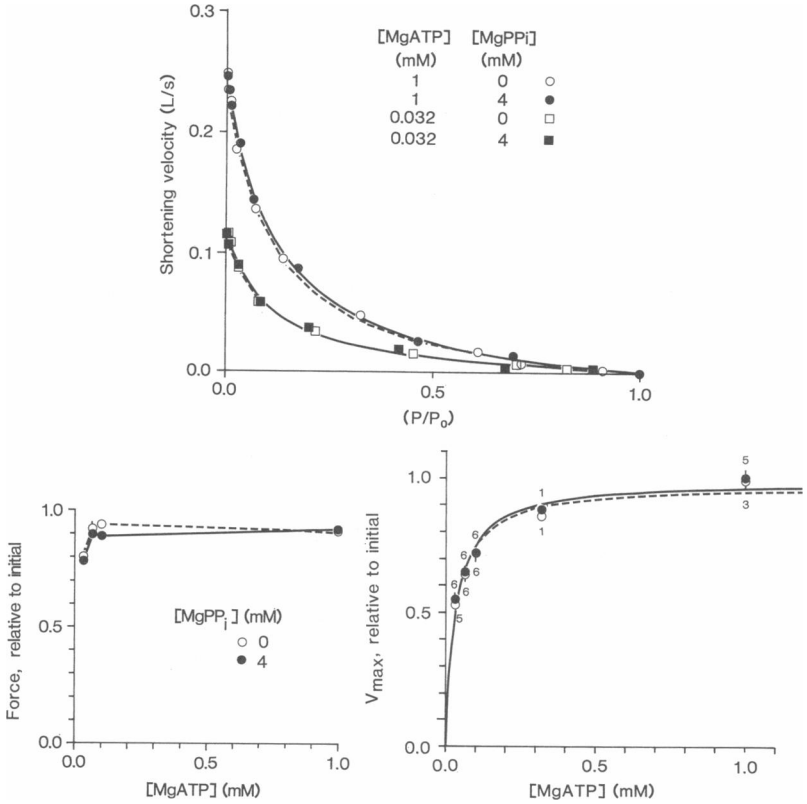


FIGURE 9 (*Upper*) Force–velocity relation of a guinea pig *Taenia coli* preparation at 1 and 0.032 mM MgATP at 0 and 4 mM $MgPP_i$. The shortening velocity, in muscle lengths per second, was determined 100 ms after the release. The afterload (P) is given relative to the isometric force (P_o). The Hill (1938) equation is fitted to the data. (*Lower*) Isometric force (*left diagram*) and maximal shortening velocity (V_{max}, *right diagram*) at different MgATP concentrations at 0 and 4 mM $MgPP_i$. The velocity data are fitted by the Michaelis-Menten equation. The number of observations are shown in the lower right diagram. Error bars are not shown when smaller than size of symbols.

TABLE 1. **Force–velocity data**

MgATP (mM)	1	1	0.032	0.032
$MgPP_i$ (mM)	0	4	0	4
V_{max} (L/s) at 100 ms	0.259 ± 0.008	0.264 ± 0.014	0.141 ± 0.007	0.145 ± 0.009
200 ms	0.152 ± 0.007	0.152 ± 0.010	0.076 ± 0.004	0.078 ± 0.004
500 ms	0.080 ± 0.005	0.078 ± 0.005	0.037 ± 0.004	0.038 ± 0.002
a/P_o at 100 ms	0.103 ± 0.004	0.108 ± 0.005	0.087 ± 0.007	0.096 ± 0.003
b (L/s) at 100 ms	0.028 ± 0.002	0.028 ± 0.002	0.011 ± 0.002	0.013 ± 0.002
n	8	5	5	6

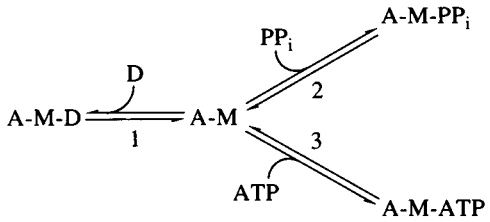
The maximal shortening velocity (V_{max}), expressed in muscle lengths/s, at 1 and 0.032 mM MgATP with 0 and 4 mM $MgPP_i$. V_{max} was determined at three different points in time after the quick release.

V_{max} values for 1 and 0.032 mM MgATP at 0 and 4 mM $MgPP_i$ are summarized. V_{max} at the different points in time (100, 200 and 500 ms) after the release were all reduced by ~50% at the lower MgATP concentration. The alteration in V_{max} was accompanied by a decrease in the parameter b of the Hill equation, whereas a/P_o was slightly decreased.

In Fig. 9 (*lower*) the isometric force (*left*) and V_{max} (*right*) data at different [MgATP] in the absence and the the presence of 4 mM $MgPP_i$ are shown. The data are normalized to the values of an initial force–velocity relation determined for each fiber preparation at optimal [MgATP] (1 mM). Force was essentially unaffected by varying [MgATP], except at 0.032 mM where an ~10% decrease was observed. The V_{max} values decreased at lower [MgATP]. At 0.032 mM the decrease was ~50%. The velocity data in the figure are fitted by the Michaelis-Menten equation ($V_{max} = r_{max}[MgATP]/(k_m + [MgATP])$). Note that V_{max} denotes the maximal shortening velocity at the respective MgATP concentration and r_{max} the fitted maximal V_{max} at saturating [MgATP]. The r_{max} values were 0.99 and 0.98 for 0 and 4 mM $MgPP_i$, respectively, which corresponds to 0.26 muscle lengths/s (cf. Table 1). The apparent K_m values were 0.032 mM for both 0 and 4 mM $MgPP_i$, showing that $MgPP_i$ does not influence the MgATP dependence of the force–velocity relation.

DISCUSSION

Force generation in muscle is considered to be associated with release of phosphate (P_i) and a transition from weakly attached actin-myosin-ADP-P_i states (A-M-D-P) to strongly bound actin-myosin-ADP (A-M-D) and actin-myosin (A-M) states (cf. Eisenberg and Hill, 1985). Binding of ATP and the formation of the A-M-ATP complex leads to dissociation. In this study we have investigated some mechanical properties and biochemical reactions of the strongly bound cross-bridge states in smooth muscle fibers. We have used the non-hydrolyzable ATP analogue, pyrophosphate (PP_i), to influence the interaction between actin and myosin. Scheme 1, below, summarizes the relevant attached cross-bridge states and reactions.



(Scheme 1)

Solution studies using the proteolytic subfragment 1 or myosin (S-1) from smooth muscle have shown that myosin binds strongly to actin in the absence of nucleotide and is dissociated from actin by ATP, a behavior that is qualitatively similar to that of striated muscle myosin (Marston and Taylor, 1980; Greene et al., 1983). When ATP is removed in chemically skinned smooth muscle preparations a state with increased relative stiffness is observed (Arner and Rüegg, 1985; Arheden et al., 1988), suggesting formation of rigor cross-bridges (A-M states in scheme 1). Structural evidence for increased attachment of cross-bridges in smooth muscle in the absence of ATP has been obtained with electron microscopy (Somlyo et al., 1988) and x-ray diffraction (Arner et al., 1988). In contrast to the case in striated muscle, removal of ATP in the relaxed states does not lead to a marked rigor force development in smooth muscle, although stiffness increases (Arheden et al., 1988) and electron microscopy reveal attached cross-bridges (Somlyo et al., 1987). A "high-rigor" force can be obtained by removing ATP at the plateau of an active contraction (cf. Fig. 1), showing that rigor cross-bridges in smooth muscle can remain attached when positively strained. However, the rigor force gradually decays towards a level of ~25–30% of the maximal active force.

This force decay is not due to ATP-induced detachment or dephosphorylation, because the experiments were done in the presence of an ATP depleting system and similar results were obtained in unphosphorylated and thiophosphorylated fibers. The rigor force decay reaches an apparent plateau ~30 min after removal of ATP. The rate constant for the force decay in rigor at this stage is very low (r_2 from Fig. 6: ~0.05 min^{-1}).

In striated muscle, $MgPP_i$ has been reported to bind to the nucleotide site of myosin and weaken the actin-myosin interaction both in vitro and in skinned muscle fibers (White, 1970; Greene and Eisenberg, 1980; Biosca et al., 1986; Schoenberg and Eisenberg, 1985). At present, very little biochemical data from solubilized smooth muscle contractile proteins are available regarding $MgPP_i$. The nucleotide analogue AMP-PNP, which in some aspects have effects similar to $MgPP_i$ in striated muscle fibers (Schoenberg and Eisenberg, 1985), weakens the binding of S-1 to actin to about the same extent (400- to 500-fold) in striated and smooth muscle (Greene and Eisenberg, 1980; Greene et al., 1983).

Kuhn (1978) has suggested that the nucleotide analogue AMP-PNP induces a slippage of cross-bridges along the actin filament leading to a decay of rigor force by affecting the interaction between myosin and actin. According to a cross-bridge model proposed by Schoenberg (1985), almost all of the cross-bridges with bound nucleotide analogue (AMP-PNP, PP_i) are attached because the equilibrium between attachment and detachment is in favor of the attached state. This "equilibrium behavior" predicts that force will decay to zero in times reflecting cross-bridge detachment rate constants. This has been supported by data from skinned striated muscles (Schoenberg and Eisenberg, 1985) although other studies report a less complete relaxation (White, 1970; Tregear, 1988).

We find that adding $MgPP_i$ to the smooth muscle fibers in rigor causes a decay of force. This effect is qualitatively similar to that in striated muscle fibers (White, 1970; Schoenberg and Eisenberg, 1985; Fig. 4 of this study) although the rate is considerably lower and the extent of the force decay is less in the smooth muscle. As discussed above, the steady-state rigor force in smooth muscle decays very slowly, which suggests that the A-M state (scheme 1) can maintain force at this force level. In the presence of $MgPP_i$, a population of A-M-PP_i cross-bridges are formed (reaction 2, scheme 1). In order to explain that the $MgPP_i$-induced force decay was irreversible and that the steady-state level was independent of the $MgPP_i$ concentration, it must be assumed that the A-M-PP_i state can undergo an irreversible transition to a lower force level. The irreversible decrease of force induced by $MgPP_i$ in the smooth muscle fibers in rigor was associated with a decrease in absolute stiffness (Fig. 3). However, stiffness decreased less than force resulting in an increase in relative stiffness (i.e., stiffness/force). Stiffness measurements in smooth muscle are complicated due to several factors, e.g., the lack of defined sarcomeric structures and the presence of nonlinear series elastic elements (SEC) outside of the contractile component. When force is varied in active smooth muscle by altering [Ca^{2+}], which presumably alters the number of active cross-bridges, the stiffness vs. force relation is nearly linear with an intercept close to origo (Pfitzer et al., 1982; Arner and Rüegg, 1985; Arheden and Hellstrand, 1991). Thus, at different active force levels, relative stiffness (stiffness/force) is similar. In rigor, both force and stiffness are reduced compared with those at maximal active force although stiffness is less reduced (Arner and Rüegg, 1985; Arheden and Hellstrand, 1991). The relative stiffness (stiffness/force) increases. It is reasonable to assume that most cross-bridges are attached in rigor, which then suggests that stiffness can decrease without detachment. This is most likely due to the presence of a nonlinear SEC in smooth muscle giving a dependence of stiffness on force. In our experiments $MgPP_i$ caused a fall in force with an increase in relative stiffness. When stiffness measurements in $MgPP_i$ were compared with rigor measurements at the same force level, after washout of $MgPP_i$, no difference could be detected. If it is assumed that stiffness of attached individual rigor and $MgPP_i$-crossbridges are the same and the presence of a nonlinear SEC is taken into account, the data are consistent with a decrease in force without detachment. This may suggest that in the presence of $MgPP_i$, rigor force decreases due to detachment of cross-bridges with reattachment in positions of lower strain, while the total number of attached cross-bridges is essentially unaltered. This interpretation is similar to that proposed for striated muscle (White, 1970; Schoenberg and Eisenberg, 1985).

The $MgPP_i$-induced force decay in smooth muscle could not be described by a single exponential function and did not reach zero force. Two exponential decays and a constant were required to fit the data. The highest rate constant (r_1 at 4 mM $MgPP_i$; Fig. 6) was ~0.025 s^{-1}. According to Schoenberg and Eisenberg (1985) the decay of force after stretch of striated muscle in the presence of $MgPP_i$ is not monoexponential and is best described by rate constants in the range 0.1–100s^{-1} (4 mM $MgPP_i$). The distribution of rate constants was interpreted to reflect a strain dependence of either the analogue binding or the cross-bridge dissociation. We have not made the corresponding stretch analysis in smooth muscle. However, the force during the first 16 ms after the rapid (0.3 ms) stretch, used to determine stiffness, reached about the same level (70%) relative to

the initial (T_1) force in the presence of $MgPP_i$ and in rigor. The rapid release of PP_i from caged PP_i gave a force decay on a timescale of minutes. It thus appears that $MgPP_i$-induced force decay in smooth muscle does not occur to a large extent in the millisecond–second time scale.

In fibers shortened in rigor, the rate of relaxation in the rigor solution was slowed as well as the rate of $MgPP_i$-induced relaxation (*triangles* in Fig. 6). This suggests that the rate of force decay of rigor and $MgPP_i$ cross-bridge states is dependent on strain. The initial decay of force, minutes after rigor is initiated (cf. Fig. 1), could reflect a strain dependence of the force decay of rigor cross-bridges. The finding that force in the presence of $MgPP_i$ did not decay to zero, at least not within 4 h, could thus reflect that a new equilibrium is reached at a lower strain level where cross-bridges detach very slowly or detach and reattach at the same strain level.

In order to obtain an estimate of the dissociation constant for $MgPP_i$ in the muscle fiber we chose to determine the rate of force decay at a defined force level, the steady-state rigor force (P_r). Since the effects of $MgPP_i$ were not influenced by diffusion limitation and the binding of $MgPP_i$ presumably is considerably faster than the rate of force decay, the reaction 2 (scheme 1) reaches an equilibrium soon after the addition of $MgPP_i$. The initial strain distribution of the A-M-PP_i states after addition of $MgPP_i$ should be similar to that of the A-M states immediately before. The initial rate of force decay will depend on the number of cross-bridges in the A-M-PP_i state and on the rate at which the individual cross-bridges with bound PP_i detach and reattach at lower strain levels, because the force decay of A-M states is very low. According to these assumptions, the dissociation (or binding constant) for $MgPP_i$ to the rigor cross-bridges can be estimated from the initial rate of force decay, because the concentration of A-M-PP_i states depends on the $MgPP_i$ concentration (reaction 2, scheme 1). Although the relation between the initial rate and $MgPP_i$ concentration did not reach a plateau, it was clearly nonlinear and could be adequately described by the Michaelis-Menten equation (*lower panel* in Fig. 6). This seems to exclude the possibility that the initial rate of force decay is limited by the rate of binding of $MgPP_i$, because that would have resulted in a linear relation between the $MgPP_i$ concentration and the initial rate of force decay. In striated muscle the dependence on the $MgPP_i$ concentration of the rate of relaxation after a stretch cannot be described by the Michaelis-Menten equation, which has been suggested to reflect cooperativity in the detachment between myosin heads (Anderson and Schoenberg, 1987). Our data could be described by the Michaelis-Menten equation and thus it seems that the effect in smooth muscle does not necessitate the assumption of cooperativity. The estimated dissociation constant (k_d) for $MgPP_i$ of 1.6 mM in the smooth muscle fiber is in the same range as in striated muscle fibers (Schoenberg and Eisenberg, 1985; Anderson and Schoenberg, 1987), where the binding of $MgPP_i$ is considered to be similar to that in solution (k_d of 2.5 mM; Biosca et al., 1986).

The binding of smooth muscle S-1 to actin in the presence of AMP-PNP in solution is dependent on ionic strength, although to a lesser extent than in striated muscle S-1 (Greene et al., 1983). The binding of smooth muscle S-1 in the presence of AMP-PNP to actin in solution was two- to threefold stronger at 45 compared with 150 mM ionic strength (Fig. 3 in Greene et al., 1983). Low ionic strength increases the stiffness of relaxed smooth muscle fibers and leads to a decrease in the maximal shortening velocity (Arheden et al., 1988). We found that the effect of $MgPP_i$ in the smooth muscle fibers was dependent on the ionic strength (Fig. 7). The initial rate of force decay decreased at low ionic strength and was about threefold lower at 45 mM compared with 150 mM ionic strength. These results suggest that ionic strength influences the interaction of PP_i-cross-bridges with actin in the muscle fiber.

Myosin light chain phosphorylation is considered to be the main regulatory system in smooth muscle (Hartshorne and Siemankowsky, 1981). Solution studies have suggested that the major effect of phosphorylation is an ~1000-fold increase in the forward rate of phosphate release (Sellers, 1985). Phosphorylation also increases the binding of smooth muscle heavy meromyosin (HMM) to actin in the presence of ATP or ADP by about two- to fourfold, an effect that is considered too small to account for the regulation of actomyosin ATPase (Sellers et al., 1982; Greene and Sellers, 1987). In the thiophosphorylated state the rate of relaxation after the addition of $MgPP_i$ was ~25% lower than in the nonphosphorylated state (Fig. 8, *lower panel* at 0 MgADP), suggesting that phosphorylation is associated with a small effect on the interaction of the strongly bound myosin-PP_i-states with actin.

MgADP did not influence the rigor force in the smooth muscle fibers in concentrations up to 0.32 mM but inhibited the $MgPP_i$-induced force decay (Fig. 8). This suggests that MgADP and $MgPP_i$ compete for the binding site on myosin. Half-maximal inhibition was observed at ~10 μM MgADP at 4 mM $MgPP_i$ (Fig. 8). This decrease in the initial relaxation rate corresponds to a reduction of $MgPP_i$ from 4 to 1 mM. The apparent K_i from these data calculated as described by Schoenberg and Eisenberg (1987) is ~2 μM for both nonphosphorylated and thiophosphorylated smooth muscle fibers. This calculated value is lower than that estimated from studies on smooth muscle HMM (50 μM; Greene

and Sellers, 1987). The data thus suggest that the binding of MgADP is stronger in muscle fibers in rigor, compared with HMM in solution. It should, however, be considered that our K_i value was determined indirectly through competition with $MgPP_i$ and thus might be restricted to these conditions. The absence of an effect of phosphorylation on the MgADP binding is similar to results from HMM in solution (Greene and Sellers, 1987). For comparison, the dissociation constant for MgADP binding in attached cross-bridges in striated muscle has been estimated, by competition with $MgPP_i$, to be 60 μM (Schoenberg and Eisenberg, 1987), which is close to the value determined in solution. In smooth muscle fibers, MgADP has been shown to decrease force and maximal shortening velocity (V_{max}) and slow the rate of ATP-induced relaxation from rigor (Arner et al., 1987b). The estimated K_i for the effect on V_{max} was in the range 0.1–0.3 mM. The apparently stronger binding of MgADP in rigor fibers, compared with data from solution and from fibers during shortening, suggests that the mechanical constraints influence the interaction of MgADP with the smooth muscle cross-bridges.

During active shortening, cross-bridges reach the A.M state (scheme 1) after release of phosphate and ADP. Detachment from this state is induced by the binding of ATP (reaction 3). The second-order rate constant for the ATP-induced detachment is in the order of $2 \cdot 10^6$ M^{-1} s^{-1} in solution (Marston and Taylor, 1980) and has been estimated to be $\sim 0.1\text{–}2.5 \cdot 10^5$ M^{-1} s^{-1} under isometric conditions in smooth muscle fibers (Somlyo et al., 1988). At decreased [MgATP] the rate of ATP-induced detachment decreases and A.M cross-bridges must be mechanically dissociated during shortening as the filaments slide past each other. This introduces an internal load that opposes shortening. We found a k_m of 32 μM for the MgATP dependence of V_{max} in thiophosphorylated smooth muscle fibers. This is ~10-fold lower than k_m values reported for striated muscle fibers (250 μM, Cooke and Bialek, 1979; 470 μM, Ferenczi et al., 1984). A correlation between V_{max} and k_m for MgATP has been found in fast and slow striated muscle fibers where V_{max} differ by about threefold (Stienen et al., 1988). This correlation also appears to exist for the 10-fold slower smooth muscle. It has been suggested that the shortening velocity is rate limited by the rate of product release (Siemankowski et al., 1985). The low k_m could reflect that steps, e.g., product release, preceding the ATP-induced detachment determine the low shortening velocity of smooth muscle. A slow sliding of filaments, as in smooth muscle, would require that the rate of the ATP-induced detachment should be reduced to a larger extent in order to influence shortening velocity.

No influence of $MgPP_i$ could be noted on the shortening velocity (Fig. 9). This is different from results from striated muscle where $MgPP_i$ acts as a competitive antagonist to MgATP with an apparent k_i of 3 mM (Pate and Cooke, 1985). The MgATP dependence of V_{max} in the smooth muscle most likely reflects an influence on the MgATP-induced cross-bridge detachment reactions. The lack of influence of $MgPP_i$ or high K_i under these conditions in smooth muscle, could then suggest that the $MgPP_i$ states detach rapidly when negatively strained without opposing shortening or that the binding of $MgPP_i$ is weak in negatively strained cross-bridges.

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