# THE INHIBITION OF MUSCLE CONTRACTION BY ADENOSINE 5′ ($\beta$, $\gamma$-IMIDO) TRIPHOSPHATE AND BY PYROPHOSPHATE

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ABSTRACT We have studied the inhibition of the contraction of glycerinated rabbit psoas muscle caused by ligands that bind to the ATPase site of myosin. Two ligands, adenosine 5′ ($\beta$, $\gamma$-imido) triphosphate (AMPPNP) and pyrophosphate ($PP_i$), decreased the force and stiffness developed in isometric contractions and the velocity of shortening of isotonic contractions. The force exerted by isometric fibers was measured as a function of MgATP in the presence and absence of a constant concentration of the ligands. As the MgATP concentration decreased, the inhibition of tension caused by the ligand increased, reaching ~50% at 25 $\mu$M MgATP and either 2 mM $MgPP_i$ or 2 mM MgAMPPNP. The maximum velocity of shortening was also measured as a function of MgATP concentration in the presence of 1 and 2 mM $MgPP_i$ and 2.5 and 5 mM MgAMPPNP. Both ligands acted as pure competitive inhibitors with $K_i$ = 3.0 mM for $PP_i$ and 5.1 mM for MgAMPPNP. These data show that both ligands are weak inhibitors of the contraction of fibers. The results provided information on the energetics of actin-myosin-ligand states that occur in the portion of the cross-bridge cycle where MgATP binds to myosin. A simple analysis of the inhibition of velocity suggests that MgAMPPNP binds to the actomyosin complex at this step of the cycle with an effective affinity constant of $\sim 2 \times 10^2$ $M^{-1}$.

## INTRODUCTION

Muscle contraction is the result of a cyclic interaction between cross-bridges extending from the myosin filaments and sites on adjacent actin filaments. A myosin cross-bridge attaches to an actin site, executes a power-stroke causing a shear displacement between the parallel filaments, and then detaches to begin a new cycle. Free energy driving the reaction comes from the hydrolysis of MgATP. A central question in muscle biophysics is to understand the process by which this interaction converts chemical energy into mechanical energy.

The interaction of actin, myosin, and MgATP has been studied extensively in solution (reviewed by Taylor, 1979; Eisenberg and Greene, 1980). Myosin hydrolyzes MgATP rapidly in a cyclic interaction with actin. MgATP that is bound to the myosin nucleotide site is hydrolyzed primarily, but not exclusively, when the myosin is dissociated from actin. Myosin, with attached hydrolysis products, then binds to actin where the products rapidly dissociate. Following product release the binding of MgATP to the actomyosin complex causes dissociation of myosin from actin. This last process would be inhibited by ligands that compete with MgATP for the nucleotide site of myosin.

The general pathways for the actin-myosin-nucleotide interactions observed in solution are assumed to be similar to those found in a functioning muscle (Eisenberg and Greene, 1980). Problems arise, however, in extrapolating solution data to the muscle fiber. A major difficulty is introduced by the geometrical constraints on the actomyosin interaction, which are certainly imposed by the structural array of actin and myosin filaments in muscle fibers. The solution studies do not include these constraints. The rates of many steps observed in solution are altered when the proteins are incorporated into the filament array where they are performing mechanical work (reviewed, Sleep and Smith, 1981). Understanding the differences between solution studies and the interaction in the fiber has been difficult because of the problems encountered in measuring the kinetics of the biochemical interactions occurring in the fiber.

One approach to measuring the properties of a specified interaction occurring in the fibers is to observe the contraction of permeable fibers at different substrate concentrations (Cooke and Bialek, 1979; Cox and Kawai, 1981; Kawai, 1978; Wilson and White, 1983; Ferenczi et al., 1984; Chaen et al., 1981; Moss and Haworth, 1984). Additional data allowing one to link specific kinetic states with muscle physiology would be of use in understanding the chemomechanics of contraction. To this end, a straightforward extension of this approach is to measure the effect on contraction of ligands that compete for the

MgATP site of myosin. Two such nonhydrolyzable ligands are Mg adenosine 5′ ($\beta$, $\gamma$-imido) triphosphate (MgAMPPNP) and Mg pyrophosphate ($MgPP_i$) (Yount et al., 1971; Danker, 1983). An extensive body of work has characterized the interactions of MgAMPPNP with myosin subfragment-1 (S-1) and acto S-1 in solution (Yount et al., 1971; Konrad and Goody, 1983; Trybus and Taylor, 1982; Greene and Eisenberg, 1980). These data are not compatible with a simple scheme for the binding of MgAMPPNP to actomyosin. The association constant of MgAMPPNP for the protein complex is stronger than expected from the rates of association and dissociation, and the rate of dissociation of the ternary complex by low concentrations of MgATP is about two orders of magnitude less than calculated from other known constants. Binding of MgAMPPNP to rigor fibers results in an apparent lengthening of the fiber that is probably due to cross-bridge conformations different from the rigor complex (Marston et al., 1979; Marston, 1980). This effect is reversible with transition back to the rigor complex coupled to work production (Kuhn, 1981; Kuhn, 1978*b*). The energetics of the interaction of $MgPP_i$ with S-1 and acto S-1 in solution have also been characterized, although little is known about the kinetics of this interaction (Greene and Eisenberg, 1980; Danker, 1983). Binding of $MgPP_i$ to rigor fibers results in a force decrease similar to that observed with MgAMPPNP (White, 1970).

To further understand the relationship between acto S-1 states in solution and those in the fiber we have measured the effect of two ligands MgAMPPNP and $MgPP_i$ on isometric and isotonic contractions of permeable muscle fibers as a function of MgATP concentration at varying MgAMPPNP and $MgPP_i$ concentrations. Both ligands inhibit isometric tension as well as $V_{max}$, the velocity of contraction at zero force.

## METHODS

Thin bundles of rabbit psoas fibers 1–2 mm diameter were dissected and tied to thin rods with surgical thread. The bundles were incubated in 50% glycerol, 50% 0.15 M KCL, 5 mM $MgCl_2$, 10 mM EGTA, and 20 mM 2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES), pH 7.0 (vol/vol). After 24 h at 0°C the solution was changed and the fibers were stored at −20°C until used. Storage lasted typically from 2 wk to 2 mo and did not appear to affect mechanical properties.

For mechanical measurements single fibers were dissected on a cold stage and mounted between the two arms of the tensiometer using perspex dissolved in acetone as a glue. The length of the mounted fiber was 1 cm. Sarcomere lengths were determined by diffraction. Initial sarcomere lengths varied from 2.3 to 2.5 $\mu$m. Two tensiometers, which gave comparable results, were used during the course of the study. Earlier measurements were made using a moving coil to measure fiber tension and to apply isometric contractions as described by Cooke and Bialek (1979). Later measurements were made using the tensiometer described by Crowder and Cooke (1983). The muscle was mounted between a solid-state force transducer (Akers, 801; Aksjelskapet Microelectronikk, Horten, Norway) and a light, stiff bereylium arm connected to a rapid motor (General Scanning, Inc., Watertown, MA) for changing muscle length. The frequency response for the transducer with fiber loaded was 2 kHz. Tension was monitored by a Compupro Computer using Tekmar A/D boards (Tekmar Co., Cincinnati, OH). For isotonic contractions the tension of the fiber was compared with a reference tension by the computer, and a signal to either lengthen or shorten the fiber was sent to the motor. This feed back loop operated at a frequency of 1 kHz.

All experiments were performed with a basic rigor buffer bathing the fibers containing 0.15 M KCl, 5 mM $MgCl_2$, 2 mM EGTA, 1.9 mM $CaCl_2$, 20 mM TES, 20 mM creatine phosphate (CP), and 30–90 $\mu$M (2.5–7.5 mg/ml) creatine kinase (CK), pH 7.0, pCa ~5. The temperature was regulated at 10 ± 0.2°C. In all force-velocity experiments, the $MgCl_2$ concentration was adjusted to maintain a free $Mg^{++}$ concentration of 5 mM using standard binding constants for $Mg^{++}$ to ATP, AMPPNP, and $PP_i$ of $1.2 \times 10^4 M^{-1}$, $3.8 \times 10^4 M^{-1}$, and $2.8 \times 10^5 M^{-1}$, respectively (Smith and Martell, 1975; Yount et al., 1971). Ionic strength was maintained constant by decreasing the KCl concentration. A creatine kinase-creatine phosphate feeder system was used in all experiments to maintain the level of MgATP inside contracting fibers. CK was purchased from Sigma Chemical Co. (St. Louis, MO) or was prepared by the method of Kuby (Kuby et al., 1954). As MgAMPPNP and $MgPP_i$ inhibit CK (Nihei et al., 1961; Watts, 1973), the concentration of CK added to the contraction buffer was adjusted with ligand concentration (up to 90 $\mu$M CK) to insure adequate ATP perfusion of contracting fibers. AMPPNP was purchased from Sigma Chemical Co. Commercially available AMPPNP is contaminated with small amounts of ATP and ADP (which is converted to ATP by CK-CP) (Penningroth et al., 1980). Using a luciferin-luciferase assay (Sigma Chemical Co.) we determined that 0.6% of total nucleotide in our sample of AMPPNP was ATP or ADP. Samples of MgAMPPNP were incubated with CK and CP and the amount of MgATP was assayed by measuring light output generated by luciferin-luciferase according to the instructions supplied by Sigma Chemical Co. AMPPNP ran as a single species when chromatographed on thin-layer polyethelyeneimine in the presence of 1 M LiCl, pH 9.0. In the analysis of all experiments involving added MgAMPPNP, the concentrations of MgATP and MgAMPPNP were adjusted to account for the 0.6% MgATP contamination.

Force velocity data were analyzed using a variation of the Hill equation (Hill, 1938). Let

$$(a + P)V = (P_o - P)b, \tag{1}$$

where $V$ equals the velocity, $P_o$ equals the isometric force, $P$ equals the contraction force at velocity $V$, and $a$, $b$ are parameters. At $P = 0$, $V_{max} = bP_o/a$ and setting $\alpha = a/P_o$, the equation can easily be rewritten

$$V = \alpha V_{max}(1 - P/P_o)(\alpha + P/P_o). \tag{2}$$

Data analysis is simplified by use of the form in Eq. 2. At each MgATP and competing ligand concentration, a minimum of 24 force-velocity data points from a minimum of 6 fibers were used. The two parameters, $V_{max}$ and $\alpha$, were determined by a nonlinear least-squares fit of the individual data points to Eq. 2 using a Gauss-Newton–type interation. A grid search for starting values of $\alpha$ and $V_{max}$ was used in an attempt to insure the global minimum sum of the squared residuals. All data points were weighted equally. Numerical calculations were performed using an Amdahl 470V/8 computer (Amdahl Corp., Sunnyvale, CA). The computer code used routines from Statistical Analysis Systems Institute (release 82.3), Carey, NC. Straight lines in Fig. 5 are least-squares linear approximations to the fitted values of $V_{max}$ with error bars representing 95% confidence limits from the hyperbolic fit. Means and standard deviations shown in Fig. 4 were determined by grouping the individual force-velocity data points into five bins and averaging within each bin in both the force and velocity directions (Cooke and Bialek, 1979). Least-squares hyperbolic fits to the isometric tension data in Fig. 2 were obtained in a fashion similar to that described above.

## RESULTS

As expected, the ligands $MgPP_i$ and MgAMPPNP inhibit the contraction of glycerinated rabbit psoas fibers. Single

fibers were mounted between a force transducer and a fast motor, which could alter fiber lengths. Force was recorded as a function of time. The fiber was bathed in a solution containing the ligand and a variable concentration of MgATP. The concentration of MgATP inside the fiber was maintained using a CK-CP regeneration system. It was found that high levels of either ligand inhibited CK. To adjust for this, the maximum velocity of contraction was measured as a function of the CK concentration at 25 $\mu$M MgATP and the desired ligand concentration. As the concentration of CK increased in the absence of ligand the velocity of contractions also increased, reaching a plateau at ~20 $\mu$M CK. A similar result was obtained by Cooke and Bialek (1979). However, 60 $\mu$M CK was required to reach the plateau in the presence of either 5 mM MgAMPPNP or 2 mM $MgPP_i$. Accordingly the concentration of CK was 30 $\mu$M in experiments run in the absence of ligands, 90 $\mu$M at high ligand concentrations and 60 $\mu$M at intermediate ligand concentrations. CP was constant, 20 mM, at all ligand concentrations.

Fig. 1 *A* shows a typical time course for tension development. Tension rose rapidly to a maximum and then decreased by 10–20% to a level that remained relatively stable. Measurements of isotonic contractions were made during the period of steady tension. The time course of tension development was similar for all fiber preparations. One or two series of isotonic load clamps were imposed on the muscle. A series consisted of up to four load clamps as shown in Fig. 1 *B* and 2. The experimental protocol used for measuring the effect of the ligand on isometric tension was different from that used to measure the effect on isotonic contraction velocity. Due to the difficulty in accurately measuring the fiber diameters, the effect of ligands on fiber tension was determined by comparing the tensions measured before and after the addition of the ligand to the medium that was bathing a contracting fiber. The fiber was mounted on a tensiometer and first immersed in the basic rigor buffer containing a given concentration of MgATP. After the tension had stabilized, ligand was added to the medium and the tension fell to a new steady state value within ~15 s. The absolute magnitude of fiber tension as a function of MgATP concentration in the absence of ligand was not statistically different from that presented previously (Cooke and Bialek, 1979), i.e., in the absence of ligand isometric tension increased as the MgATP concentration decreased reaching a maximum at =25–50 $\mu$M MgATP and then decreasing with increasing ATP concentration.

Fig. 3 *A* and *B* give the ratio of fiber force in the presence of competing ligand to force in the absence of ligand as a function of MgATP concentration. At high MgATP concentrations, the ligands had no effect on fiber tension. As the MgATP concentration decreased, the inhibition by the ligands increased, as expected for competitive inhibition. When the results of Figs. 3 *A* and *B* are plotted in double reciprocal fashion, i.e., the inverse of

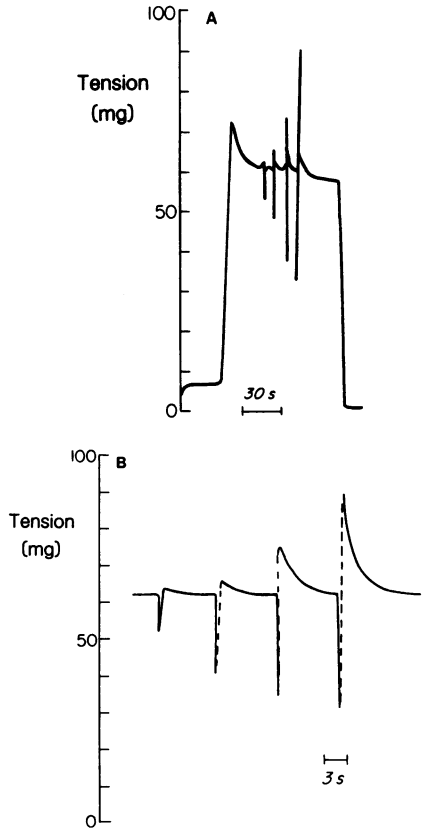


FIGURE 1 (*A*) Time course of isometric tension development in a single contracting glycerinated psoas muscle fiber for 100 $\mu$M MgATP and no competing ligand. Deviations at 30 to 60 s following activation represent four isotonic releases for measurements of contraction velocity. Force drops at the end when the fiber is broken and a check is made to insure that there has been no drift in the base line of the force transducer. (*B*) Time course of tension during the load clamps shown with an expanded time scale. The sharp downward deflection is the load clamp, and the following upward deflection is the rise in tension that occurs when the fiber is reextended. Data from a similar series of load clamps is shown in more detail in Fig. 2.

fractional force vs. the inverse of MgATP concentration, the data did not fit a straight line but displayed a concave downard curvature as shown in Fig. 3 *C*. The curvature seen in Fig. 3 *C* shows that force is not a simple hyperbolic function of the MgATP concentration. However, the data of Figs. 3 *A* and *B* could be fit by a hyperbolic function if one assumes that force extrapolates to a nonzero value when the MgATP concentration equals zero. This fit is shown in Figs. 3 *A* and *B* and discussed in the next section.

The velocity of fiber contraction during load clamps showed a slight decline with time in a manner that was similar to the decline in tension. The most reproducible measurements of contraction velocity were obtained by immersing the fiber in a contracting solution that contained the desired levels of both MgATP and ligand. One or two series of up to four load clamps were then applied to the fiber at precise intervals, usually 30 s following the

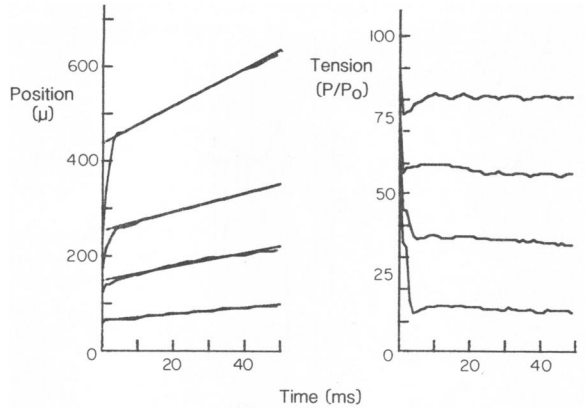


FIGURE 2 Original record showing displacement and tension for four isotonic releases. When the tension reached an approximate steady state, four isotonic releases were performed to: 0.8 $P_o$, 0.6 $P_o$, 0.35 $P_o$, and 0.12 $P_o$. Following each release the fiber was extended to its original length (1 cm) at a constant velocity of 1 mm/s. After the extensions the fiber was held isometric for 2 s to allow the tension to return to its original value, and the next release was then performed. Up to two sets of up to four releases were performed on each fiber. *Left*: Decrease in fiber length as a function of time for isotonic releases at four different forces. Straight lines represent least-squares linear fits to data; slopes give velocities of contraction. *Right*: Force ($P$) divided by the isometric tension at the beginning of the series ($P_o$) as a function of time for contractions shown on the *left*. The traces with greater slopes shown on the left correspond to releases to lower tensions shown on the *right*. The MgATP concentration was 50 μM.

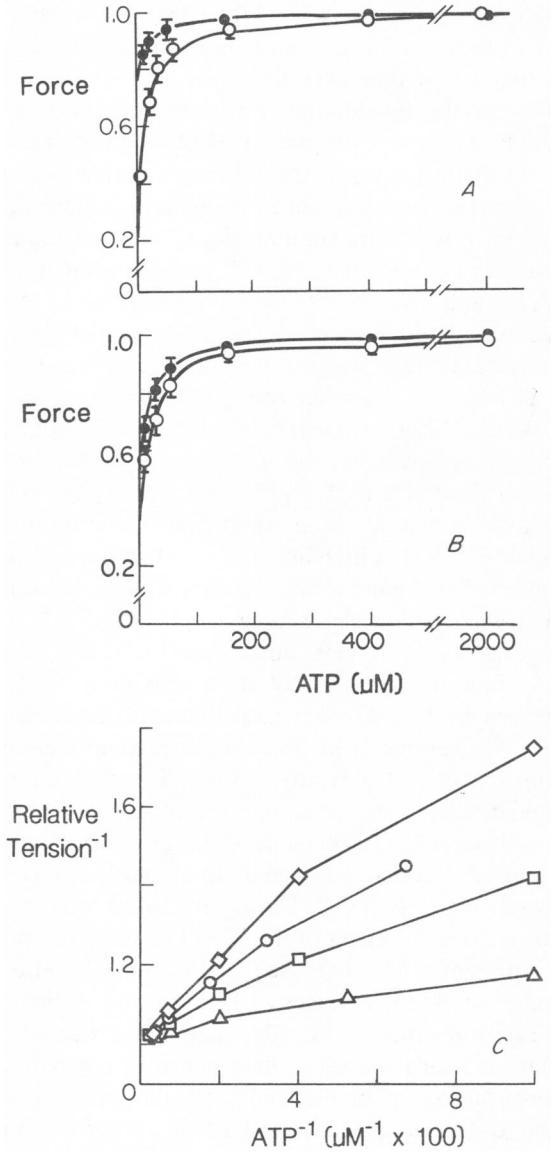


FIGURE 3 The relative isometric force of single glycerinated psoas muscle fibers shown as a function of the MgATP concentration in the presence of MgAMPPNP and $MgPP_i$. At each MgATP concentration, forces represent the ratio of force in the presence of competing ligand to force in the absence of competing ligand. The absolute magnitude of the isometric tension rises by ~50% as the ATP concentration is lowered from 1 mM to 25 μM as shown by Cooke and Bialek (1979). (*A*) 1 mM (○) and 0.35 mM (●) MgAMPPNP. (*B*) 2 mM (○) and 0.5 mM (●) $MgPP_i$. The medium contained the nucleotides in rigor buffer plus 1.8 mM $CaCl_2$, 20 mM CP, and variable amounts of CK, 30–90 μM, as described in Methods. Each data point represents the mean of six different measurements. The solid lines are least-squares hyperbolic fits to the data as described in the text. (*C*) The data of *A* and *B*, above, are replotted in a double reciprocal fashion. 1 mM (○) and 0.35 mM (△) MgAMPPNP, 2 mM (◇) and 0.5 mM $MgPP_i$ (□).

activation of the fiber. The fiber length and fiber tension recorded during one series of four isometric releases are shown in Fig. 2. The length of the fiber first decreased rapidly due to series elasticity of the fiber following the release to a new tension. The length then showed a linear decrease with time. The linear portion of the length vs. time trace between 10 and 40 s was fitted to a straight line by linear regression. The slope of this line gives the velocity of contraction. This portion of the length-time trace was approximately linear for all of the experimental conditions that were studied. The tensions were also averaged over the same time period to determine the mean fiber tension during the releases. Release tension was varied between 0.05 and 0.95 times $P_o$ to construct complete force velocity curves shown in Fig. 4. Isotonic velocities of contraction measured during the series of four releases shown in Fig. 2 were similar to those in which only one isotonic release was performed during the activation. The effect of the previous fiber history on the data was examined by measuring velocity attained in a load clamp to 0.3 $P_o$ as a function of the time at which the load clamp was applied. In a series of four load clamps the velocity measured in the first and fourth release did not differ by more than 5%. Although the velocity measured decreased by ~10% over the first 2–3 min of the contraction, this decrease appeared to be similar in all fibers and was not influenced by isotonic releases.

The ligands inhibited the velocity of contraction to approximately the same degree that they inhibited the tension. Fig. 4 shows force-velocity relations recorded in the presence and absence of 5 mM MgAMPPNP. The maximum velocities of contraction were obtained by fitting the data to Eq. 2, shown by the solid lines in Fig. 4, and extrapolating to zero tension. The maximum velocity was

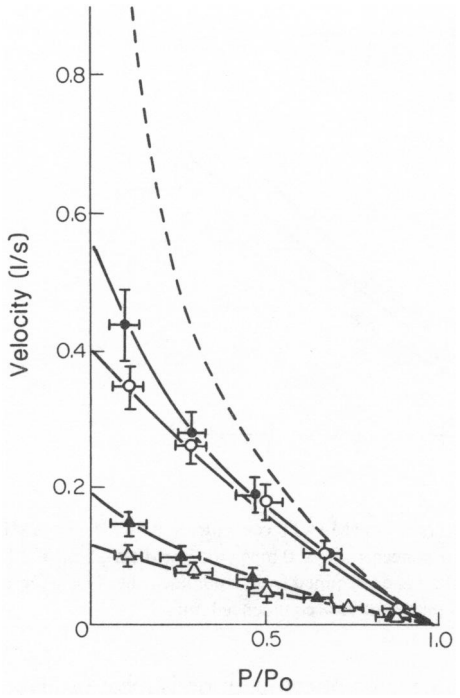


FIGURE 4 The velocity of contraction of single muscle fibers is shown as a function of the relative force exerted by the fibers. The contraction medium contained 25 $\mu$M MgATP (▲), 31 $\mu$M MgATP, and 5 mM MgAMPPNP (△), 100 $\mu$M MgATP (●), 100 $\mu$M MgATP, and 5 mM MgAMPPNP (○). The dashed line is the force-velocity relation at saturating MgATP concentrations, 5 mM (Cooke and Bialek, 1979). The medium also contained 20 mM CP and 30–90 $\mu$M CK. The solid lines are Hill's equation, Eq. 2, fit to the data by a nonlinear, least-squares method. Means and standard deviations are from between 4 and 14 (average = 9) distinct force-velocity measurements as described in Methods.

recorded as a function of MgATP concentration for two different ligand concentrations and the results plotted in double reciprocal fashion, see Fig. 5. The inhibition of the contraction velocity by $MgPP_i$ was very similar to that observed for MgAMPPNP; 2 mM $MgPP_i$ producing approximately the same effect as 5 mM MgAMPPNP. In both the absence and presence of the ligands, all of the data on the inhibition of contraction velocity can be fit within experimental accuracy by the equations derived to explain the competitive inhibition of a simple enzymatic reaction obeying Michaelis-Menten kinetics: $V = V_{max}\,(ATP)/[K_m\,(1 + C_i/K_i) + ATP]$, where $V_{max}$ is the maximum contraction velocity at infinite MgATP concentration, $K_m$ is the Michaelis constant that describes the dependence of velocity upon MgATP concentration, $C_i$ is the concentration of ligand, and $K_i$ is its inhibition constant. This equation predicts that plots of the inverse of the velocity vs. the inverse of the substrate concentration should be straight lines as is observed in the data shown in Fig. 5. The value of $K_i$ can be determined from the intercept on the abscissa. For MgAMPPNP the two intercepts provide values for $K_i$ of 5.0 mM (2.5 mM MgAMPPNP) and 5.2 mM (5 mM MgAMPPNP). The corresponding values of $K_i$ for $MgPP_i$ are 2.9 mM (1 mM $MgPP_i$) and 3.1 mM (2 mM $MgPP_i$). the fact that the values of $K_i$ determined at two different concentrations of ligand agree within experimental error provides additional support for the observation that both ligands appear to act as pure competitive inhibitors of the maximum velocity of contraction. Averaging the data at the two ligand concentrations, we obtain a $K_i$ of 3.0 mM for $MgPP_i$ and a $K_i$ of 5.1 mM for MgAMPPNP.

## DISCUSSION

The ligands, MgAMPPNP and $MgPP_i$, are not hydrolyzable by actomyosin and complete with MgATP for the active site slowing the overall cross-bridge turnover rate through the actomyosin ATPase cycle (Yount et al., 1971; Marston et al., 1976). Because the binding of MgATP in the cycle dissociates the actomyosin bond, competition by the ligands should slow the rate of dissociation. During isotonic contractions, undissociated myosin heads must be detached mechanically as the actin filament continues to move; this will slow the velocity of contraction. Thus, qualitatively the observed inhibition of velocity by the ligands is in accord with current models of cross-bridge function. A similar decrease in velocity has been observed as the concentration of MgATP is lowered. Although the tension of a fiber is the result of enzymatic activity, it does not follow classical behavior. For instance, as the substrate concentration decreases the tension at first increases and then decreases (Cooke and Bialek, 1979; Chaen et al., 1981; Ferenczi et al., 1984). One explanation for the behavior of isometric tension as substrate or ligand concentrations vary is that actomyosin states can exert tension when the nucelotide site of myosin is either filled with ligand or is empty as has been suggested by studies on rigor fibers (Marston et al., 1979; Kuhn, 1978*a*). Assuming that ligand-bound states can exert force, one might expect the data of Figs. 3 *A* and *B* to fit a hyperbola with a nonzero intercept on the force axis as substrate approaches zero. The data of Figs. 3 *A* and *B* do in fact fit such a curve, as shown by the solid lines. With all units in micromoles and $x$ representing the concentration of MgATP, the hyperbolae in Fig. 3 *A* are given by $(x + 13.7)/(x - 17.7)$ for 0.35 mM MgAMPPNP, $(x + 7.8)/(x + 18.7)$ for 2 mM MgAMPPNP. The hyperbolae define a Michaelis constant, $K_m$, for MgATP of 3 $\mu$M and an inhibition constant, $K_i$, for MgAMPPNP of 1 mM. The inhibition of isometric tension by $MgPP_i$ was similar to that observed for MgAMPPNP. These data, shown in Fig. 3 *B* could also be fitted with hyperbolae with nonzero intercepts. For 0.5 mM $MgPP_i$ the hyperbola is given by $(x + 8.8)/(x + 17.1)$ and for 2 mM $MgPP_i$ by $(x + 7.8)/(x + 21.3)$. The fits define a similar $K_m$ for MgATP of 6 $\mu$M, and a $K_i$ of 2 mM.

The cyclic actomyosin-nucleotide interaction that produces force and movement in the fibers is obviously complex, and it is thus rather surprising that experimentally both ligands appear to be pure competitive inhibitors

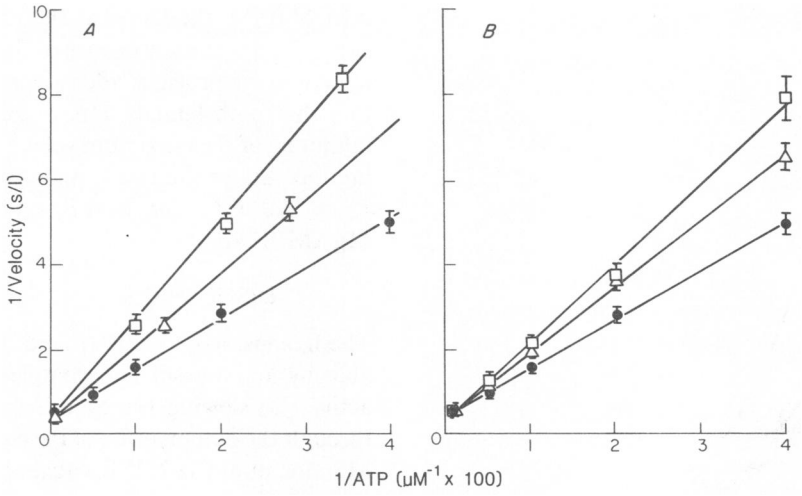


FIGURE 5 The inverse of the velocity of contraction is shown as a function of the inverse of the MgATP concentration for (*A*) MgAMPPNP concentrations: 0 mM, (●); 2.5 mM, (△); and 5 mM, (□), and for (*B*) pyrophosphate concentrations: 0 mM (●), 1 mM (△), 2 mM (□). The straight, solid lines are least-squares linear regression fits to the data. Each value of $V_{max}$ is determined from a nonlinear fit of between 24 and 62 (average = 36) force-velocity measurements as described in Methods. Error bars represent 95% confidence limits.

of the maximum velocity and contraction. It is in fact equally surprising that the dependence of the velocity of contraction on MgATP concentration also follows Michaelis-Menten kinetics. Both the data of Cooke and Bialek (1979) and that obtained here can be described by this formalism with Michaelis constant, $K_m$, of 0.25 mM and a $V_{max}$ of 2.1 1/s. Ferenczi et al. (1979) using frog muscle found an approximate fit to Michaelis-Menten kinetics with a $K_m$ of 0.47 mM, and values of 0.2–0.8 mM have been obtained by Kawai (1982) and by Shimizu and Tanaka (1984) for the $K_m$ associated with the response to sinusoidal oscillation. Note that very similar results are obtained in another motile system. The beat frequency of sea urchin sperm flagella decreases as the MgATP concentration is lowered, following Michaelis-Menten type kinetics with $K_m$ = 0.22 mM (Brokaw, 1975). The beat frequency is also competitively inhibited by MgAMPPNP with a $K_i$ of 3.1 mM (Okuno and Brokaw, 1981). The kinetics followed by both substrate and MgAMPPNP as well as the values obtained for $K_m$ and $K_i$ are very similar to the results obtained here. This suggests that basic similarities between the mechanisms of force production in the two systems may exist.

In the case of classical competitive inhibition of enzymatic activity, $K_i$ is equal to the dissociation constant of the ligand from the enzyme site. However, in the case of the inhibition of contraction velocity the connection between the strength of ligand binding and the observed inhibition depends on a number of aspects of the cross-bridge cycle. Although a rigorous discussion of this question requires consideration of a rather complicated model of cross-bridge kinetics, some simple arguments can suggest a connection between the affinity of ligand binding and the inhibition of contraction velocity. We assume that MgATP competes with the ligand at one point in the cross-bridge cycle. This region of competition is most probably a small portion of the powerstroke, and we initially assume it occurs over a region of length 2 nm. However the arguments presented below do not depend strongly on the exact value. If MgAMPPNP binds to a myosin site in this region, then the myosin is dissociated mechanically and will rebind to actin where it again enters the region of nucleotide competition. This assumption can be changed and we consider alternative models following the discussion below. Within the framework of these assumptions we now ask whether the observed inhibition of fiber contraction velocity can be explained if nucelotides bind to the cross-bridge in this region with the same affinities and rates as to actomyosin subfragment-1 in solution. We consider contraction at 25 $\mu$M MgATP in the presence and absence of 5 mM MgAMPPNP. At 25 $\mu$M MgATP the velocity of contraction is experimentally observed to be ten times slower than at saturating MgATP, so that a considerable fraction of nucleotide free A · M states should be available for ligand binding (here A signifies actin and M signifies either myosin or myosin subfragments). The fractions of cross-bridges that bind MgATP or MgAMPPNP are estimated from their relative rates of binding to A · M in solution. MgATP binds to the A · M state with a rate of $\sim 2 \times 10^6$ $M^{-1}s^{-1}$ (Taylor, 1979; Goldman et al., 1984; Kawai, 1982) or at 25 $\mu$M MgATP, with an effective rate constant of 50 $s^{-1}$. In the absence of MgAMPPNP and at 25 $\mu$M MgATP, approximately one-half of the myosin heads are detached by MgATP at the experimentally observed $V_{max}$ of 0.2 lengths/s. The corresponding rate for MgAMPPNP is $1.2 \times 10^4$ $M^{-1}s^{-1}$ (Trybus and Taylor, 1982) or at 5 mM MgAMPPNP with an effective rate constant of 60 $s^{-1}$. Thus whenever an A · M bridge becomes available, MgATP and MgAMPPNP compete almost equally for the myosin nucleotide site. At first this

may seem compatible with an observed twofold inhibition of fiber velocity under these conditions. However, one has to consider the fate of the MgAMPPNP bound to the myosin. If MgAMPPNP is not dissociated from the myosin in another step of the cycle, then the M · AMPPNP complex will be detached from actin mechanically and will rebind to actin returning to the region where MgATP and ligand again compete for the myosin site. Assuming that the affinity constant of $10^4$ $M^{-1}$ for binding of MgAMPPNP to A · M measured by equilibrium methods (Greene and Eisenberg, 1980; Konrad and Goody, 1982) applies at the end of the powerstroke, the rate at which MgAMPPNP dissociates from the A · M · AMPPNP state can be defined. The above model can be easily analyzed to provide the concentrations of the three states within the region of nucleotide binding as a function of position and fiber velocity. MgAMPPNP competes very effectively with MgATP and almost all of the myosin heads (97%) bind to MgAMPPNP. This would cause a dramatic decrease in the contraction velocity. The correct decrease in velocity is predicted if one assumes that the affinity constant of MgAMPPNP for the A · M state is $2 \times 10^2$ $M^{-1}$. With this affinity the rate of MgATP binding is effectively decreased by a factor of two and approximately one-half of the myosin heads bind MgATP and are dissociated at the measured $V_{max}$ of 0.1 lengths/s. An alternative analysis is to determine the rate of MgAMPPNP release from A · M from the data of Konrad and Goody (1982). In the presence of MgATP, the A · M · AMPPNP complex is dissociated at a rate of $1.9 \times 10^4$ $M^{-1}s^{-1}$. At 25 $\mu$M ATP, this yields an effective rate constant of 0.5 $s^{-1}$. At 5 mM AMPPNP, the maximum contraction velocity is 0.1 lengths/s and a cross-bridge traverses the 2-nm region of ligand competition in ~0.02 s. Thus with a rate constant of 0.5 $s^{-1}$, only 1% of the A · M · AMPPNP states are dissociated as a cross-bridge crosses the region of nucleotide competition. Once bound, MgAMPPNP is very difficult to remove given the rate of MgATP binding to A · M · AMPPNP measured in solution, again suggesting a substantially greater inhibition of $V_{max}$ than observed. If the region of competition is extended to 10 nm, the cross-bridge remains in it for five times longer, and the only result is that now 5% of the A · M · AMPPNP states can be dissociated by ATP in a single pass.

Within the framework of the assumptions presented above, we reach the conclusion that the observed inhibition of velocity implies that the affinity of MgAMPPNP for actomyosin must be more than an order of magnitude weaker than its affinity measured by equilibrium methods in solution or in rigor fibers. Note that the affinity of MgAMPPNP for acto S-1 measured by the dependence of the rate of acto S-1 dissociation by MgAMPPNP has about the same magnitude as that required to explain the inhibition of velocity (Konrad and Goody, 1982; Trybus and Taylor, 1982).

Our estimation of the ligand affinity is reached under the assumption that MgATP and ligand compete at one step of the cycle and that bound ligands are not dissociated at other steps in the cycle. The model is not unique and other schemes can explain the data. Specifically if MgAMPPNP is dissociated from A · M at some other step in the cycle, then the twofold inhibition observed at 25 $\mu$M MgATP and 5 mM MgAMPPNP is expected, because their relative rates of binding to A · M are approximately equivalent.

In the above analysis, we considered the ligand MgAMPPNP because more data are available on its interaction with the contractile proteins. However, in many respects $MgPP_i$ and MgAMPPNP are equivalent: their affinities for A · M in solution, and their $K_i$'s for the inhibition of tension or contraction velocity are similar. Thus the discussion of MgAMPPNP may well apply to $MgPP_i$. Additional data on the kinetics of the interaction of $MgPP_i$ with actin and myosin would be required to resolve this question precisely.

The affinity of MgAMPPNP for acto S-1 measured by equilibrium methods in solution would detect the affinity of the acto S-1 state that binds MgAMPPNP most tightly. Our analysis suggests that this state is not the one that occurs at the end of the powerstroke. If the state with highest affinity for MgAMPPNP occurs a few nanometers before the end of the powerstroke, then the binding of MgAMPPNP to rigor muscle would result in effectively lengthening the fiber as experimentally observed. Furthermore, with this assumption it is possible to explain the data obtained with fibers and to resolve ambiguities in the affinity constant for MgAMPPNP to A · M noted in solution studies (Trybus and Taylor, 1982; Konrad and Goody, 1982) within the framework of a single model of the actomyosin-nucleotide interaction. Details of this model will be published elsewhere.

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