Mechanics of glycerinated muscle fibers using nonnucleoside triphosphate substrates

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ABSTRACT We have investigated the ability of the photoaffinity, nonnucleotide ATP analogues, 2-[(4-azido-2-nitrophenyl) amino] ethyl triphosphate (NANTP) and 2-[(4-azido-2-nitrophenyl) amino] propyl triphosphate (PrNANTP), to support active contraction in glycerinated rabbit psoas fibers. At millimolar concentrations, in the absence of calcium, both analogues relaxed fibers. In the presence of calcium, MgNANTP produced isometric tension and stiffness that were one-half to two-thirds the values obtained in MgATP. Maximum shortening velocity and the calcium-activated, myofibrillar catalyzed rate of hydrolysis were approximately the same for MgNANTP as for MgATP. With MgNANTP as the substrate, increasing concentrations of the diphosphate analogue, MgNANDP, inhibited shortening velocity but did not change isometric tension. The addition of increased concentrations of orthophosphate (P_i) decreased tension while shortening velocity increased. Thus, the effects of the hydrolysis products of NANTP were quite similar to those observed previously for ADP and P_i in the presence of MgATP. Taken together, these observations show that MgNANTP binds to, and functions in the active site of myosin in a manner quite analogous to MgATP. Thus, the aryl azido group should serve as a valid photoaffinity label for the purine portion of the active site. In contrast, MgPrNANTP, which differs from MgNANTP only in an extra CH_2 spacer between the nitrophenyl ring and the triphosphate moiety did not support isometric tension or active shortening in the presence of calcium. Fiber stiffness increased in the presence of calcium and MgPrNANTP, with a calcium-activated, myofibrillar MgPrNANTPase which was about half that obtained with MgATP. Thus, in the presence of MgPrNANTP, cross-bridges appeared to be cycling through states that were attached to actin, but not producing force.

INTRODUCTION

The force and motion observed in contracting muscle results from a cyclic interaction between actin, myosin, and the energy source, MgATP. Despite intensive investigation, the mechanism by which this chemical interaction converts chemical energy into mechanical work remains unresolved. One important technique that has been extensively employed in attempting to understand actomyosin biochemistry and mechanics has been the use of ATP analogues. For example, one such analogue (AMPPNP) binds to actomyosin but it is not hydrolyzed (Yount et al., 1971). An alternative approach to modification of the phosphate groups has been modification at either the base or ribose portion of ATP. The use of such analogues has allowed the stabilization of possible intermediate states in the cross-bridge cycle, and when coupled with structural and mechanical studies of fibers, has given insight into the working powerstroke of cross-bridge states (see review by Cooke, 1986).

Nakamaye et al. (1985) have recently synthesized an entirely new class of nonnucleotide ATP photoaffinity analogues represented by 2-[(4-azido-2-nitrophenyl) amino] ethyl triphosphate (NANTP) and as presented here, the new homologue, 2-[(4-azido-2-nitrophenyl) amino] propyl triphosphate (PrNANTP). The structures given in Fig. 1 show these analogues lack both a purine ring and ribose moiety, and differ dramatically from ATP. Nonetheless, MgNANTP is hydrolyzed by myosin and by actomyosin in solution at a rate and with a metal ion dependence similar to ATP (Nakamaye et al., 1985). The subunit compositions of the active site of skeletal (Nakamaye et al., 1985) and smooth muscle myosin (Okamoto et al., 1986) have been determined by the use of NANTP as a photoaffinity label. In addition, Trp-130 has been shown to be part of the active site in skeletal muscle (Okamoto and Yount, 1985). These studies rest upon the assumption that MgNANTP binds to, and fucntions in, the active site in a manner analogous to MgATP. The most convincing way to verify proper steric binding in the active site of myosin would be to demonstrate that the mechanical properties of muscle fibers contracting in the presence of MgNANTP are similar to those in the presence of MgATP. Additionally, given the distinct structural differences between NANTP, PrNANTP, and ATP, a comparison of results with the three substrates can provide additional insights on the three-dimensional structure of the myosin nucleotide binding site.

Despite the obvious chemical differences, we show here the MgNANTP and MgATP produce remarkably similar mechanical behavior in active, chemically skinned, rabbit psoas fibers. In addition their responses to the diphosphate and orthophosphate hydrolysis products

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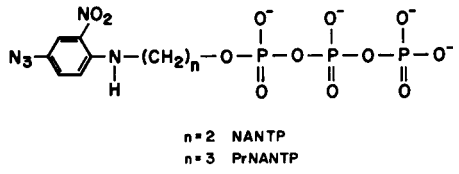


FIGURE 1 Structures of NANTP and PrNANTP.

are also very similar suggesting that the steps involved in product release are closely related. The homologue, PrNANTP, has an extra CH_2 group between the nitrophenyl ring and the triphosphate group, and thus is only ~1.5 Å longer than NANTP. We find that with this very minor modification, PrNANTP does not support active tension or shortening, despite the fact that stiffness measurements suggest cross-bridge attachment to actin in the presence of calcium. These results point to the exquisite requirements for a substrate to support tension development and suggest that PrNANTP may be used to stabilize new intermediates in the cross-bridge cycle, whose structure can then be determined.

METHODS

Thin strips of psoas muscle were dissected from a rabbit, tied to sticks at body length, chemically skinned, and stored in glycerol as described previously (Cooke et al., 1988). For mechanical measurements, single fibers were dissected and mounted in a well between a solid-state force transducer (Akers 801, Horten, Norway) and a rapid motor (General Scanning, Watertown, MA) for changing muscle length during isotonic releases. Fingernail polish (Wet 'n Wild, Color no. 453, Pavion Ltd., Nyack-on-Hudson, NY) diluted 1:10 with acetone was used as glue. Individual, mounted fibers were 3–4 mm in length. For experiments involving NANTP or PrNANTP, the fiber was then immersed for 2 min in a rigor buffer containing 0.16 M KAc, 7 mM $MgAc_2$, 1 mM EGTA, 40 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.0 in order to wash out any residual ATP present in the glycerinated fibers. Except as noted below and in Results, the solution in the well was then exchanged for a relaxing buffer similar to the above rigor buffer, which in addition contained 0.1 mM P^1, P^5 diadenosine pentaphosphate along with varying amounts of K_2HPO_4, KH_2PO_4, ATP, creatine phosphate, NANTP, NANDP, and PrNANTP, with KAc concentration (~0.13–0.15 M) adjusted to maintain a constant ionic strength of 195 mM. For experiments involving NANDP, $MgAc_2$ concentration was increased to 12 mM. For experiments examining whether NANTP or the magnesium complex is the true substrate, the "magnesium-free" experimental buffer contained 100 mM KAc, 40 mM MOPS, 10 mM EDTA, 10 mM $CaCl_2$, 0 mM $MgAc_2$, pH 7, with either 250 μM ATP or 250 μM NANTP.

NANTP was prepared by the method of Nakamaye et al. (1985). PrNANTP was prepared in a similar manner except 3-amino propanol replaced ethanolamine in the first step of the synthesis. The di- and triphosphate NAN analogues were assumed to have H^+ and metal ion binding constants identical to ADP and ATP. Except for the diphosphate analogue, binding constants were taken from Chase and Kushmerick (1988). The Naperian logarithms of the binding constants for NANDP were taken as follows: [MgNANDP]/[Mg][NANDP] = 3.2, [CaNANDP]/[Ca][NANDP] = 2.8, [MgHNANDP]/[Mg] · [HNANDP] = 1.5, [KNANDP]/[K][NANDP] = 0.7, [HNANDP]/[H][NANDP] = 6.6, [H_2NANDP]/[H][HNANDP] = 4.2 (Smith and Martell, 1975). Concentrations of the various ionic species necessary to calculate final ionic strength were determined by solving the full nonlinear system of balance equations by a Newton iteration technique. For experiments involving active contractions, the fiber was subsequently activated by addition of $CaCl_2$ to the well in which the fiber rested (~1 mM) until maximal isometric tension was obtained. At all steps, the solution bathing the fiber was gently stirred by taking up and ejecting into the well ~10 μl of buffer through a micropipette with diaphragm connected to a loudspeaker coil vibrating at a frequency of 2 Hz. All mechanical experiments were conducted at 10°C in subdued light.

Isometric tension was normalized with respect to fiber area by measuring the fiber diameter at five to seven locations along the fiber at 100× magnification, averaging the values, and assuming the fiber to be cylindrical. For force-velocity data, the fiber was released at a preset constant fraction of isometric tension for 40 ms by a computer-controlled feedback loop between the force transducer and motor arm. To correct for possible drift in the force transducer, the fiber was then rapidly shortened, allowing it to go slack, so that the transducer output at a true zero in force could be established. The fiber was subsequently rapidly restretched to its original length. Velocity was determined by a least-square linear fit to the position of the motor arm during the period 10–40 ms after initial release. As has been noted previously, there is deterioration in mechanical reproducibility of chemically skinned fibers as a function of time (Podolsky and Teichholz, 1970). We found that only the first three to five releases of a given fiber could be reliably used in data analysis. In experiments not involving PrNANTP, isometric stiffness was determined from the peak-to-peak tensions resulting from applying a 0.5% (peak-to-peak) sinusoidal oscillation of the muscle length at a frequency of 1,000 Hz. Because fibers were found not to contract actively in PrNANTP, stiffness in the presence of PrNANTP was determined from the change in tension resulting from a rapid (0.5 ms), 0.5% extension of fiber length. The mechanical apparatus and other experimental protocols used were as described previously (Cooke et al. 1988; Pate and Cooke, 1989).

Force-velocity data were fit to the Hill equation (Hill, 1938),

$$V = \frac{V_{max}\alpha(1 - P/P_o)}{(\alpha + P/P_o)}, \qquad (1)$$

on a model PB286 computer (Packard Bell Electronics, Woodland Hills, CA) running the MS-DOS operating system (Release 3.3, Microsoft Corporation, Redmond, WA) and using the nonlinear least-squares routine NLIN in the Statistical Analysis Subroutines package (Release 6.03, SAS Institute, Research Triangle Park, NC). A grid search for starting values was employed in an attempt to insure convergence to the global minimum sum of squared residuals. With the exception of experiments involving MgPrNANTP and magnesium-free EDTA buffers (no active shortening was observed in either case), each fit used a minimum of 20 force-velocity data points. The extrapolated velocity at zero tension was taken as V_{max}. All other data are given as mean ± SEM.

For some control experiments at 4 mM ligand concentration, it was desired to examine the mechanical properties of a single fiber, first in the absence, and then in the presence of either 15 mM P_i or 20 mM creatine phosphate, or similarly first at the standard ionic strength of 195 mM and then subsequently at either μ = 225 mM or μ = 165 mM. In these experiments, after activation, isometric tension was determined and the fiber allowed to shorten isotonically at a low load (5 or 10% P_o). P_i or creatine phosphate concentrations were raised by adding an aliquot of a buffer as above, containing 4 mM MgATP or 4

mM MgNANTP, but with KAc replaced by either 52 mM creatine phosphate or 67 mM P_i (μ = 195 mM). In other experiments, ionic strength was lowered to 165 mM by addition of an aliquot of an activating buffer that lacked KAc, or raised to 225 mM by addition of an aliquot of buffer containing 1 M KAc, 40 mM MOPS, 1 mM EGTA, 1 mM $CaCl_2$, pH 7. Isometric tension was again determined and the fiber allowed to shorten isotonically at the prior load. This protocol allows each fiber to serve as its own control in detecting any small changes in tension, while avoiding the mechanical perturbations to the fiber which would result if the mounted fiber were pulled through a surface meniscus during the change of solutions. More importantly, the use of low release loads, with each fiber serving as its own control, is a more accurate way of detecting small changes in shortening velocity than extrapolation to zero load using the Hill fit (Cooke et al., 1988; Pate and Cooke, 1989). We note that the protocol for changing P_i or creatine phosphate concentration resulted in a calculated change in ionic strength and substrate concentration of <1%. The protocol for raising ionic strength resulted in a calculated 3% change in substrate concentration; the protocol for lowering ionic strength resulted in less than a 1% change. No effort was made to adjust for these small changes. After each addition changing the concentration of P_i, creatine phosphate, or ionic strength, an additional small amount of $CaCl_2$ was titrated into the well to insure that the fiber remained maximally activated.

Myofibrils used to determine MgATPase, MgNANTPase, and MgPrNANTPase rates were obtained using a modification of the method of Etlinger et al. (1976) as described in Cooke and Franks (1980). Myofibrils were diluted to 140 μg/ml in the appropriate calcium containing buffer, identical to that used for the corresponding mechanical measurements. Phosphate (P_i) generated was determined using the malachite green colorimetric assay (Komada et al., 1986) at 0, 1, and 2 min. after initiation of the experiment. A least-squares, linear fit to [P_i] as a function of time was taken to give the hydrolysis rate, normalized assuming 45% of the myofibrillar protein was myosin (Cooke and Franks, 1980). The rate of hydrolysis was measured at room temperature (~20°C) in subdued light.

RESULTS

The abilities of MgNANTP and MgATP to generate isometric tension in glycerinated rabbit psoas fibers are compared in Fig. 2. After mounting between the force transducer and motor arm, the fiber was immersed in an ATP-free rigor buffer and washed for 30 s to remove residual ATP. The solution was then exchanged for one containing 4 mM MgNANTP as described in Methods, relaxing the fiber. Addition of calcium resulted in a rise of isometric tension. Subsequent activation in 4 mM MgATP gave a value for P_o of 0.17 N/mm^2. This value is close to ones previously obtained by us and others using slightly differing conditions (Kawai, 1986; Chase and Kushmerick, 1988; Pate and Cooke, 1989), indicating that the decreased active tension observed in the presence of MgNANTP is not simply the result of irreversible damage by the photoaffinity analogue. Tension, stiffness, and shortening velocities with MgATP or MgNANTP as substrate are summarized in Table 1. For data presented in this table, each fiber was activated only one time in either MgNANTP or MgATP to

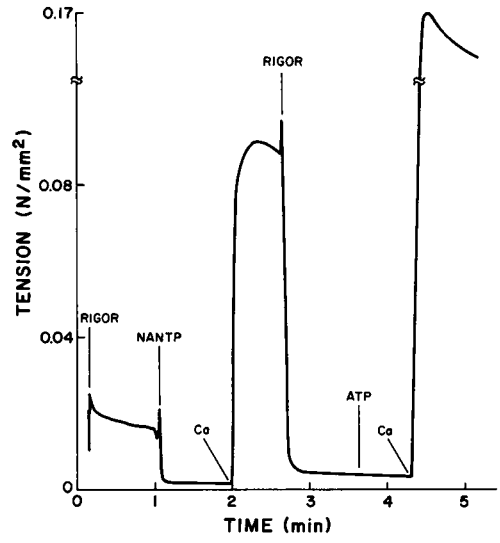


FIGURE 2 Tension as a function of time for a fiber activated first in MgNANTP and subsequently in MgATP. The fiber was first washed in a nucleotide-free rigor buffer, then relaxed in a buffer containing 4 mM MgNANTP. After activation by addition of Ca^{2+}, an isometric tension of 0.09 N/mm^2 was obtained. MgNANTP was subsequently washed out using rigor buffer. Immersion in buffer containing 4 mM MgATP relaxed the fiber. After addition of Ca^{2+}, normal isometric tension of 0.17 N/mm^2 was obtained. Solution changes/additions were as noted.

minimize the mechanical degradation with time observed in glycerinated fibers. As the table shows, 4 mM MgNANTP relaxes fibers, though not quite as well as 4 mM MgATP, while producing ~50%–60% of the active tension and the active sinusoidal stiffness. When the substrate concentration is lowered to 250 μM, the relative abilities of MgATP and MgNANTP to support concentration are similar to those obtained at higher substrate concentrations.

Fig. 3 shows the results of experiments involving isotonic releases of single fibers. Fig. 3 *A* plots the position of the motor arm to which one end of the fiber is attached as a function of time after release to various tensions (Fig. 3 *B*), along with least-squares, linear fits to the position data, with the slope taken as the velocity of shortening. MgNANTP concentration was again 4 mM. Position traces show a slight downward concavity indicating a progressive slowing of the fiber as has been observed previously for glycerinated fibers activated in MgATP (Brenner, 1986; Julian et al., 1986; Cooke et al., 1988). Fig. 3 *C* shows force-velocity data long with the nonlinear, least-squares Hill fits at 4 mM MgNANTP (*lower plot*) and 4 mM MgATP (*upper plot*). To facilitate display, data are normalized with respect to isometric tension in 4 mM MgATP; thus, the lower tensions at zero velocity are for fibers in 4 mM MgNANTP. Extrap-

TABLE 1 **Mechanical parameters of contraction in the presence of ATP, NANTP, and PrNANTP**

	MgATP	MgNANTP	MgATP	MgNANTP	MgPrNANTP
	4 mM	4 mM	250 μM	250 μM	4 mM
Relaxed tension (N/mm^2)	0.004 ± 0.0006 (9)	0.011 ± 0.003 (12)	0.01 ± 0.003 (7)	0.005 ± 0.002 (4)	0.004 ± 0.001 (2)
Active tension (N/mm^2)	0.19 ± 0.03 (9)	0.12 ± 0.02 (13)	0.18 ± 0.03 (7)	0.15 ± 0.03 (4)	0.01 ± 0.003 (4)
Relaxed stiffness (N/mm^2)	0.41 ± 0.05 (5)	0.78 ± 0.09 (4)	0.69 ± 0.19 (4)	0.46 ± 0.09 (3)	0.30 ± 0.10 (2)
Active stiffness (N/mm^2)	9.6 ± 2.4 (4)	5.6 ± 0.7 (4)	12.0 ± 1.4 (4)	6.5 ± 1.0 (4)	2.1 ± 0.2 (4)
V_{max} (1/s)	1.24 ± 0.07 (44)	1.31 ± 0.07 (46)	0.41 ± 0.04 (28)	0.38 ± 0.07 (20)	0.0 ± 0.0 (8)

Active and relaxed tension and stiffness along with V_{max} for psoas fibers in the presence of MgATP, MgNANTP, and MgPrNANTP. Values given as mean ± SEM. Number of data points in parenthesis.

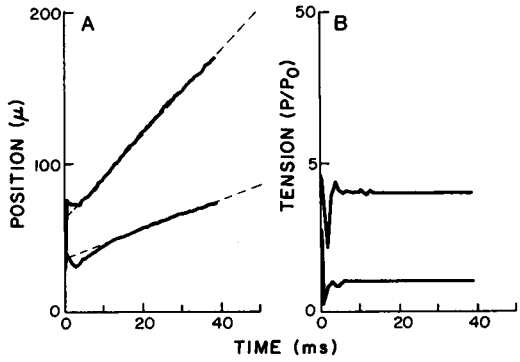
olation to zero force gives values for V_{max} of 1.31 ± 0.07 and 1.24 ± 0.07 muscle lengths/seconds, respectively. Analysis of variance for the nonlinear model (Eq. 1) with NANTP and ATP induced tensions normalized to their respective isometric values, indicates no difference in the two force-velocity data sets at the 0.05 level. When substrate concentration is lowered to 250 μM, V_{max} decreases by approximately two-thirds for both MgNANTP and MgATP. Analysis of variance for the nonlinear model again indicated no difference in the data sets at the 0.05 level. No active tension or shortening was observed for fibers activated in a buffer containing ATP or NANTP but lacking magnesium. Thus, just as is the case for ATP, it is the metal complex of NANTP which is the true chemomechanical substrate.

As indicated in Methods, the binding constants of H^+ and Mg^{++} for NANTP and PrNANTP are not known. In calculating ionic strengths we have assumed them to be identical to those for ATP. The mechanics of glycerinated fibers using MgATP as substrate are known to be weakly dependent upon small changes in ionic strength. This raises the possibility that if the mechanics of glycerinated fibers using MgNANTP as substrate are exceptionally sensitive to ionic strength, the differences we observe may simply be due to inaccuracies in calculating ionic strength. Table 2 gives values of isometric tension and shortening velocity at 10% P_o for μ = 165 and 225 mM, relative to those obtained at the standard ionic strength of 195 mM. Values were determined using the control protocols described in Methods. The data indicate that altering ionic strength by 30 mM causes only minor (<8%) changes in either P_o or shortening velocity. Although data are presented at only one release load, less extensive analyses at other loads also indicate no major changes. This suggests that most, if not all, of the mechanical differences between MgATP and MgNANTP are the result of substrate differences, not ionic strength sensitivity.

Fig. 4 shows results from a fiber activated in 4 mM MgPrNANTP, followed by subsequent buffer exchange and activation of the same fiber in 4 mM MgATP. The protocol is similar to that in Fig. 2, with solution changes and additions indicated. As is evident, the fiber relaxes in MgPrNANTP in the absence of calcium. Unlike the case for MgNANTP, however, little active tension is generated, nor does the fiber actively shorten upon addition of calcium. Surprisingly, fiber stiffness does increase upon calcium activation from a resting value comparable to that observed in 4 mM MgATP to a value approximately one-quarter that observed in fibers activated in 4 mM MgATP (Table 1). Subsequent activation of the fiber in 4 mM MgATP results in active tension and shortening velocities that are equal to control fibers, indicating that the effects seen in MgPrNANTP do not result from irreversible, nonspecific damage to the muscle fiber.

Calcium activated myofibrillar hydrolysis rates were also determined at 250 μM MgATP, MgNANTP, or MgPrNANTP. Values obtained were 1.10 ± 0.05 (3 obs), 0.98 ± 0.01 (3 obs), and 0.44 ± 0.12 (3 obs) triphosphate hydrolyzed/myosin head/second, respectively. The MgATP control values are similar to ones we have previously obtained using slightly different conditions (Cooke and Pate, 1985).

Previous work with skinned rabbit psoas fibers has indicated that varying concentrations of the products of MgATP hydrolysis also modify P_o and V_{max}, providing information on various kinetic steps in the contractile cycle. Hence, we investigated the effects of varying concentrations of NANDP and P_i on the contraction of



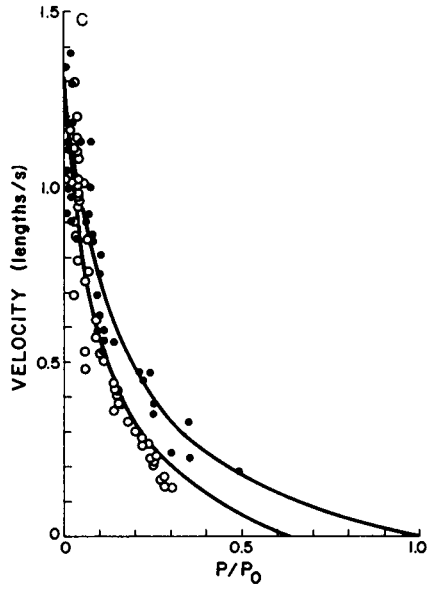


FIGURE 3 (*A*) Length and (*B*) tension of a single fiber as a function of time for a series of two isotonic releases. Traces in *A* show the position of the motor arm attached to the fiber. The dashed lines are least-squares fits to position data taken over the period 10–40 ms. Traces in *B* show tension as a function of time, normalized to isometric tension. The displacement trace with the greater slope (velocity) corresponds to the lower release tension. The fiber was activated and the two isotonic releases were made within 20 s. After release, the fiber was reextended at a velocity of 1 mm/s. Traces from a single fiber, length = 3.1 mm, 4 mM MgNANTP. (*C*) Shortening velocity plotted as a function of tension for 4 mM MgATP (●) and 4 mM MgNANTP (○) from a series of experiments as in *A* and *B*. Solid lines are least-squares Hill fits (Eq. 1) as described in Methods. Tensions are normalized with respect to the isometric value at 4 mM MgATP.

psoas fibers in 4 mM MgNANTP. Results are summarized in Table 3. The presence of 15 mM P_i caused a 30% decrease in P_o, whereas there was a 5% decrease in isometric tension in the presence of 4 mM added NANDP (2.8 mM MgNANDP). Addition of 4 mM NANDP decreased V_{max} by ~30%. Addition of 15 mM P_i increased V_{max} by 34%. Force-velocity curves in the

TABLE 2 Isometric tension and shortening velocity as a function of ionic strength

Ionic strength	165 mM	225 mM
Relative tension	1.05 ± 0.03	0.93 ± 0.03
Relative velocity	0.93 ± 0.03	1.01 ± 0.02

Isometric tension and shortening velocity at 10% P_o at 165 and 225 mM calculated ionic strength, 4 mM MgNANTP. Mean ± SEM (4–6 observations) are given relative to those obtained at μ = 195 mM (0.12 N/mm^2 and 0.77 lengths/s, respectively) as described in the text.

presence of increased concentrations of P_i or NANDP are shown in Fig. 5. All observed differences are significant at the 0.05 level, with the exception of the change in P_o with NANDP (*t*-test or analysis of variance of the force-velocity data using the model of Eq. 1).

DISCUSSION

This paper reports the rather remarkable observation that a molecule, NANTP, which lacks both ribose and a

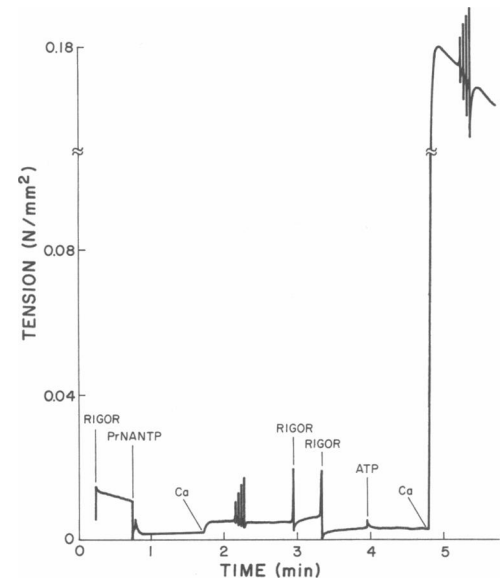


FIGURE 4 Tension as a function of time for a fiber activated first in MgPrNANTP and subsequently in MgATP. The fiber was first washed in a nucleotide-free rigor buffer, then relaxed by addition of 4 mM MgPrNANTP. After activation by addition of Ca^{2+}, no rise in isometric tension was obtained. The transient deviations in the plot were attempts at isotonic releases. MgPrNANTP was subsequently washed out by exchanging the solution for rigor buffer. Addition of 4 mM MgATP relaxed the fiber. After addition of Ca^{2+}, normal isometric tension of 0.16 N/mm^2 was obtained, with subsequent transients showing normal shortening velocities of 0.8, 1.0, and 1.2 muscle lengths/s for isotonic releases of 10, 5, and 1% P_o, respectively. Solution changes/additions were as noted.

TABLE 3 **Effects of NANDP and P_i on tension and V_{max} in 4 mM MgNANTP**

	4 mM NANDP	15 mM P_i
Tension (N/mm^2)	0.11 ± 0.02 (4)	0.08 ± 0.03 (6)
V_{max} (1/s)	0.88 ± 0.03 (25)	1.75 ± 0.05 (43)

Tension and V_{max} in elevated concentrations of P_i and NANDP. The numbers in parentheses for tension row are the number of observations; V_{max} are the number of data points used in the least-squares fit to the Hill Eq. 1.

nitrogen base supports contraction in muscle fibers to almost the same extent as does ATP. The analogue shown in Fig. 1 consists of a substituted phenyl ring, which replaces the adenine base, connected via a spacer side chain to the triphosphate. The length of the spacer group is such that it is similar to the distance spanned by the ribose of ATP. Thus, the phenyl ring is located in approximately the same position as the adenine ring structure of ATP. In spite of the fact that this molecule has only a vague resemblance to ATP, it functions quite well in supporting both relaxation, and active tension in muscle fibers, achieving a maximum shortening velocity in active fibers that is experimentally identical to that achieved by ATP.

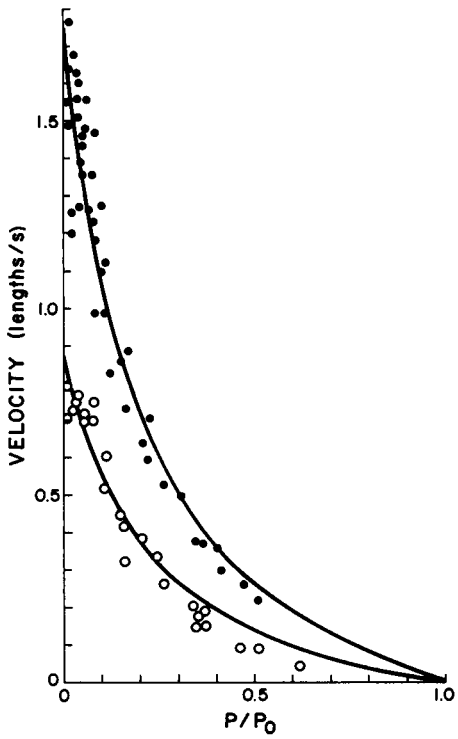


FIGURE 5 Contraction velocity plotted as a function of tension normalized to the isometric value using buffers containing 4 mM MgNANTP and either 4 mM NANDP (○) or 15 mM Pi (●). Solid lines are least-squares Hill fits (Eq. 1) as described in Methods. Values of isometric tensions and maximum shortening velocity are given in Table 3.

The properties observed for NANTP cannot be due to small levels of contaminating ATP. ATP is not a precursor in the synthesis of the analogue and thus cannot be a contaminant. Nor could significant levels of ATP be brought in by the fibers. Fibers were washed in a rigor solution, and they showed no ability to shorten or generate force before NANTP was added to the solution. Upon addition of the MgNANTP plus Ca^{++}, tension developed rapidly and at the same rate as attained by ATP. Thus, one is led to the conclusion that the analogue does support almost normal function in muscle fibers.

Experiments in which the levels of substrate or products were varied suggest that a number of steps in the contractile cycle are very similar in the presence of ATP or NANTP. As the concentration of ATP is lowered the maximum shortening velocity of glycerinated rabbit psoas muscle decreases with a K_m for half maximal velocity of 150 μM MgATP (Cooke and Pate, 1985). At low levels of substrate, the value of V_{max} is determined by the rate at which substrate binds to a myosin head at the end of the cross-bridge powerstroke, dissociating the head from actin (Cooke and Bialek, 1979; Pate and Cooke, 1989). We found that the maximum shortening velocity measured at low substrate concentration was inhibited to the same extent for ATP and NANTP, suggesting that the rates of binding of NANTP and ATP to myosin at the end of the powerstroke are similar.

The ability of the hydrolysis product, NANDP, to bind to myosin and inhibit V_{max} was also similar to the inhibition of V_{max} by ADP in the presence of ATP (Cooke and Pate, 1985) again suggesting similar affinities of the products. Addition of NANDP to fibers activated in MgNANTP does not increase tension. This is different from previous observations with ATP and ADP, where ADP has been shown to potentiate isometric tension by 20% under comparable conditions, probably due to the inhibition of the release of cross-bridges from force producing states within the powerstroke (Abbot and Mannherz, 1970; Cooke and Pate, 1985). This is the only effect we have found to be qualitatively different from that observed with ATP, and suggests that MgNANDP is a better analogue of ADP when binding to states at the end of the powerstroke than it is when binding to states within the powerstroke, thus providing additional evidence for strain dependent association rates for binding of nucleotides to myosin.

Addition of P_i to active fibers inhibits tension, presumably by reversing steps that occur near the beginning of the powerstroke (Hibberd et al., 1985; Pate and Cooke, 1985). Cross-bridges are transferred from myosin·ac-

tin·ADP states that produce force to weakly-bound myosin·actin·ADP·P_i states that produce little force. The extent of force inhibition is dependent on the populations of these states that occur at the beginning of the powerstroke. The inhibition by P_i observed in the presence of NANTP was similar to that observed by ourselves and others with MgATP as substrate (Herzig et al., 1981; Hibbard et al., 1985; Kawai, 1986; Kentish, 1986; Webb et al., 1986; Nosek et al., 1987; Brozovich et al., 1988; Chase and Kushmerick, 1988; Pate and Cooke, 1989), suggesting that the population and transition rates between these states is similar for the two substrates. Thus, a number of transitions at both the beginning and end of the powerstroke appear to be similar for these two substrates.

We have previously observed that increased [P_i] increases shortening velocity at physiological MgATP concentration (Pate and Cooke, 1989). The magnitude of the increase (10%) was less than that observed in the present experiments using MgNANTP as substrate. To assess whether this effect was due to some nonspecific action of P_i, several additional controls were performed. To avoid the error introduced by extrapolation of the Hill fit (Eq. 1) to zero velocity, fiber velocities were compared at a single low tension using protocols discussed in Methods. Single fibers were allowed to shorten at 5% P_o with no added P_i and then subsequently at 15 mM P_i using MgNANTP as substrate. These experiments gave a ratio of velocities at 5% P_o in the presence and absence of P_i of 1.29 ± 0.03 (4 obs), with values well within the scatter envelope of the data in Figs. 4 and 5. To separate a NANTP effect from an ATP effect, the same protocol was employed with 4 mM MgATP as substrate. The ratio of shortening velocities was 1.25 ± 0.04 (11 obs), indicating the same result with the low substrates. The possibility that the effect of P_i was nonspecific and resulted from the replacement of monovalent acetate anions used to maintain ionic strength with divalent HPO_4^{2-} anions was considered. Control protocols (Methods) gave a ratio of velocities at 5% P_o in the absence and presence of the divalent ion, creatine phosphate (20 mM), of 1.00 ± 0.01 (3 obs) suggesting the substitution of divalent for monovalent anions was not the cause of the increase in shortening velocity. NANTP is not a substrate for creatine phosphokinase (Nakamaye et al., 1985). Thus, we conclude that the increase in shortening velocity is a result of the inclusion of P_i in the experimental buffers, and that the magnitude of this effect is more variable than previously reported.

The ability of NANTP to support contraction is rather surprising in light of our knowledge of the nature of protein nucleotide interactions. Most enzymatic systems have a relatively high degree of specificity for one type of nucleotide, and the actomyosin, nucleotide chemomechanical interaction is no exception. Although CTP supports active tension and shortening in muscle fibers comparable to that of ATP, the other nucleotides ITP, UTP, and GTP do not function well (Pate and Cooke, 1988). GTP generates only ~10% of the tension and shortening velocity achieved by ATP. ITP, in which the only change is a substitution of a carbonyl group for the amino group of adenine of ATP also supports only 10% of the force and velocity of contraction observed for ATP. These results suggest that interaction occurring at the adenine 6-amino group of ATP are critical for the optimal development of force and shortening velocity. The fact that NANTP, which lacks a comparable amino group still supports contraction shows that these interactions are less important than suggested by the results obtained with ITP and GTP.

The addition of a single CH_2 group to the hydrocarbon chain of NANTP to produce PrNANTP induced a very different behavior in the fibers. Although PrNANTP was capable of relaxing fibers, it did not support active tension in the presence of calcium. Surprisingly, the rise in stiffness indicated that cross-bridges were attached to actin. The rate of hydrolysis for this analogue (44% of ATP) showed that it cycled through a series of steps which have some similarity to those traversed by ATP. The series of steps, however, does not contain those states which generate force in the presence of ATP or NANTP. One function of the study of nucleotide analogues, and a long sought goal of the field of muscle contraction, is to isolate different states in the contractile cycle so that they can be studied by structural methods. It would appear that the states produced by PrNANTP are excellent candidates for structural studies in that cross-bridges are attached to actin in a manner which does not lead to force production.

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