MUSCLE CROSS-BRIDGE KINETICS IN RIGOR AND IN THE PRESENCE OF ATP ANALOGUES

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ABSTRACT Recently we reported preliminary mechanical experiments on freshly skinned rabbit psoas fibers that suggested that while almost all of the cross-bridges are attached to actin in the presence of 4 mM adenyl-5'-yl-imidodiphosphate (AMP-PNP) (ionic strength, 0.13 M), there is an equilibrium between the attached and detached states, so that, in the presence of 4 mM AMP-PNP, fibers should not be able to maintain tension (Schoenberg, et al., 1984, *in* Contractile Mechanisms in Muscle, Pollack and Sugi, editors., Plenum Publishing Corp., NY). Since this suggestion was at variance with published results of Clarke and Tregear (1982, *FEBS [Fed. Eur. Biochem. Soc.] Lett.* 143:217), we reinvestigated the ability of rabbit psoas fibers to support tension following a 2-nm stretch in rigor and in the presence of the nucleotide analogues, PPi and AMP-PNP, for analogue concentrations ranging from 0.25 to 4 mM. We find that, whereas in rigor there is very little tension decay following a stretch, in 4 mM nucleotide analogue solution, the force generated by stretch quickly decays to zero. The force decay is not exponential; rather, it can be described by rate constants that range from ~0.1 to 100 s^{-1} in 4 mM PPi, and 0.01 to 10 s^{-1} in 4 mM AMP-PNP. This large range of decay rate constants may be partially related to the dependence of either analogue binding or cross-bridge dissociation upon strain, since we find that the rate constants for force decay decrease with decreasing size of stretch or with decrease of analogue concentration below the maximum studied (4 mM). In general the results are consistent with an equilibrium model for cross-bridge binding to actin, where the rate constants for cross-bridge detachment determine the rate of tension decay.

INTRODUCTION

Recently we showed that cross-bridges with ATP bound to the active site can bind to actin in a relaxed muscle (Brenner et al., 1982; Schoenberg et al., 1984). We found that at low ionic strength, where large numbers of ATP-bound cross-bridges are attached in the absence of Ca^{2+}, the apparent stiffness depends upon the velocity of stretch with which the stiffness is measured. We postulated that this type of behavior was due to a rapid equilibrium attachment and detachment of cross-bridges. With rapid stretches, all the attached cross-bridges would be strained and the muscle would appear stiff. With slow or moderate stretches, cross-bridges would detach before being strained significantly and they would, on average, reattach to neighboring actins in positions of less strain so that the muscle would not appear as stiff as when stiffness was measured with a rapid stretch.

We also made observations on the apparent stiffness of fibers where ATP had been replaced with adenyl-5'-yl-imidodiphosphate (AMP-PNP) or pyrophosphate (PPi) (Schoenberg et al., 1984; Brenner, B., Chalovich, J. M., Greene, L.E., Eisenberg, E., and Schoenberg, M., submitted for publication). The behavior of the fibers in those experiments was quite similar to, although on a slower time scale than, the behavior in ATP solution, and tended to support the idea of equilibrium attachment and detachment of cross-bridges in AMP-PNP and PPi solution just as in ATP solution. However, one of the major predictions of our model for the equilibrium attachment of cross-bridges (see Schoenberg, 1985), is that any force induced by stretch or other means should decay to zero in times reflecting the cross-bridge detachment rate constants. This prediction seemed to be questioned by a number of reports in the literature (White, 1970; Kuhn, 1978; Clarke and Tregear, 1982), suggesting that in the presence of AMP-PNP or PPi, some cross-bridge tension could persist for exceedingly long times. For this reason, we decided to reinvestigate the question of the decay of force following a rapid stretch of a freshly skinned rabbit psoas fiber bathed in PPi, AMP-PNP, or rigor solution.

In contrast with previous reports in the literature, we find nearly complete relaxation of tension within tens of seconds in the presence of 4 mM MgPPi, and within minutes in the presence of 4 mM MgAMP-PNP. These results support our equilibrium cross-bridge model and are opposed to models where some of the cross-bridges turn over very slowly or not at all (Kuhn, 1978; Clarke and Tregear, 1982).

The force decay following stretch does not occur with a single exponential. Rather, it can be explained by cross-bridges detaching with rate constants ranging from ~0.1 to

100 s^{-1} in the presence of 4 mM PPi and ~0.01 to 10 s^{-1} in the presence of 4 mM AMP-PNP. However, since the rate constants we measured depend upon nucleotide analogue concentration over the entire range of concentrations studied, the detachment rate constant we measure must involve an analogue binding step in addition to the actual cross-bridge dissociation step. For this reason, the rate constants for the actual dissociation step will be even faster than the rate constants observed.

METHODS

Fiber Preparation

3–4-kg rabbits were anaesthetized with 250 mg of ketamine hydrochloride, exsanguinated, and the lateral edge of the psoas muscle was removed, stretched 20%, and pinned to a Sylgard foundation in an acrylic chamber filled with dissecting solution. The dissecting solution (after Eastwood et al., 1979) contained 150 mM K propionate, 3 mM Mg acetate, 3 mM Na_2ATP, 5 mM EGTA, 0.5 mM dithiothreitol (DTT), and 5 mM KH_2PO_4, pH 6.8 at 5°C. Shortly after the fiber bundle was pinned, two cuts, separated by 6–12 mm were made along the lateral edge and a single fiber was seized at the distal cut edge and slid out of the bundle by pulling in a direction parallel to the bundle. The fiber was transferred in a dish to the experimental chamber, and each end was pressed upon small carbon fibers that extended from the force transducer and displacement generator. Before permanent attachment, the fiber was rotated about its axis to measure, under 50 × magnification, the maximum and minimum diameters. After the diameter measurement, the experimental chamber was lowered, briefly exposing the muscle fiber and transducer tips to air, and the fiber was glued in place with cyanoacrylic glue (Histoacryl, B. Braun Melsungen, Federal Republic of Germany or CA-3, 3M Co., St. Paul, MN). Fibers with evidence of twisting or runoff of glue onto the fiber were rejected. After mounting, sarcomere length was adjusted to 2.6 μm. All experiments were performed at 5°C.

Experimental Solutions and Procedure

The experimental solutions are shown in Table I. Na_2ATP, Li_4AMP-PNP, and DTT were obtained from Sigma Chemical Co., St Louis, MO. EGTA and imidazole were obtained from Eastman Kodak Co. (Rochester, MN). All chemicals were analytic grade or purer. In making 4 mM PPi solution, the $MgCl_2$ was always added last, drop by drop, to stirred solution to avoid precipitation. Solutions containing intermediate amounts of analogue were made from an appropriate mixture of 4 mM solution and diluent.

The muscle was almost always put into rigor before exposure to nucleotide analogue solution. This was accomplished by lowering the temperature to 0°C, and then washing through 30 ml of cold quick rinse solution followed by 10 ml of cold rigor solution. This technique allowed induction of rigor with little tension development (<2–5 dyn), little sarcomere length shortening (<0.5%), and little disturbance to the diffraction pattern. Dye studies showed that after 30 ml of rinse, the amount of original solution remaining in the experimental chamber was <1 part in 10^4, suggesting that the residual ATP contaminating the rigor solution should be $\ll 1$ μM (see Results). At the end of the experiment, the maximum isometric tension, P_0, was measured at 5°C, in a solution containing 80 mM potassium propionate, 3 mM CaEGTA, 2 mM $MgCl_2$, 3 mM MgATP, 10 mM phosphocreatine, 300 Sigma units of creatine phosphokinase, and 10 mM imidazole, pH 7.

Transducers and Displacement Generator

The force transducer was a modified Akers gauge transducer (Aksjelskapet Mikroelectronikk, Horten, Norway). It was first shortened as 2 mm were ground from the distal end of the transducer and then extended as a 6 mm carbon-epoxy fiber extension was epoxied in place. The force transducer was shortened to increase the frequency response and later extended to increase the sensitivity and decrease the thermal drift when the transducer tip was submerged in water. The brass housing and the active elements of the force transducer were pretreated with SS4004 primer and coated with RTV 60 silicone rubber compound (General Electric, Waterford, NY) to make them impervious to saline solution and insensitive to light. The overall compliance of the transducer/displacement system was <0.03 μm/dyn. The sensitivity of the force transducer was 50 mV/dyn. The natural frequency with the tip in water was 3.2 kHz and the damping time constant was 0.93 ms. The higher frequency noise was equivalent to 0.25 dyn, while the long term drift over 1 h was <2 dyn.

Sarcomere length was sensed using a Schottky barrier photodiode in a manner similar to that described in Schoenberg and Wells (1984), except that the laser was oriented perpendicular to the fixed chamber, and the fiber rotated relative to the laser beam to maximize the diffracted light intensity. The length of fiber illuminated was ~250 μm. The detector was positioned 130 mm from the muscle fiber. A sarcomere length change of 1 nm/half sarcomere gave a signal of 30 mV. The electronic noise in the system was equivalent to <0.1 nm/half sarcomere but the vibrational noise was ~0.3 nm/half sarcomere. The response time of the system was <100 μs.

The displacement generator was a loud speaker voice coil, previously described in Schoenberg and Wells (1984). Normally the control circuitry was operated such that the feedback loop forced the speaker position signal to follow a command signal, thereby controlling the overall length change applied to the muscle fiber segment. Alternatively, using a TTL controlled analogue switch, the speaker position signal could be removed from the feedback loop and replaced by the sarcomere length signal, thereby allowing direct feedback control of sarcomere length. Before operating in sarcomere length-control mode, a step was first made in overall length-control mode and the sarcomere length signal to be fed back was adjusted to be zero when the speaker position was zero, and to have about the same amplitude, in millivolts, as the speaker displacement signal. This allowed TTL switching between the two modes without readjustment of the gain controls of the feedback loop. In general, the gain was kept low to minimize the number of instances of fiber destruc-

TABLE I
COMPOSITION OF SOLUTIONS (IN MILLIMOLES PER LITER)

Solution	KCl	Imidazole	EGTA	EDTA	Excess Mg^{++}	MgATP	MgPP or MgAMPPNP	DTT
Relaxing	150	10	1	—	2	1	—	0.5
Quick rinse	100	20	5	15	—	—	—	—
Rigor	155	10	1	—	2	—	—	0.5
4 mM Analogue	90	10	1	—	2	—	4	0.5
Analogue diluent	102	10	1	—	2	—	—	0.5

pH 7.0 ± 0.05 at 5°C.

tion due to loop instability. This often resulted in having 1–2 cycles of oscillation in sarcomere length following the step so that tension decay is generally displayed starting from ~3 ms.

Use of Sarcomere-Length Feedback Control

Fig. 1 *A* shows the force decay, overall length change, and sarcomere length change of a 250-μm central region of the fiber when a small, ~2-nm/half sarcomere, stretch is applied to one end of the muscle fiber segment. In this typical record from an experiment not using sarcomere length control, the sarcomere length change is initially ~2 nm/half sarcomere, but by the end of the experimental time it has decreased to ~1.5 nm. Clearly the tension decay record should be corrected for the sarcomere length decay. Since, for a linear system, a 2-nm stretch will produce 1.33 times as much force as a 1.5-nm stretch, a first order correction for the point where the sarcomere length is only 1.5 nm is to multiply the force at that point by 1.33. In early experiments the force decay curves were corrected, point by point, in this manner and the results have previously been reported briefly (Schoenberg and Eisenberg, 1984).

An alternative, more direct approach to obtaining the true force decay for an isometric fiber after a rapid stretch is to use feedback control to directly prevent any sarcomere shortening of the region of fiber under observation. This is accomplished by pulling, or releasing, the end of fiber, as illustrated in Fig. 1 *B*, to compensate for sarcomere length changes that would have occurred without intervention. This tends to increase the noise in the records, but has the advantage that the force decay curve then requires no correction and the only uncertainties are those related to the accuracy and noise of the sarcomere length signal. All the figures show data obtained with sarcomere length control. However, since data obtained with the earlier correction technique (Schoenberg and Eisenberg, 1984) were not significantly different from those obtained with sarcomere length control, data from five fibers studied with the earlier method were averaged into the summary of results shown in Table II.

The 1–2-dyn long-term drift in the force transducer caused a small uncertainty in the absolute zero of force. Because of this, the zero of force in Fig. 1, as well as the other figures, was taken as the force just before stretch. In actuality, this zero force was <1 dyn in ATP solution, <2 dyn in AMP-PNP or PPi solution, and generally 3–4, but in any case, <6 dyn in rigor. All these forces are small compared with the 60 dyn of Ca^{2+}-activated isometric force produced by a typical fiber.

TABLE II
HALF-TIMES FOR TENSION DECAY, $\tau_{1/2}$, IN NUCLEOTIDE ANALOGUE SOLUTIONS

Concentration of analogue	PPi (n = 8)	AMP-PNP (n = 4)
mM	*s*	*s*
4	0.24 (± 0.09)	3.5 (± 2.9)
1	1.7 (± 0.4)	27 (± 16)
0.25	20 (± 9)	102 (± 75)

Controls

Since some of our results differ from those reported previously, we did a number of controls to try to find the reason for the experimental discrepancies. Our experiments were routinely done on freshly skinned, unglycerinated rabbit psoas fibers. However, several control experiments showed that fibers stored at −20°C for 2 mo in 50% glycerol/dissecting solution, without DTT, give similar results. Secondly, we routinely put fibers into rigor by first rinsing with chilled EDTA (quick rinse) solution. This preserved order in the striation pattern, and resulted in fibers with little initial tension (i.e., little so-called rigor tension). Again, in a series of control experiments we found that fibers put into rigor in the presence of Mg^{2+} or Ca^{2+} and Mg^{2+} so that they have large rigor tensions, while not having as uniform a striation pattern, do show more or less the same qualitative behavior. Finally, we also did several experiments with 225 μM p,p-di(adenosine-5')pentaphosphate (Ap_5A) (Sigma D-6392), 10 mM glucose, and 10 U/ml of hexokinase (Sigma H-5875) added to the

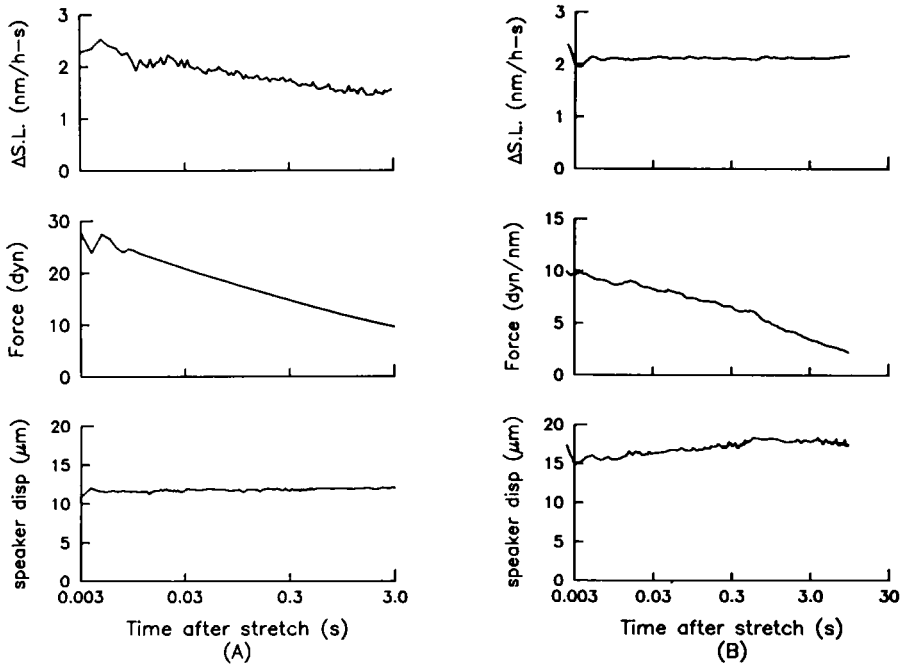

FIGURE 1 Sarcomere length, speaker position, and force decay after a stretch; (*A*) with speaker position control, (*B*) with sarcomere length control. Note sarcomere length does not stay constant in *A*. (*A*) Experiment 100683; diameters, 100 × 120 μm. P_o, 65 dyn. (*B*) Experiment 101584; diameters, 100 × 75 μm. P_o, not measured.

bath to reduce any possible ATP contamination. This again produced similar results.

RESULTS

Force Decay After Stretch

Figs. 2 and 3 show the force decay after a 2-nm stretch in rigor, MgPPi, and MgAMP-PNP solutions. The noise in the traces is due to amplification by the feedback loop of noise in the sarcomere length signal. Nonetheless, it is readily seen first, that the force decay in rigor is quite slow and second, that the force decay is greatly accelerated by increasing concentrations of AMP-PNP or PPi. The decay is not exponential (see below). To clearly observe both the early and late portions of the decay, force has been plotted as a function of the logarithm, base 10, of the time after the start of stretch. With such a log plot, each decay curve will have an initial flat region in the time region corresponding to times less than the fastest decay time constants. Starting the plot from $t = 3$ ms after the start of stretch (the time after which the sarcomere length was essentially uniform and fiber vibration had died down) the initial flat region is clearly seen for all concentrations of AMP-PNP. It is also seen for 0.25 mM PPi and perhaps 1 mM PPi. However, in 4 mM PPi, the initial flat region lies to the left of $t = 3$ ms. This suggests that by 3 ms in 4 mM PPi solution, some of the strained cross-bridges have already detached and reattached to actins where they are less strained.

In Figs. 2 and 3, it is seen that the force induced by stretch has decayed close to zero by 300 s, for both 1 and 4 mM AMP-PNP and PPi. In a few experiments lasting for longer times, these forces decayed even closer to zero. For stretches lasting several thousand seconds, even the force in 0.25 mM analogue solution decayed to within a few dynes of zero by the end of the experiment. The same was true for the so-called rigor tension induced upon going from relaxing solution into rigor. This tension, which was small when the rigor solution contained EDTA and larger when it contained magnesium, always decayed, upon AMP-PNP or PPi addition, very close to zero in times comparable to those seen in Figs. 2 and 3.

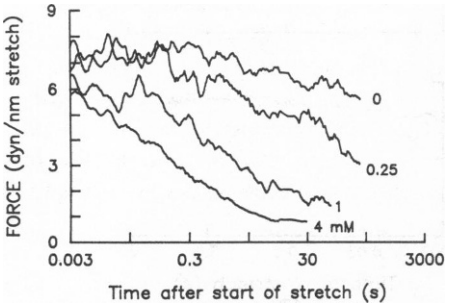

FIGURE 2 Force decay after a 2-nm stretch in MgPPi solution. Ordinate, force/unit stretch. Numbers next to each curve give MgPPi concentration in millimoles per liter. Experiment 121784; diameters, 90 × 100 μm. P_0, 58 dyn.

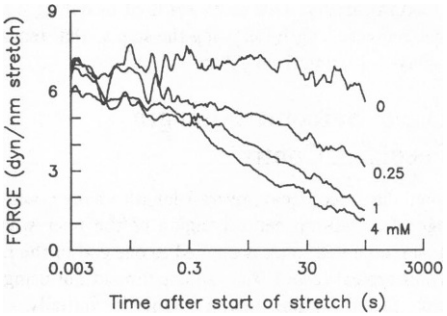

FIGURE 3 Force decay after a 2-nm stretch in MgAMP-PNP solution. Ordinate, force/unit stretch. Numbers next to each curve give MgAMP-PNP concentration in millimoles per liter. Experiment 091384; diameters, 65 × 80 μm. P_0, 65 dyn.

The fact that the force induced by rigor or stretch decays relatively quickly almost to zero at high nucleotide analogue concentrations contradicts the reported results of Clarke and Tregear (1982). In their experiments with glycerinated rabbit psoas muscle, they found fibers could maintain significant tension for up to 18 h in 1 mM AMP-PNP. In a separate series of experiments, (Schoenberg, unpublished observations) an attempt was made to reproduce the results of Clarke and Tregear using apparatus, solutions, and solution changing techniques identical to their own. Despite this, experiments done using AMP-PNP freshly obtained from Sigma Chemical Co. produced results indistinguishable from those reported in the body of this paper. However, experiments performed with a preparation of AMP-PNP (Sigma Lot 112F-7150) stored at −20°C for two years did show a maintained tension of ~5–6 dyn in the presence of 0.5–2 mM AMP-PNP. This large a maintained tension was never seen with fresh AMP-PNP.

Number of Attached Crossbridges During Force Decay

While our results are at variance with those of Clarke and Tregear, they are, in general, compatible with our equilibrium crossbridge model (Schoenberg, 1985) that was used to explain the behavior of fibers in the presence of ATP and absence of Ca^{2+}. One of the predictions of our equilibrium cross-bridge model is that the number of attached cross-bridges should remain constant during the period of force relaxation. That this is indeed the case was confirmed in the experiment shown in Fig. 4. After the initial 2-nm stretch, a second test stretch of ~2 nm was applied, either shortly (10 ms) after the initial stretch (before much tension decay), or 600 ms after the initial stretch (at a time when the tension had decayed by more than half). It is seen from the equal magnitude of the tension response to the two equal-sized test stretches (the magnitude being a measure of the number of attached cross-bridges), that the number of attached cross-bridges is constant during the tension decay. Since the magnitude of the initial force

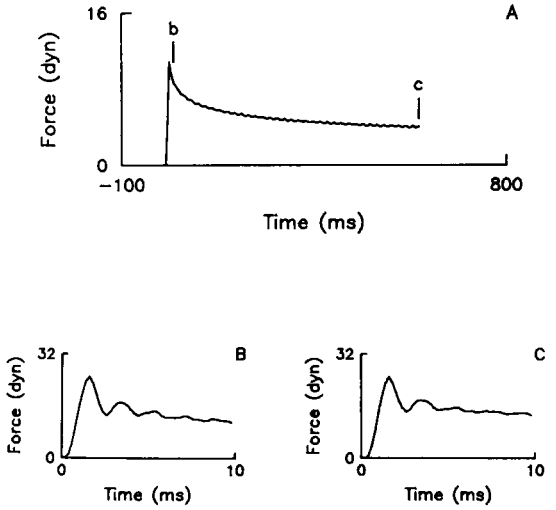

FIGURE 4 Force response to a second test stretch applied either 10 or 600 ms after the initial 2-nm stretch. (*A*) Force response to a 2-nm stretch in 4 mM PPi solution, linear time scale. (*B*) Force response to a second 2-nm test stretch applied at 10 ms. (*C*) Force response to a second 2-nm test stretch applied at 600 ms. Vertical lines in *A* show times of applied test stretches. Zero of force in *B* and *C* is the force extant immediately before the test stretch. Since the amplitude of the test stretch is the same in *B* and *C*, the amplitude of the force response is proportional to number of attached cross-bridges. The initial 2 ms of both traces are nearly identical, implying equal numbers of attached bridges at 10 and 600 ms. After 2 ms, a greater slope can be seen in trace *B* because of the greater underlying rate of force decay at 10 ms compared with 600 ms. Oscillations in the force traces are due to oscillations in the applied displacement, due to the low gain in the feedback loop (see Methods). Experiment 050884; diameters, 65 × 80 μm. P_o, 57 dyn.

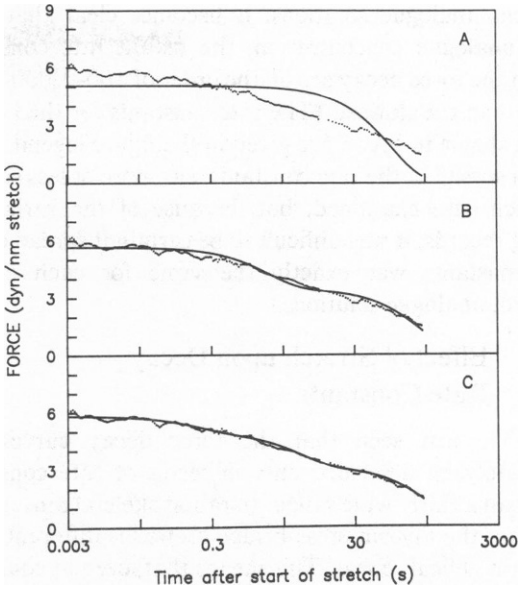

FIGURE 5 Best least squares fit of the force decay in 1 mM AMP-PNP to multiple exponentials of the form, $A_i e^{-r_i t}$. Units of A_i are dynes per nanometer. Units of r_i, s^{-1}. (*A*) single exponential: $A_1 = 5.1$; $r_1 = 9.2 \times 10^{-3}$. (*B*) two-exponential fit: $A_1 = 3.5$, $A_2 = 2.2$; $r_1 = 3.6 \times 10^{-3}$, $r_2 = 5.0 \times 10^{-1}$. (*C*) three-exponential fit: $A_1 = 3.2$, $A_2 = 1.8$, $A_3 = 0.8$; $r_1 = 2.8 \times 10^{-3}$, $r_2 = 1.9 \times 10^{-1}$, $r_3 = 4.9$. Best fit determined using a nonlinear least squares routine based upon Marquadt's compromise. Experiment 091384; diameters, 65 × 80 μm. P_o, 65 dyn.

change produced by a 2-nm stretch is the same in rigor and in all but one of the analogue solutions,[1] it seems likely that the number of cross-bridges attached in AMP-PNP or PPi solution is the same as in rigor.

Shape and Rate Constants of the Force Decay Curves

Table II summarizes the times ($\tau_{1/2}$) for the force to decay to 50% of its initial value[1] in each of the experimental solutions. It is seen that at a given concentration of nucleotide analogue, the force decay is on the order of ten times more rapid in MgPPi solution than in MgAMP-PNP solution. Contrary to what one would expect from the MgAMP-PNP and PPi binding constants measured by Marston et al (1976) and Goody et al (1976) (see Discussion), there is no evidence that the analogue effect is saturating as analogue concentration is increased from 1 to 4 mM. Actually, over the range of concentrations studied, the change in $\tau_{1/2}$ with change in analogue concentration is more than proportional. The reason for greater than proportional behavior is not understood.[2]

Fig. 5, derived from a fiber bathed in 1 mM AMP-PNP solution, shows a typical force decay curve. Although there is considerable variation from fiber to fiber in the precise shape of the force decay curves (likely related to noise and inaccuracies in the sarcomere length signal) it is clear from Fig. 5 that the force decay following a rapid stretch is not described by a single exponential. While it may not be possible to totally rule out the two-exponential fit from a single record, examination of a large number of records suggests that at least three, or alternatively, a spread of rate constants, are necessary to fit the decay. The range that the decay rate constants span can be deduced from the rate constants derived from the multiexponential fits. Upon examining a number of decay curves for fibers in the

[1]The initial force response in 4 mM PPi was, on average, only 80–90% of that in rigor. As discussed above, the most probable explanation for this is that some of the strained crossbridges have already detached and reattached during the 3 ms before the initial force is estimated. This means that the correct half-time for force decay in 4 mM PPi is probably somewhat shorter than the value given in Table II, since the true initial force is probably 10–20% higher than the value used in the half-time computation. This problem could have been ameliorated by taking the initial force as the force after stretch of a rigor muscle. This was not done since, because of experimental scatter in the data, self-normalization of each record produced much more reproducible half times than when comparisons were made to a rigor control.

[2]Currently we are modeling to see if the greater than proportional behavior can be accounted for by assuming that the cross-bridge with bound analogue is attached to actin at a different angle from the rigor cross-bridge (Marston et al., 1976, 1979), or whether more complicated behavior, for example, cooperativity between cross-bridges bound to actin (Kuhn, 1978), needs to be invoked.

different analogue solutions, it becomes clear that at a given analogue concentration, the fastest rate constants seen in the force decay are on the order of 100–1,000 times faster than the slowest. (The rate constants for the typical record shown in Fig. 5 are given in the figure legend.) The overall spread of the rate constants was more or less similar for each case examined, but, because of the variability among records, it was difficult to be certain if spread of the rate constants was exactly the same for each of the different analogue solutions.

Effect of Stretch upon Decay Rate Constants

We have just seen that the force decay curves can adequately be described only in terms of rate constants that span a fairly wide range. In rabbit skeletal muscle, the repeat of the myosin cross-bridge crowns is different from the actin helical repeat. This means that, even at equilibrium, if large numbers of crossbridges are bound to actin they will have to be attached with somewhat different strains. If the cross-bridge detachment rate constants are strain sensitive, this could be the explanation for the range of force decay rate constants (Schoenberg, 1985). To see if the detachment rate constants might be strain sensitive, the experiment shown in Fig. 6 was performed. Stretches of 1, 2, and 4 nm were applied to a fiber, and the tension decays plotted as force per nanometer stretch. Since the stress-strain relationship for the fibers is linear, each curve has the same origin. It is clear that the larger the additional strain imparted to the cross-bridges by the stretch, the faster the rate of tension decay. The half-time for tension decay was ~10-fold shorter with a 4-nm stretch than with a 1-nm stretch. Similar results were obtained in three additional experiments.

ATP Contamination

It was important to be certain that the force relaxation seen in our experiments was not due to ATP contamination. To study this question, we needed a sensitive assay for ATP contamination. One very sensitive assay is the amount of active tension redeveloped by a fiber following quick release of the fiber to near zero tension. As Fig. 7 shows, a fiber in rigor solution redevelops almost no active tension following a release. The addition of just 2.5 μM ATP to the rigor solution results in a significant increase in the amount of tension redevelopment. Adding additional ATP results in additional force development. To deduce the contaminant ATP in pure rigor solution, we can make a plot of redeveloped force versus added ATP concentration and extrapolate back to the abscissa intercept. The data in Fig. 7 give an approximately linear relationship when plotted in this manner and, from the abscissa intercept, it is clear that the contaminant ATP with the fiber in pure rigor solution is <1 μM.

Having shown that the contaminant ATP in rigor must be <1 μM, we can now ask whether the somewhat small tension relaxation seen in rigor after stretch might possibly be due to, or influenced by, ATP contamination. If the contaminant ATP in rigor were as large as 1 μM, and if this contaminant ATP were the cause of the force decay seen in rigor, then adding, say, 5 μM additional ATP should increase the force decay by a factor of 6. Since, on the contrary, the rate of force decay is identical in rigor solution and in rigor solution with 5 μM ATP added, and only much larger concentrations of added ATP influence the rate of force decay (records not shown), the tension decay in pure rigor solution is not due to, or influenced by, the level of contaminant ATP present in normal rigor solution.

Similar controls were done for the fibers in PPi and AMP-PNP solution. Plots of redeveloped force versus added ATP concentration suggested that in 1 mM PPi solution the contaminant ATP was also <1 μM, while in 1 mM AMP-PNP solution it was perhaps 2–4 μM. Using the same reasoning as previously, if the contaminant ATP concentration in 1 mM AMP-PNP were as large as 4 μM, and this amount of contaminant ATP was influencing the force decay, then adding 10 μM ATP should more than triple the contaminant ATP effect. Since, in fact, adding

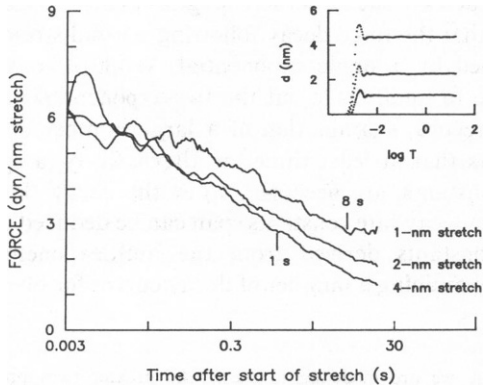

FIGURE 6 Force decay after different amplitude stretches. Vertical lines show the half times for tension decay for the 1-nm and 4-nm stretches, 8 s and 1 s, respectively. Actual stretches shown in inset. Experiment 100484; diameters, 90 × 80 μm. P_0, not measured.

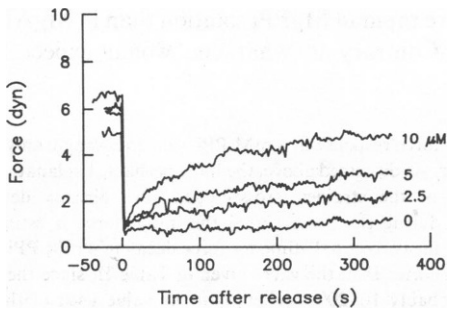

FIGURE 7 Force redevelopment after release to near zero force in rigor solution containing different amounts of added ATP. Numbers next to each curve give concentration of added ATP in micromoles per liter. Experiment 110884; diameters, 70 × 65 μm. P_0, 68 dyn.

10 μM ATP has no effect upon the force decay in 1 mM AMP-PNP, whereas adding much larger concentrations of ATP does have an effect (not shown), it appears that the force decay seen in Figs. 2 and 3 is not at all related to, or influenced by, the levels of contaminant ATP present in the experiments. Experiments in which Ap_5A, glucose, and hexokinase were added to the experimental solutions without having an effect (see Controls) also support this conclusion.

DISCUSSION

Relation to Previous Studies

Previously we have shown that at low ionic strength, in the absence of Ca^{2+}, but in the presence of ATP, large numbers of cross-bridges attach to actin in relaxed muscle at 5°C. It was clear from those studies, however, that on the time scale of seconds, or even milliseconds, it is not proper to think of the cross-bridges with bound ATP as statically attached to actin; rather, on the time scale of a millisecond, an individual cross-bridge will detach and reattach many times. This simple assumption regarding equilibrium between attached and detached states of the cross-bridge, in the fiber, as in solution (Stein et al., 1979), makes it possible to account for much of the complex mechanical behavior of the muscle bathed in Ca^{2+}-free ATP solution (see Schoenberg, 1985).

The present study was undertaken to see whether this simple equilibrium cross-bridge model was appropriate only for cross-bridges with ATP bound to the active site, or whether it could also explain the behavior of cross-bridges with other moieties bound to the active site. Studying two nonhydrolyzable ATP analogues, AMP-PNP and pyrophosphate, we again have found behavior quite consistent with the simple equilibrium cross-bridge model. This model predicts that when a muscle is rapidly stretched, the actin and myosin-containing filaments slide past one another and each attached cross-bridge is rapidly strained, producing a rise in tension. After the stretch, however, as the cross-bridges continue to detach and reattach as demanded by the equilibrium nature of the attachment, they will, on average, reattach to actins in positions where they are less strained than immediately after the stretch, and the force induced by the stretch will decay toward zero. Consistent with this, we find that in rigor, where the cross-bridge detachment rate constants are thought to be quite slow, there is very little tension decay within the first seconds after a quick stretch (see Figs. 2 and 3). On the other hand, in the presence of the nucleotide analogues, AMP-PNP and PPi, which in vitro greatly enhance the rate of dissociation of myosin from actin, the rate of force decay after stretch is greatly enhanced. The force decay after stretch also shows two additional properties of the simple equilibrium cross-bridge model; namely, the force decays toward the equilibrium force, $F = 0$, (Figs. 2 and 3) and the force decay occurs without net change of attached cross-bridge number (Fig. 4). Our finding that the force induced by stretch appears to decay to very close to zero, even at subsaturating analogue concentrations, suggests that all of the cross-bridges can bind nucleotide analogue and undergo detachment/reattachment, i.e., there is no population of extremely slowly detaching cross-bridges.

Although our results are internally consistent and agree, at least qualitatively, with the simple cross-bridge model outlined above, our finding that PPi or AMP-PNP completely relaxes the tension of rabbit psoas fibers contradicts the results of Clarke and Tregear (1982) who report that glycerinated rabbit psoas fibers can maintain as much as 15 dyn of tension for up to 18 h in the presence of 1 mM AMP-PNP. We found almost no tension maintained in the presence of millimolar amounts of AMP-PNP or PPi, except when we added ATP to the analogue solution. However, even this effect was small (1–2 dyn additional force for 10 μM ATP added to 1 mM AMP-PNP), and it is not clear that this offers an explanation for the force maintenance seen by Clarke and Tregear. On the other hand, the finding (see Results) that a batch of AMP-PNP stored for two years at −20°C could produce ~5 dyn of maintained tension does raise the possibility that one of the breakdown products of AMP-PNP, possibly ATP (Penningroth et al., 1980), can cause significant tension maintenance.

In addition to the literature on mammalian muscle, there is also a considerable body of literature concerned with nucleotide analogue effects on insect fibrillar flight muscle. Most investigators studying insect fibrillar flight muscle and rabbit psoas (White, 1970; Kuhn, 1978; Clarke and Tregear, 1982) report similar results in the two preparations. With regard to the question of maintained tension in the presence of AMP-PNP, Clarke and Tregear, using single Lethocerus fibers, found results similar to what they found with rabbit fibers. White, the first to observe decay of force and speed-dependent stiffness in the presence of nucleotide analogue, also often found significant tension maintenance in the presence of 5 mM MgPPi with Lethocerus bundles, although some of his records (his Fig. 4, for example) show little tension maintenance and appear quite compatible with our own. Kuhn, studying Lethocerus bundles, found results very similar to our own. The isometric tension values he reports as a function of AMP-PNP concentration, often thought of as steady tensions, but, in fact, defined as the tension remaining after 20 min of tension decay starting from a rigor force of ~20 dyn, are only slightly higher than we would expect, based upon our rabbit data, and a large fraction of this difference is accounted for by the difference in termperature of the two experiments (Schoenberg, M., and E. Eisenberg, unpublished data). On the basis of this, it therefore seems likely that the equilibrium crossbridge behavior of rabbit skeletal and insect fibrillar flight muscle is similar, with little significant tension maintenance in the presence of nucleotide analogue. It should be emphasized that the issue of

tension maintenance is by no means just a quantitative one. Whereas our result, nearly zero tension maintenance, can be understood in terms of a fairly simple model, an explanation for any maintained tension would likely require a number of ad hoc assumptions (see Pate and Brokaw, 1980).

An area of agreement among most investigators regards the number of cross-bridges attached in nucleotide analogue solution. Since the stiffness is the same in AMP-PNP, PPi, and rigor solutions, it appears that the same number of cross-bridges are attached in each case. Since in rigor there is evidence that nearly all of the cross-bridges are attached (Lovell and Harrington, 1981; Cooke and Franks, 1980; Cooke and Thomas, 1980), presumably the same is true for nucleotide analogue solution. One caveat to this is that, if the cross-bridge elasticity resides in the myosin subfragment-2 region of the molecule, we could not detect detachment of only one of the two myosin heads.

Cross-bridge Detachment Rate Constants with Nucleotide Analogue Bound

If our interpretation of our results is correct, namely that the decay of tension following stretch is due to the redistribution of myosin cross-bridges as they detach and reattach to different actins, the rate of tension decay provides direct information about the cross-bridge detachment rate constants. This point is considered at length in Schoenberg (1985) but, intuitively, the reason one can get the detachment rate constants is that under conditions where the equilibrium favors attachment, as in our experiments, the cross-bridge attachment rate constants do not enter into the force-decay rate; because the attachment rate constants are faster than the detachment ones, detachment is rate limiting and therefore determines the rate of tension decay.

This being the case, our results imply that following a stretch in nucleotide analogue solution, cross-bridges detach with rate constants ranging from ~0.1 to 100 s^{-1} in 4 mM PPi solution and 0.01 to 10 s^{-1} in 4 mM AMP-PNP solution. A reason for this wide range of rate constants might be related to the mismatch between the pitch of the actin helix, which is 38.5 nm, and the repeat distance of myosin cross-bridge origins, 14.3 nm. Because of this mismatch, under conditions where most of the cross-bridges are attached, the cross-bridges will have to be attached with somewhat different strains. Fig. 6 suggests that the rate with which cross-bridges detach increases with increasing strain. Modeling efforts are currently underway to see if the rather wide range of detachment rate constants (2–3 decades) after a 2-nm stretch can be entirely explained in terms of the somewhat modest effect seen in Fig. 6.

Ultimately, we would hope to be able to relate the detachment rate constants measured in the fiber to the dissociation rate constants measured in solution. However, the fiber situation is quite complex. We found that the rate of detachment increased with increasing analogue concentration over the entire range of concentrations studied. This suggests that the detachment rate constant measured in our experiments is a composite one, involving two steps: an analogue binding step and the actual cross-bridge dissociation step. Since in the absence of analogue, i.e., in rigor, the force decay is very slow, presumably the analogue binding step always precedes the dissociation step. In this case, the rate constants for the actual cross-bridge dissociation step are faster than the rates of force decay we measure at subsaturating analogue concentration. We also found that the rate constants for cross-bridge detachment increase with increasing strain. It is not clear whether one, or in fact, both of the steps involved in cross-bridge detachment are strain sensitive. Marston et al., 1979, have previously reported that analogue binding is increased by increased strain, and it also seems likely that the cross-bridge dissociation step might be accelerated by strain. Therefore, while it is of considerable interest that the dissociation rate constants measured in solution for myosin subfragment-1 (S1) with bound analogue (Marston, 1982) fall within the range of rate constants we have measured, it is difficult to be more precise at present.

It was actually surprising that the apparent cross-bridge detachment rate constants increased as nucleotide analogue concentration was increased from 1 to 4 mM. Marston et al. (1976) found a dissociation constant of 30 μM for the binding of MgAMP-PNP to rabbit fibers, implying nearly complete saturation of analogue binding at ~300 μM concentration. Similarly, Goody et al.(1976) using insect flight muscle and a titration based upon changes in the ratio of two equatorial x-ray reflections, also found a dissociation constant in that range for AMP-PNP (90 μM), and a slightly higher dissociation constant, 450 μM, for PPi. Although early in vitro studies again found dissociation constants in the submillimolar range, (Hofmann and Goody, 1978; Greene and Eisenberg, 1980), very recent in vitro estimates of the dissociation constants of AMP-PNP and PPi binding to rabbit actin-S1 (Biosca et al., 1985) suggest much weaker binding of nucleotide analogue to the S1-actin complex. Biosca et al. found dissociation constants of ~2 mM for both AMP-PNP and PPi in the absence of troponin-tropomyosin; the dissociation constants may be even higher in the presence of troponin-tropomyosin (Williams and Greene, 1983). Although the reason for the wide range of reported binding constants is unclear, our data imply that analogue binding is far from saturation at 1 mM, and probably also at 4 mM analogue concentration, and would seem to be compatible only with dissociation constants at least in the millimolar range.

Cross-bridge Detachment Rate Constants in Rigor

In rigor, we see very little evidence of cross-bridge detachment over the time scale of our experiments. Figs. 2 and 3

show little force decay in the absence of analogue. We had previously reported that the decay of force in rigor might be as large as 20% in 3 s, but with improved technique, particularly with regard to attaching the fibers, the decay within 3 s is usually found to be 0–5%. This suggests that some of the force decay often seen in rigor may be due to sarcomere slippage because of poorly attached ends. However, there appears to be a small, but nontheless real (10–20%), detachment of cross-bridges on the time scale of minutes.[3]

Actually, it is now clear why there is not more force decay in rigor. In solution, the S1 dissociation rate constant from actin is on the order of 0.01 s^{-1} (Marston, 1982). In the simplest crossbridge models this would lead to >50% force decay in 100 s. To explain the rather slow rigor decay, it may be necessary to postulate cooperative cross-bridge binding as suggested by Kuhn (1978) or possibly, since in rigor both the cross-bridge heads are thought to be attached, it could be that in the fiber both heads of a cross-bridge must dissociate before the tension supported by a cross-bridge decays. In this regard, it is interesting that in the presence of nucleotide analogue our measured detachment rate constants are closer to the in vitro S1 dissociation rate constants, possibly implying a greater amount of single-headed binding in the presence of analogue.

In summary, we find that, whereas in rigor, force induced by stretch decays relatively little over the first few minutes, it decays nearly to zero within tens of seconds in the presence of high concentrations of PPi, and within minutes in high concentrations of AMP-PNP. This behavior, which fits qualitatively with the fairly simple cross-bridge model proposed in Schoenberg (1985), is very similar, albeit on a slower time scale, to the behavior seen when cross-bridges have ATP bound to the active site.

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[3]The amount of force decay in rigor was actually somewhat more variable than most of our other results. A few fibers, which by all our criteria appeared to be well attached, showed significant force decay, 20–40%, on the minute time scale. It is not clear whether this represents real variability between fibers, or whether it could be due to a possible 10–20% uncertainty in the sarcomere length signal superimposed upon a 10–20% real effect.