Effect of adenosine triphosphate analogues on skeletal muscle fibers in rigor

Mark Schoenberg
Laboratory of Physical Biology, National Institutes of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT It is commonly believed, for both vertebrate striated and insect flight muscle, that when the ATP analogue adenyl-5'-yl imidodiphosphate (AMPPNP) is added to the muscle fiber in rigor, it causes the fiber to lengthen by 0.15%. This has been interpretated (Marston S. B., C. D. Roger, and R. T. Tregear. 1976. *J. Mol. Biol.* 104:263–267) as suggesting (*a*) that in rigor the crossbridge is fixed to, i.e., almost never detaches from the actin filament; (*b*), that the crossbridge remains fixed to the actin filament after AMPPNP addition; and (*c*) that the ability of AMPPNP to cause apparent lengthening of a muscle fiber is due to its ability to cause a conformational change in the myosin crossbridge that has an axial component of ~1.6 nm/half-sarcomere. The present study, done only on chemically-skinned rabbit psoas fibers, confirms that AMPPNP can cause muscle fibers to lengthen by 0.15% but only for a narrow set of experimental conditions. When experimental conditions are varied over a wider range, it becomes apparent that the extent of lengthening of a rigor muscle fiber upon AMPPNP addition depends almost entirely on the strain present in the rigor fiber before AMPPNP addition. Addition of AMPPNP to an unstrained rigor fiber (one supporting zero tension), induces zero length change while addition of AMPPNP to very highly strained rigor fibers induces length changes >0.15%. The data thus do not support the hypotheses that the crossbridges remain fixed to the actin filament after AMPPNP addition and that the size of the apparent length change induced by AMPPNP is related to the size of the axial component of a conformational change. Instead, the data support the idea that the ability of AMPPNP to cause lengthening of a rigor muscle fiber is related to its ability to accelerate the rate at which strained crossbridges detach from actin and reattach in positions in lesser strain. The data do not rule out a conformational change upon AMPPNP binding, they simply make clear that any attempt to measure a force response conceivably due to a conformational change, would be more than obscured by the force changes due to crossbridges detaching and reattaching in positions of lesser strain.

INTRODUCTION

In 1976, Marston, Roger, and Tregear, studying glycerinated insect flight muscle, observed that addition of the ATP analogue, adenyl-5'-yl imidodiphosphate (AMPPNP) to a rigor fiber exerting force caused the force to decrease by ~3 dyn (3 mg-force). Subsequent removal of the 0.5 mM MgAMPPNP caused tension to increase by ~1 dyn. Although this effect was small, it was none-the-less concluded (*a*) that in the presence of AMPPNP crossbridges do not detach from actin, and (*b*) that AMPPNP binding to a rigor crossbridge causes a conformational change in the crossbridge that effectively lengthens the muscle fiber. The above conclusions were considerably strengthened by the reports of Marston et al., 1979, and Clarke, 1982 that (*a*) the fall in tension upon AMPPNP addition was actually as large as 6 dyn, (*b*) the 6 dyn fall upon AMPPNP addition was also followed by a 6 dyn rise upon its removal, and (*c*) the half times for the tension fall and rise were 1 and 13 s respectively, about what would be expected for diffusion of AMPPNP into and out of fiber. The conclusions were also strengthened by the report (in this work cited) that virtually identical results were obtained from both rabbit psoas and insect flight fibers.

Although the above results are certainly consistent with the idea that when AMPPNP is added to a rigor fiber the crossbridges never detach but simply undergo a lengthening conformational change, there is actually considerable evidence against this. For one, the ultrastructural changes seen when AMPPNP is added to a rigor fiber are incompatible with the idea that crossbridges never detach from actin (Reedy et al., 1987). Secondly, if crossbridges in the presence of AMPPNP are never detaching, then any tension induced by stretch should persist forever. Actually, experimentally, the tension induced by stretch soon decays away (Clarke and Tregear, 1982; Schoenberg and Eisenberg, 1985). Thirdly, if crossbridges were never detaching in the presence of AMPPNP, then the fiber stiffness measured with either a slow or rapid stretch would be identical. In reality, fiber stiffnesses measured with a slow stretch are very much

less than those measured with rapid stretches (Schoenberg, 1988). Finally, AMPPNP, under conditions of moderate or high ionic strength, is known to increase the mobility of crossbridge heads (Fajer et al., 1988). This has been interpreted as suggesting that AMPPNP, under some conditions, actually causes dissociation of crossbridges from the actin filament. Because the mechanism by which AMPPNP causes dissociation of myosin from actin in solution is by increasing the detachment rate constant, it seems reasonable that the same mechanism should apply in fibers.

Because the above evidence suggests quite strongly that in the presence of AMPPNP crossbridges are not fixed to the actin filament but instead continually detach and reattach to it, the present study was undertaken to reinvestigate the early experiments that led to the opposite conclusion. While the current set of experiments confirm most of the experimental findings of Marston et al., 1976, and also the more recent ones of Tregear, 1988, doing the experiments over a somewhat wider range of conditions gave results that are not consistent with the hypothesis that the apparent lengthening of a muscle fiber upon AMPPNP addition is due to a conformational change. Instead, the results support the idea (Schoenberg and Eisenberg, 1985) that the apparent lengthening caused by AMPPNP addition is the result of AMPPNP's ability to increase the crossbridge detachment rate constants.

METHODS

All experiments were performed, at 5°C, on freshly dissected or 1-d old single chemically-skinned rabbit psoas fibers. These fibers had their sarcolemma made permeable to the bathing medium using a procedure similar to that of Eastwood et al., 1979 (see Schoenberg, 1988*a*, for details). Most of the techniques and experimental apparati are similar to those described in Schoenberg and Eisenberg, 1985 and Schoenberg and Eisenberg, 1987. Li_4AMPPNP (A-2647) was purchased from Sigma Chemical Co., St. Louis, MO. All solutions containing AMPPNP, unless specifically noted, also contained 225 μM p,p-di(adenosine-5')pentaphosphate (Ap_5A) (Sigma Chemical Co., D-6392), 2 mM *d*-glucose, and 10 units/ml of hexokinase (Sigma Chemical Co., H-5875). The force transducer, made from an AE801 force gauge (AME, Horten, Norway), had a 1-cm long carbon fiber extension epoxied to the tip. It had a sensitivity of 50 mV/dyn and a resolution of 0.5 dyn. The drift over a 4 h period was ~1 dyn. For increased accuracy, all tensions reported in the present work are reported relative to the resting tension in relaxing solution. At the sarcomere length used in the present studies (2.5 μm), this was ~0.5 dyn.

In all experiments, before AMPPNP addition to the fiber, the fiber was first put into low-tension or high-tension rigor (Kawai and Brandt, 1976). To put the fiber into low-tension rigor, relaxing solution at 0°C was quickly replaced by cold rigor solution containing 15 mM EDTA and no Mg^{2+} (see Schoenberg and Eisenberg, 1985, for more details). After several chamber volumes were washed through, the EDTA-containing rigor solution was replaced with a rigor solution that contained 2 mM magnesium acetate, 3 mM (ethylenebis [oxyethylenenitrilo])-tetraacetic acid (EGTA), 90 mM potassium propionate, and 10 mM imidazole at pH 7.0. To put the fiber into high-tension rigor, the fiber was first activated in our standard activating solution (pCa, 4.5; Schoenberg and Eisenberg, 1985), after which the activating solution was replaced by a rigor solution containing 2 mM magnesium acetate, 85 mM potassium propionate, and 40 mM imidazole at pH 7.0. This resulted in a rigor fiber which supported a tension of ~60% P_o, where P_o is the maximum Ca^{2+}-activated tension measured in the isometric fiber at 5°C.

Whenever 4 mM AMPPNP was added to the muscle fibers it was done as follows. The existing rigor solution was replaced with so-called "pre-AMPPNP" rigor solution which contained 3 mM EGTA, 6 mM magnesium acetate, 70 mM potassium propionate, 2 mM *d*-glucose, 225 μM Ap_5A, and 10 mM imidazole at pH 7.0. Replacing the original rigor solution with this solution had no effect upon the rigor tension. After this solution replacement, 18 units (Sigma Chemical Co.) of hexokinase were added to the 1.8 ml bath. This again had no effect on rigor tension. Finally, 72 μl of 100 mM AMPPNP were added to the bath, yielding an AMPPNP solution having a final AMPPNP concentration of ~4 mM. For experiments where the final concentration was critical, two additional chamber volumes of 4 mM AMPPNP solution were washed through. In neither type of maneuver was the fiber moved through an air-water interface.

RESULTS

Fig. 1 shows the time-course of tension decay when 4 mM AMPPNP is added to a rigor fiber exerting tension. It is seen that 2,400 s after AMPPNP addition, the tension has fallen almost to zero. For Fig. 1, the fiber was first put into low-tension rigor, then stretched, and when the tension supported by the fiber became relatively steady, 4 mM AMPPNP was added. Very similar results are seen when the fiber, instead of being stretched, is put into high tension rigor before AMPPNP addition. In the experiments that follow, the main question to be addressed is whether the tension drop observed above is due to cross-

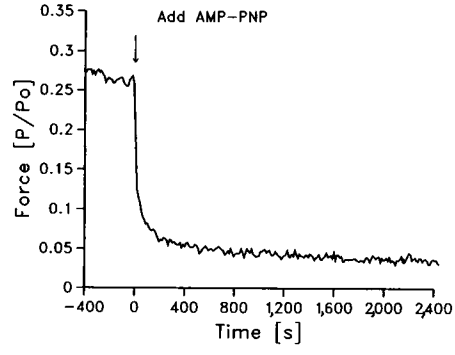

FIGURE 1 Decay of force after addition of MgAMPPNP to a rigor fiber supporting tension. Fiber was put into low-tension rigor and stretched to generate a force. When that force became relatively steady, 4 mM AMPPNP was added (arrow, time = 0). Force expressed as fraction of P_o. Fiber 071988. Fiber diameters, 63 × 73 μm. P_o, 33.5 dyn.

bridges undergoing a conformational change while fixed to the actin filament, or whether, alternatively, it is due to continually attaching and detaching crossbridges detaching and reattaching in positions of lesser strain.

If, in the presence of AMPPNP, crossbridges are fixed to the actin filament and never detach, and if, when AMPPNP is added to a rigor fiber supporting tension it causes a conformational change that has a component in the axial direction, then the size of the force drop that is seen upon AMPPNP addition should be a fixed amount determined solely by the magnitude of the conformational change. Expressed another way, the size of the force drop should be independent of the initial tension supported by the rigor fiber. The experiment of Fig. 2 was designed to test this. Fibers were mounted between a length driver and force transducer and then put into either low-tension or high-tension rigor (see Methods). Fibers put into low-tension rigor could be made to support variable amounts of force by stretching them various amounts. When the forces supported by the rigor fibers became moderately steady, the value of the force was noted, 4 mM AMPPNP was added, and then, 2,400 s after AMPPNP addition, the force level was again noted. Fig. 2 shows results from one of the eight fibers studied in this manner.

In Fig. 2, each vertical line connects two horizontal lines. The upper horizontal line indicates the rigor tension before AMPPNP addition and the lower horizontal line indicates the tension 2,400 s after addition of AMPPNP. Each trial in Fig. 2 represents a slightly different treatment of the rigor fiber. Thus, in trial 1, the fiber was put into low-tension rigor and was stretched ~3 nm/half-sarcomere so that it supported 34 dyn. 2,400 s after 4 mM AMPPNP addition, the tension was 4.9 dyn. Trials 2–5, and 7 were similar except that before AMPPNP addition, different amounts of tension were placed upon the fiber. In trial 6, the fiber was put into high-tension rigor and as a result, 40 dyn of force was supported. 2,400 s after AMPPNP addition, 6.6 dyn or tension remained. It is clear that the size of the tension drop upon AMPPNP addition is not a fixed amount. It is also not a fixed percentage of the initial rigor tension. Instead, the size of the tension drop is linearly dependent upon the amount of tension supported by the rigor fiber at the time of AMPPNP addition. An important point to note is that 2,400 s after AMPPNP addition, the fiber tension, while small, is not absolutely zero. The fiber of Fig. 2 was specifically chosen for illustration in order to show that the tension 2,400 s after AMPPNP addition can occasionally be as large as 5 dyn. In 10 of the 16 fibers studied as above, the tension remaining 2,400 s after AMPPNP addition was between 1.2 and 3 dyn. In the other six fibers, it ranged between 3.1 and 5 dyn. For the 12 fibers in which the maximum Ca^{2+} activated tension (P_o) was also measured, the force remaining after 2,400 s averaged 5.7 ± 0.7% P_o.

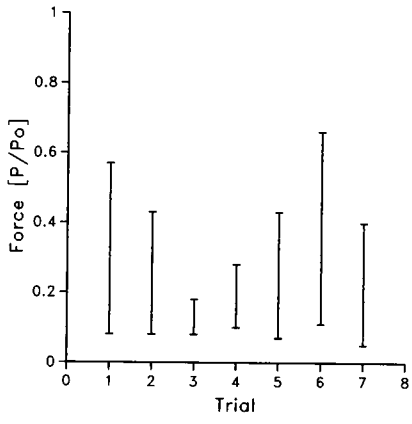

FIGURE 2 Effect of adding 4 mM AMPPNP to a rigor fiber supporting different amounts of tension. Each trial represented by one vertical and two horizontal lines. For trials 1–5 and 7, the fiber was put into low-tension rigor and then stretched variable amounts. When the force became relatively steady at the value indicated by upper horizontal line, 4 mM AMPPNP was added. 2,400 s after AMPPNP addition, the force had decayed, like in Fig. 1, to the value indicated by the lower horizontal line. In trial 6, the fiber was put into high-tension rigor and AMPPNP was added without stretch of the fiber. Note that the residual forces 2,400 s after AMPPNP addition in all cases are about the same. Fiber 050588. Fiber diameters, 125 × 107 μm. P_o = 60 dyn.

Experiments like that of Fig. 2 were also done with a second ATP analogue, pyrophosphate (PP_i). Because the rate of tension drop with PP_i is 15 times faster than with AMPPNP (Schoenberg and Eisenberg, 1985), the so-called residual tension was measured after only 900 s. The data from one of the three experiments performed with PP_i are shown in Fig. 3. Again it is noted that the size of the tension drop upon addition of an ATP analogue depends solely upon the initial tension supported in rigor. Again the residual tension is very small, averaging, for PP_i, 2.3 ± 0.7%P_o.

The above experiments are not compatible with the idea that crossbridges are fixed to the actin filament in the presence of ATP analogues. What they suggest is that the tension drop seen upon AMPPNP or PP_i addition is due to the ability of these ATP analogues to accelerate the rate at which crossbridges detach from the actin filament and reattach in positions of lesser strain. A puzzle is why the force supported 2,400 s after AMPPNP addition, or 900 s after PP_i addition, is not absolutely zero. This could mean either that there is a contaminant in the solutions that supports tension generation, that all the crossbridges are detaching extremely slowly, or the most likely possibility, that simply a small fraction of the crossbridges are detaching extremely slowly.

To expore the possibility that a force-producing con-

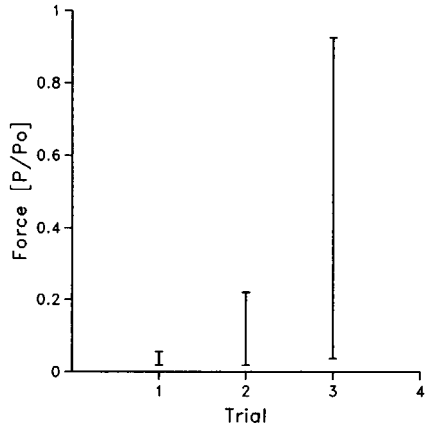

FIGURE 3 Effect of adding 4 mM Mg PP_i to a rigor fiber supporting different amounts of tension. Protocol similar to that of Fig. 2, going through low-tension rigor in trials 1 and 2, and high-tension rigor in trial 3. Again note very low residual forces, here measured 900 s after Mg PP_i addition. Fiber 062688. Diameters, 82 × 108 μm. P_o = 53 dyn.

taminant is responsible for the 2 to 5 dyns of residual tension seen 2,400 s after AMPPNP addition, several approaches were taken. In one type of experiment, hexokinase was eliminated from the bathing solutions and experiments like that of Fig. 2 were performed using 4 mM AMPPNP that had between 20 and 40 μm ATP added. The results of this experiment confirmed our previous finding (Schoenberg and Eisenberg, 1985) that contaminant ATP will indeed raise the tension seen 2,400 s after AMPPNP addition. Experiments in which 20–40 μm ATP was added to the 4 mM AMPPNP, but hexokinase and glucose were included in the solutions as usual, showed the same residual tension as experiments without ATP addition. This not only confirms the efficacy of our glucose/hexokinase system in eliminating added ATP, it also suggests that the residual tension is probably not due to ATP contamination of the AMPPNP.

A second way in which the possibility of a force producing contaminant was explored is as follows. A muscle fiber was put into low-tension rigor and stretched until it supported 15 to 20 dyn. After the force supported by the fiber became relatively steady, 4 mM AMPPNP was added to the bathing solution and 2,400 s were allowed to elapse. After 2,400 s, the muscle fiber was mechanically shortened so that the force supported by the fiber dropped to between 0 and 1 dyn. If there was a force-producing contaminant in the AMPPNP that was responsible for the tension 2,400 s after AMPPNP addition, the contaminant should cause the tension to rise slowly back to the value at 2,400 s. This did not happen, even after waiting 1,000 s, in any of the five experiments in which this protocol was followed. This again suggests that the small tension seen 2,400 s after AMPPNP addition to a rigor fiber is probably not due to a force-producing contaminant.

To explore the possibilty that all the crossbridges are detaching extremely slow or not at all 2,400 s after 4 mM AMPPNP addition, one can apply a stretch to the muscle fiber and see how quickly the force induced by the stretch decays away. If all the crossbridges are detaching extremely slow or not at all, the induced force should persist for a long time. We previously reported (Schoenberg and Eisenberg, 1985) that if stretches of 1–4 nm/half-sarcomere are applied to a muscle under the above conditions, the half-time for crossbridge detachment is on the order of 1–10 s. Furthermore, we reported that the half-time for tension decay increases as the size of the stretch decreases. One conceivably could argue that the crossbridges detach when strained by 1–4 nm, but they do not detach 2,400 s after AMPPNP addition because then they are strained less than that. To test for this possibility, in the present experiments stretches of only 0.1–0.2 nm/half-sarcomere were applied to the muscle fiber. The additional force generated by these small stretches was only on the order of 1 dyn. As Fig. 4 shows, this additional ~1-dyn force decays away with rate constants comparable with those seen with 1–4 nm stretches. The average half-time for tension decay with 0.1–0.2 nm/half-sarcomere stretches was 27 ± 4 s (N = 8) while that with 2 nm stretches was 22 ± 4 s (n = 9). It is clear then, that 2,400 s after AMPPNP addition to a rigor fiber, even while the

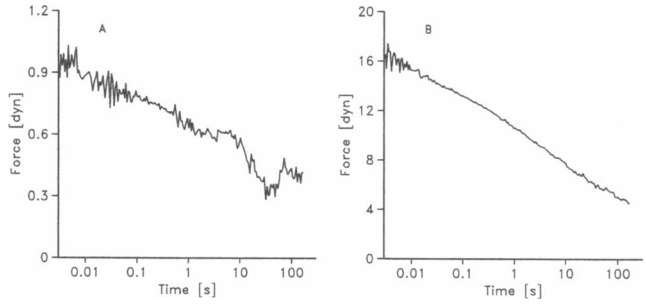

FIGURE 4 Force decay after stretch of a typical fiber bathed in 4 mM AMPPNP. (A) Small stretch, ~0.1 nm/half-sarcomere. (B) Large stretch, ~2 nm/half-sarcomere. All stretches were applied more than 2,400 s after AMPPNP addition so that the force before stretch was ~2 dyn for the first stretch (a large one) and ~1 dyn for all subsequent stretches. It is seen that the rate of force decay after stretch is only slightly slower with the very small stretch, which induces an additional force of only 0.9 dyn, as compared with the 2-nm/half-sarcomere stretch, which induces a force of ~17 dyn. In order to reduce noise, A is an average of 15 similar stretches and B is an average of 3 repeat stretches. Fiber 070788. Even with this amount of signal averaging, the trace in A is still relatively noisy. This is seen in terms of the high frequency noise and also in the apparent deviation from monotonic decay between t = 10 and t = 60 s. Diameters. 97 × 121 μm. P_o was 64 dyn.

muscle fiber supports a small amount of tension, the majority of crossbridges are continually detaching and reattaching.

The above experiments illustrate quite clearly that the size of the force drop seen upon AMPPNP addition is not a fixed amount as expected if the drop were due to the axial component of a conformational change, and they also illustrate quite nicely that crossbridges in the presence of AMPPNP are continually detaching and reattaching. Another even more graphic way of illustrating that the effect of AMPPNP upon rigor fibers is not due to the conformational change of crossbridges fixed to the actin filament is illustrated in Fig. 5. It is known that addition of AMPPNP to a rigor fiber exerting force shifts the fiber's length-tension relationship during stretch or release of the fiber to the right along the length axis. If this shift is due to crossbridges undergoing a conformational change while not detaching, then the size of the axial shift in the length-tension relationship should be the same regardless of how much tension the rigor fiber is supporting when the AMPPNP is added. On the other hand, if the apparent shift in the length-tension relationship is due to crossbridges detaching and then reattaching in positions of lesser strain, the amount of the shift should depend linearly upon the tension supported at the time of AMPPNP addition. To examine this question, the experiments of Fig. 5, *A* and *B* were performed. For the experiment of Fig. 5 *A*, a single muscle fiber was put into low-tension rigor. From this starting point of low tension (<3 dyn), a 3 nm/half-sarcomere 2 ms ramp was applied to the fiber in order to measure its length-tension relationship. After the measurement was over, and the fiber had been returned to its initial unstrained condition, AMPPNP was added. 2,400 s later, the length-tension relationship was again recorded. As Fig. 5 *A* illustrates, when the initial rigor tension is small, the size of the AMPPNP induced shift in the length-tension relationship is indistinguishable from zero. In six experiments, the average shift recorded was -0.16 ± 0.29 nm/half-sarcomere. Not only is the measured magnitude of the shift small and indistinguishable from zero, the measured sign of the effect, presumably just by chance, is in a direction different from that expected from a lengthening conformational change.

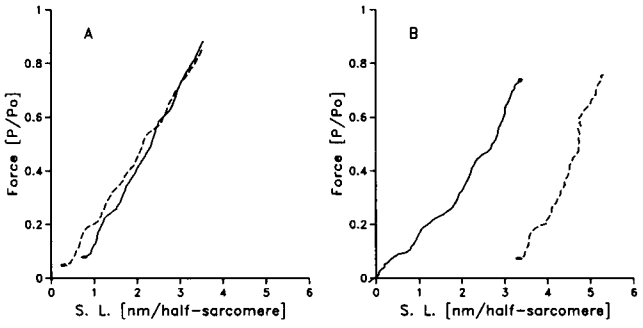

FIGURE 5 Shift in the sarcomere length–force relationship induced by adding 4 mM MgAMPPNP to a rigor fiber supporting either "nearly zero" (*A*), or "large amounts" (*B*), of tension. *Solid lines* in *A* and *B*, length–force relationship for the rigor fiber; (*dashed lines*), length–force relationship for the fiber 2,400 s after AMPPNP addition. In *A*, fiber was put into low-tension rigor. It exerted ~3.7 dyn of force. It was stretched 2 nm/half-sarcomere in 2 ms and gave the sarcomere length–force relationship shown by the solid line above. 2,400 s after AMPPNP addition, a similar stretch was applied yielding the dashed line above. Note no significant shift in the length–force relationship. We have plotted the sarcomere length–force relationship, but since sarcomere length does not change upon AMPPNP addition, very similar results would be obtained plotting the overall length–force relationship. In *B*, a fiber was put into high-tension rigor, where it supported ~30 dyn of force. A 3-nm/half-sarcomere release was applied to the muscle over 2 ms, yielding the length–force relationship shown by the solid line above. 2,400 s after subsequent AMPPNP addition, a 2-nm/half-sarcomere stretch was applied over 2 ms, yielding the dashed relationship above. Note the ~3 nm/half-sarcomere shift in the length–force relationship, corresponding to a shift of ~0.24% of muscle length. (*A*) Fiber 060288; diameters, 68 × 111 μm; P_o, 48 dyn. (*B*) Fiber 061488; diameters, 77 × 116 μm; P_o, 40 dyn.

In contrast, Fig. 5 *B* shows the effect of adding AMPPNP to a rigor fiber that is supporting a large amount of tension. Here the fiber was put into high-tension rigor, and when the force became moderately steady at ~30 dyn, a 2-nm/half-sarcomere 2-ms ramp release gave the length-tension relationship shown by the solid line. After the ramp release and restretch was complete and the fiber was at its initial length, supporting 30 dyn of tension, 4 mM AMPPNP was added. 2,400 s later, a 3-nm/half-sarcomere ramp stretch gave the length-tension relationship shown by the dashed line. It is seen that addition of AMPPNP to this fiber which was supporting a large amount of tension caused a shift in the length-tension relationship of nearly 3 nm/half-sarcomere. This is almost a factor of 2> the 1.6 nm/half-sarcomere shift (0.15%) normally attributed (Marston et al., 1976; Marston et al., 1979) to AMPPNP addition. Addition of AMPPNP to rigor fibers supporting tensions intermediate between the extremes of Fig. 5 *A* and *B* gives intermediate amounts of shift in the length-tension relationship. The main point, of course, is that the shift in the length-tension relationship caused by AMPPNP is not a fixed amount, but varies in direct proportion to the amount of rigor tension present when AMPPNP is added.

DISCUSSION

Three different hypotheses have been put forward to explain the mechanical effect of nucleotide analogue addition to rigor fibers. Marston et al. (1976) postulated

that crossbridges do not detach in the presence of AMPPNP and attributed the mechanical effect of AMPPNP addition to a conformational change in the myosin head which had a component in the axial direction. Schoenberg and Eisenberg (1985) suggested that the mechanical effect of nucleotide addition was due to the ability of nucleotide analogues to increase the crossbridge detachment rate constants above those in rigor, thereby allowing the strained crossbridges in rigor to detach and reattach in positions in lesser strain. Kuhn (1978) suggested that crossbridges could sometimes detach in the presence of nucleotide analogues, but only under conditions of high strain. In the discussion that follows, we attempt to decide between these various possibilities by considering, one at a time, a number of key factors with regard to the behavior of crossbridges having AMPPNP at the nucleotide binding site. It is hoped that by focusing on one issue at a time it will be easier to highlight general areas of agreement or disagreement.

Residual tension

Surprisingly, 15 years after the first use of AMPPNP as an ATP analogue, there is still some discussion as to the amount of tension that exists after AMPPNP is added to a rigor fiber that is supporting tension. Leigh et al., 1972, using 15 mM AMPPNP, reported a residual tension of zero after AMPPNP addition. Kuhn, 1978, using an AMPPNP concentration of 5 mM and waiting 900 s before measurement, reported a residual tension of 4 dyn. Tregear, 1988, using 1 mM AMPPNP and waiting several hours after first stretching the fiber to >40 dyn, reported a residual tension of 6 dyn. We previously reported, using 4 mM AMPPNP, a residual tension of <2 dyn (Schoenberg and Eisenberg, 1985). Here, the residual tension with 4 mM AMPPNP was 2.9 ± 0.3 dyn ($N = 12$).

Differing from the above reports of low residual tension are the reports of Clarke, 1982, and Clarke and Tregear, 1982. These studies, which used 1 mM AMPPNP, reported a residual tension of >16 dyn that persisted for more than 24 h. There is no clear explanation for this discrepancy, although there is some evidence (see Crossbridge detachment section) that the Clarke results may be artifactual.

Crossbridge detachment

Currently there are at least four independent lines of evidence suggesting that crossbridges continually detach in the presence of ATP analogues. The first of these relates to the electronmicroscopic pictures obtained by Reedy et al., 1987. They found, looking at thin sections of muscle fibers bathed in AMPPNP, that they could not interpret their images without assuming that at least some of the crossbridges had detached from the positions where they were attached in rigor and had then reattached somewhere else along the actin filament.

A second line of evidence suggesting that crossbridges detach in the presence of ATP analogues comes from examining the response of a muscle fiber to small stretches. When a small stretch is applied to a muscle fiber in the presence of AMPPNP or PP_i a force is induced (Schoenberg and Eisenberg, 1985; this report). The decay of that force has just the attributes expected if the decay is due to continually attaching and detaching crossbridges. Not only does the rate of decay increase with increasing concentration of nucleotide analogue, but the rate of decay is ~15 times faster with PP_i than the AMPPNP. This latter finding is significant because the rate constants for myosin subfragment-1 detachment from actin in solution are also ~15 times greater in the presence of PP_i compared with AMPPNP (see Schoenberg, 1988*a*, quoting unpublished data of R. Goody and also J. Biosca and E. Eisenberg). Thus the mechanical data as well as the ultrastructural data support the idea that crossbridges continually attach and detach in the presence of AMPPNP.

A third line of evidence suggesting that crossbridges detach in the presence of AMPPNP and PP_i comes from examining the apparent stiffness of muscle fibers when stiffness is measured with different speeds of stretch. With rapid stretches, (stretches so rapid that the crossbridges would not be expected to have time to detach during the stretch), fibers in PP_i or AMPPNP have the same stiffness as a fiber in rigor (White, 1970; Kuhn, 1978; Martson et al., 1976; Schoenberg and Eisenberg, 1985; Pate and Cooke, 1988). With slower stretches, (where the crossbridges have time to detach and reattach in positions of lesser strain during the stretch), the fibers appear very much less stiff (Lymn, 1975; Schoenberg and Eisenberg, 1985). The exact relationship between apparent fiber stiffness and speed of stretch (Schoenberg, 1988*a*) is particularly characteristic of continually attaching and detaching crossbridges (Schoenberg, 1985, 1988*b*), and, this relationship would be exceedingly difficult to explain in any other way.

A final line of evidence that suggests that crossbridges continually detach in the presence of ATP analogues comes from examining the effect of compounds like AMPPNP or PP_i on the number of attached crossbridges. In solution, compounds like AMPPNP and PP_i can decrease the amount of binding of myosin subfragment-1 to actin, relative to the rigor condition, by increasing the S1 detachment rate constant. In the fiber, at normal or high ionic strength, AMPPNP and PP_i likewise decrease the number of crossbridges attached (Padron and Huxley, 1984; Brenner et al., 1986; Frajer et al., 1988; Pate and

Cooke, 1988). It is, of course, reasonable to assume that the decrease in the number of crossbridges attached in fibers is, just as in solution, very likely due to AMPPNP and PP_i's ability to increase the crossbridge detachment rate-constants relative to those in rigor, and, in fact, this is what has been found experimentally (see Schoenberg and Eisenberg, 1985).

We can also ask whether there may be conditions where crossbridges in the presence of AMPPNP do not continually attach to and detach from the actin filament. Based upon the present data, this would seem very unlikely. The one particular condition where crossbridge detachment has been suggested as not occurring is the condition of low strain (Kuhn, 1978). From Fig. 4 we see, however, that even in the condition of low strain, most of the crossbridges are attaching and detaching. It thus seems likely that in the presence of AMPPNP and PP_i, the majority of crossbridges are always continually detaching and reattaching. This property of continual attachment and detachment of crossbridges is not unique to AMPPNP and PP_i. We've previously shown (Brenner et al., 1982; Schoenberg, 1988*a*,*b*) that crossbridges having ATP bound at the nucleotide binding site continually attach and detach and we have also shown (Schoenberg and Eisenberg, 1985) that even in rigor, where the crossbridges bind exceptionally tightly, there is probably also continual attachment and detachment, although in the rigor case, the attachment and detachment occurs on a much slower time-scale (see also Kuhn, 1978). Thus it seems like this phenomenon of continual attachment and detachment represents a general property of crossbridges, simply reflecting the fact that actin and myosin bind noncovalently.

Rate of crossbridge detachment

It has previously been observed that, under similar conditions, the rate constant for crossbridges detachment in the presence of AMPPNP is considerably slower than the rate constant with which myosin subfragment 1 detaches from actin in solution (Tozeren and Schoenberg, 1986; Tregear, 1988). This is, again, not a special property of the AMPPNP crossbridge, but is true for the PP_i, ADP, and also rigor crossbridge (Kuhn, 1978). Anderson and Schoenberg (1987) and Tozeren (1987) have both suggested that the reason for the slow detachment of crossbridges in fibers relative to the detachment of subfragment-1 in solution is due to the fact that crossbridges in the fiber bind, not with one, but with two heads. If both heads of the crossbridge need to detach before the crossbridge can relieve a significant fraction of the tension it supports, then the rate constant with which this occurs would indeed be much slower than the rate constant for subfragment-1 detachment from actin in solution. Experiments done under conditions where the second head doesn't bind tightly strongly support this contention (M. Schoenberg, manuscript submitted for publication).

Evidence for a conformational change when AMPPNP is added to a rigor fiber

Since, as we saw above, crossbridges having AMPPNP at the nucleotide binding site are continually attaching and detaching from the actin filament, it would seem rather difficult to obtain mechanical evidence for any conformational change that might occur at the very moment when the crossbridge head first binds AMPPNP. Thus, the data in Figs. 2, 3, and 5 are not at all what one would expect if the mechanical response to AMPPNP addition was simply due to a large scale conformational change. It is not that the data rule out a conformational change, it is simply that any force response that might be due to a conformational change is more than obscured by the force changes due to crossbridges detaching and reattaching in positions of lesser strain. These conclusions, are based upon experiments performed on rabbit psoas fibers and it is of interest to ask whether there is any mechanical evidence at all to support the idea of a conformational change when AMPPNP binds to crossbridges. We first discuss experiments done with vertebrate striated muscle and then insect flight muscle.

The evidence most often cited in support of a conformational change upon AMPPNP binding in vertebrate muscle is the work of Clarke, 1982. Clarke, 1982 reported that when 1 mM AMPPNP is added to a rabbit rigor fiber, the tension abruptly falls by 6.0 dyn (halftime, 1.0 s), and when AMPPNP is removed, the tension abruptly rises by 5.9 dyn (halftime, 13.0 s). Although such behavior is indeed suggestive of a conformational change, this finding has not been able to be reproduced by others (Kuhn, 1981; Schoenberg and Eisenberg, 1985; Tregear, 1988). Another very serious problem with the work of Clarke, 1982, on rabbit fibers is that it reports that when 1 mM AMPPNP is added to a rabbit rigor fiber, there is a residual tension of 16 dyn. This large a value for residual tension, also, has mentioned earlier, has not been reproduced by others (Kuhn, 1978; Schoenberg and Eisenberg, 1985; Tregear, 1988).

The original story with regard to a conformational change was based upon evidence from insect flight muscle. Here the evidence from a conformational change, in my opinion, is only slightly better. In contrast to the results obtained with rabbit muscle, there does appear to be a small tension rise seen upon AMPPNP removal in insect flight muscle. Unfortunately however, there appears to be considerable disagreement as to the time course of the tension change. The figures in Clarke, 1982

show a tension rerise essentially complete within 30 s while traces for insect fibers from Kuhn, 1981 and Tregear, 1988, show tension still rising as long as 1,000 s after AMPPNP addition. Additionally, the tension fall in Clarke, 1982, was reported to be totally complete within 5 s, contradicting the findings of Kuhn, 1981 and Tregear, 1988, where tension was found to be falling well beyond 5 s.

Should the very small tension rerise seen in insect flight muscle upon AMPPNP addition be considered as evidence for a conformational change in insect flight muscle? This rerise, seen by Marston et al., 1976; Kuhn, 1981; Clarke, 1982; and Tregear, 1988, has been reported as being 1, 6, 6.3, and 3 dyn, respectively. It seems then, that the force rerise seen in insect flight muscle upon AMPPNP removal, while real, is both small and somewhat variable in magnitude. A very serious problem in interpreting the force rerise seen in insect fibers as evidence for a large scale conformational change is that in most studies the force rerise occurs on a time scale of 500–1,000 s. In other words, the force rise seen in insect fibers upon AMPPNP removal appears to be more than two orders of magnitude slower than the rate at which one would expect AMPPNP to diffuse out of the fiber. In light of these considerations, one might conclude that there is virtually no convincing mechanical evidence in support of the notion that AMPPNP produces a significant conformational change upon binding to the rigor crossbridge and evidence about such a conformational change clearly will have to come from more structural techniques such as x-ray diffraction or electronmicroscopy.

In summary, then, the available evidence from rabbit psoas fibers overwhelmingly supports the idea that the apparent lengthening of rigor muscle fibers upon AMPPNP addition is due to AMPPNP's ability to significantly increase the crossbridge detachment rate constants. This enables the strained crossbridges of rigor to then more quickly detach from the actin filament and reattach in positions of lesser strain. The behavior of crossbridges in the presence of AMPPNP is well described by the equilibrium crossbridge model of Schoenberg, 1985, with the exception that the rate constants for crossbridge detachment are somewhat slower than the rate constants with which myosin subfragment-1 detaches from actin in solution. As suggested by Tozeren, 1987 and also Anderson and Schoenberg, 1987, this difference in rate constants may largely be due to the fact that in the fiber the crossbridges bind with two heads. If Tozeren, 1987, and Anderson and Schoenberg, 1987, are correct, then it is possible to understand a very large fraction of the mechanical behavior of crossbridges in terms of the known kinetic properties of actin and myosin in solution. Questions that remains are whether AMPPNP induces a large scale conformational change in the crossbridge head upon binding and whether insect fibers behave differently from rabbit fibers. It is now clear, however, that resolution of the first question must depend upon information garnered from ultrastructural techniques and not from mechanical measurements.

I am greately indebted to Dr. R. T. Tregear (and other members of his department) for the great friendship and hospitality extended to me and my family while I was a guest in his laboratory for five months during 1985. Most of the ideas for the experiments described in this paper grew out of the many stimulating and helpful discussions held with Dr. Tregear during this time.

Received for publication 7 November 1988 and in final form 13 February 1989.

REFERENCES

Barrington-Leigh, J., K. C. Holmes, H. G. Mannhertz, G. Rosenbaum, F. Eckstein, and R. S. Goody. 1972. Effects of ATP analogs on the low-angle X-ray diffraction pattern of insect flight muscle. *Cold Spring Harbor Symp. Quant. Biol.* 37:443–447.

Brenner, B., L. C. Yu, L. E. Greene, E. Eisenberg, and M. Schoenberg. 1986. Ca^{2+}-sensitive cross-bridge dissociation in the presence of magnesium pyrophosphate in skinned rabbit psoas fibers. *Biophys. J.* 50:1101–1108.

Clarke, M. L., and R. T. Tregear. 1982. Tension maintenance and crossbridge detachment. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 143:217–219.

Eastwood, A. B., D. S. Wood, K. L. Bock, and M. M. Sorenson. 1979. Chemically skinned mammalian skeletal muscle. 1. The structure of skinned rabbit psoas. *Tissue & Cell.* 11:553–566.

Fajer, P., E. A. Fajer, N. Brunsvold, and D. D. Thomas. 1988. Effects of AMPPNP on the orientation and rotational dynamics of spin-labeled muscle cross-bridges. *Biophys. J.* 53:513–524.

Kawai, M., and P. W. Brandt. 1976. Two rigor states in skinned crayfish single muscle fibers. *J. Gen. Physiol.* 68:267–280.

Kuhn, H. J. 1978. Cross-bridge slippage induced by the ATP analogue AMP-PNP and stretch in glycerol-extracted fibrillar muscle fibers. *Biophys. Struct. Mech.* 4:159–168.

Kuhn, H. J. 1981. The mechanochemistry of force production in muscle. *J. Musc. Res. Cell Motil.* 2:7–44.

Lymn, R. W. 1975. Low-angle X-ray diagrams from skeletal muscle: the effect of AMP-PNP, a non-hydrolizable analog of ATP. *J. Mol. Biol.* 99:567–582.

Marston, S. B., C. D. Rodger, and R. T. Tregear. 1976. Changes in muscle crossbridges when β,γ-imido-ATP binds to myosin. *J. Mol. Biol.* 105:263–276.

Marston, S. B., R. T. Tregear, C. D. Rodger, and M. L. Clarke. 1979. Coupling between the enzymatic site of myosin and the mechanical output of muscle. *J. Mol. Biol.* 128:111–126.

Padron, R., and H. E. Huxley. 1984. The effect of the ATP analogue AMPPNP on the structure of crossbridges in vertebrate skeletal muscles: x-ray diffraction and mechanical studies. *J. Musc. Res. Cell Motil.* 5:613–655.

Pate, E., and R. Cooke. 1988. Energetics of the actomyosin bond in the filament array of muscle fibers. *Biophys. J.* 53:561–573.

Reedy, M. C., M. K. Reedy, and R. S. Goody. 1987. The structure of insect flight muscle in the presence of AMPPNP. *J. Musc. Res. Cell Motil.* 8:473–503.

Schoenberg, M. 1988. The kinetics of weakly- and strongly-binding crossbridges: implications for contraction and relaxation. *In* Molecular Mechanism of Muscle Contraction. H. Sugi and G. H. Pollack, editors. Plenum Publishing Corp., New York. 189–202.

Schoenberg, M. 1988. Characterization of the myosin adenosine triphosphate (M · ATP) crossbridge in rabbit and frog skeletal muscle fibers. *Biophys. J.* 54:135–148.

Schoenberg, M., and E. Eisenberg. 1985. Muscle cross-bridge kinetics in rigor and in the presence of ATP analogues. *Biophys. J.* 48:863–871.

Schoenberg, M., and E. Eisenberg. 1987. ADP binding to myosin crossbridges and its effect on the crossbridge detachment rate-constants. *J. Gen. Physiol.* 89:905–920.

Tozeren, A. 1987. The influence of doubly attached crossbridges on the mechanical behavior of skeletal muscle fibers under equilibrium conditions. *Biophys. J.* 52:901–906.

Tozeren, A., and M. Schoenberg. 1986. The effect of cross-bridge clustering and head-head competition on the mechanical response of muscle fibers under equilibrium conditions. *Biophys. J.* 50:875–884.

Tregear, R. T. 1988. Mechanical properties of demembranated muscle fibers in the presence of MgAMPPNP. *In* Molecular Mechanism of Muscle Contraction. H. Sugi and G. Pollack, editors. Plenum Publishing Corp., New York. 513–526.

White, D. C. S. 1970. Rigor contraction and the effect of various phosphate compounds on glycerinated insect flight and vertebrate muscle. *J. Physiol. (Lond.)* 208:583–605.