POSSIBLE COOPERATIVITY IN CROSSBRIDGE DETACHMENT IN MUSCLE FIBERS HAVING MAGNESIUM PYROPHOSPHATE AT THE ACTIVE SITE

MATTHEW L. ANDERSON AND MARK SCHOENBERG
Laboratory of Physical Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT When rabbit psoas muscle fibers bathed in solutions containing the ATP analogue magnesium pyrophosphate ($MgPP_i$) are first stretched rapidly and then held isometric, a force is generated during the stretch which decays during the subsequent isometric period (Schoenberg, M., and E. Eisenberg. 1985. *Biophys. J.* 48:863–871). Previously we showed that the force decay is due to crossbridge heads detaching and reattaching in positions of lesser strain, the rate of decay of force reflecting the crossbridge detachment rate constants. Since the crossbridge detachment rate constants with $MgPP_i$ bound to the active site are so much faster than without analogue bound, at subsaturating concentrations of analogue, if the heads act independently and nucleotide association and dissociation is rapid, the rate of force decay should simply be proportional to the number of heads with bound analogue. That is, the analogue concentration dependence of the rate of force decay should have the same form as the Michaelis-Menten equation. Here we report that the concentration dependence of the rate of force decay is not described by the Michaelis equation, but is instead sigmoidal. This suggests possible cooperativity in the detachment of the crossbridge heads, the amount of cooperativity being described by an interaction coefficient of ~2. One idea put forward to explain the data is that both of the heads of a crossbridge may need to bind analogue before the crossbridge can relax a substantial fraction of the tension it supports.

INTRODUCTION

Previously we demonstrated in relaxed fibers the existence of "weakly-binding rapid-equilibrium" myosin crossbridges which continually attach to and detach from the actin filament (Brenner et al., 1982; Schoenberg et al., 1984; Schoenberg, 1987). We also showed similar behavior with ATP analogues such as AMP-PNP and pyrophosphate at the active site (Schoenberg and Eisenberg, 1985; Brenner et al., 1986), even though the equilibrium between attached and detached crossbridge moieties was shifted more towards attachment and the attachment and detachment rate constants were much slower. We analyzed this behavior (Schoenberg, 1985) and showed that when attached equilibrium crossbridges are strained by a rapid stretch, a tension is induced that decays with rates that reflect the crossbridge detachment rate constants. This is because the tension induced by stretch is relieved by strained crossbridges detaching and then reattaching in positions of lesser strain.

If the individual crossbridge heads are acting independently, and the rate of nucleotide binding and dissociation is rapid, then it is possible to show that the concentration dependence of the rate of force decay should have the same form as the Michaelis-Menten equation. Because crossbridge heads may not always act independently, and because previous evidence from our laboratory suggested that the rates of force decay in the presence of $MgPP_i$ might not be a Michaelis-Menten function of analogue concentration (Schoenberg and Eisenberg, 1985), we have explored in greater detail the concentration dependence of the rate of force decay in the presence of $MgPP_i$. We find that only the binding of $MgPP_i$, not free PP_i, can increase the crossbridge detachment rate constants. We also find that the concentration dependence of the rate of force decay does not follow the simple Michaelis expression but is sigmoidal. We can explain the data in terms of a cooperative model in which it is postulated that both of the heads of a myosin crossbridge need to bind analogue before the crossbridge is likely to relax a substantial fraction of the tension it supports.

METHODS

Individual fibers from the lateral edges of rabbit psoas muscles were isolated, demembranated (made permeable

to the bathing medium), and mounted as described in Schoenberg and Eisenberg, 1985. The sarcomere length was adjusted to 2.5 μm and the temperature to 5°C. Fibers were put into rigor as described in Schoenberg and Eisenberg, 1985 and then equilibrated with solutions containing different concentrations of pyrophosphate. The base solution contained 10 mM imidazole, 3 mM EGTA, 90 mM Kpropionate, and 0.5 mM dithiothreitol, pH 7.0. The total concentration of $MgCl_2$ was always 2 mM more than the total concentration of PP_i. Ionic strength was kept at ~110 mM by removal of 5 mM Kpropionate for each 1 mM $MgPP_i$ added. Upon replacement of the rigor solution with one containing pyrophosphate and magnesium, the tension extant in rigor decayed very close to zero, i.e., to values < 0.05 P_0, where P_0 is the maximum Ca^{2+}-activated tension.[1]

The basic experimental maneuver was to apply a quick, 2-nm/half-sarcomere stretch to the fiber (rise-time, 0.5 ms). This induced a force, the decay of which was followed during the subsequent period when the fiber was held isometric. As reported previously, and as seen in Fig. 1, the force decay is multiexponential, with the fastest and slowest decay rate constants differing by two to three orders of magnitude. Because of this, it was convenient to plot force versus the logarithm of time after stretch and to use the reciprocal of the half-time for tension decay as a measure of the rate of decay. When there are multiple exponentials, use of the half-time as a measure of the rate of the decay is somewhat arbitrary. However, since within experimental accuracy the decay curves plotted versus the log of time appear to have similar shape regardless of the concentration of PP_i, 1/half-time is a useful measure of rate of decay because it can be more precisely determined than can rate constants obtained by fitting to multiple exponentials. The choice of 1/half-time as a measure of rate is not critical for the interpretation of the experiments as the results were essentially the same regardless of whether the rate of decay was calculated from the time for the force to decay by one-half, three-quarters, or one-quarter.

The mechanical movements were controlled using feedback from a sarcomere length detector to eliminate artifacts due to end compliance of the glued fibers (Schoenberg and Eisenberg, 1985). For each experiment, observations were made for concentrations of PP_i ranging from 0.25 mM, where there was little effect, to 4 mM, the maximum concentration where $MgPP_i$ could reliably be kept in solution. To improve the accuracy of the measurements, three to four individual traces from repeat identical maneuvers were generally averaged before the decay half-time was measured.

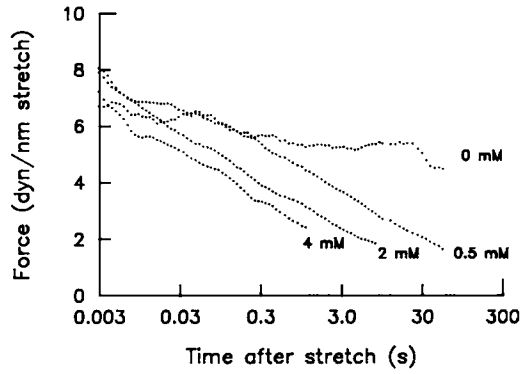

FIGURE 1 Force decay after stretch of a single rabbit psoas fiber bathed in solutions containing varying amounts of $MgPP_i$. (Ordinate) Force per nm/stretch—stretch amplitude, nominally 2-nm/half-sarcomere. (Abscissa) Time after start of stretch (logarithmic scale). The concentration of total PP_i for each solution is given next to the appropriate curve. For each solution, the total Mg was in excess by 2 mM.

RESULTS

Fig. 1 shows a typical set of curves for the decay of force after stretch from a single rabbit psoas fiber bathed in solutions containing 0, 0.5, 2, and 4 mM PP_i. It is seen that in the absence of PP_i the rate of decay is quite slow, but with increasing concentration of analogue, the rate of force decay is greatly accelerated. It is, in fact, $MgPP_i$, and not PP_i itself, that accelerates the rate of force decay. We find (data not shown) that in the absence of magnesium, increasing PP_i has little or no effect.

From experiments like that in Fig. 1 the rate of force decay was determined at a variety of different concentrations of PP_i. The solid circles in Fig. 2 summarize the data. The vertical lines give standard errors of the mean and the numbers in parentheses give the number of measurements for each point. Since we found that $MgPP_i$ and not PP_i is the moiety responsible for the increase in the rate of force decay, the concentration dependence data should be plotted versus [$MgPP_i$]. This is done in Fig. 3. While Mg^{+2} binds quite tightly to PP_i^{-4}, at pH 7.0 the dominant ionic species of PP_i is more than 85% HPP_i^{-3} (Martell and Schwarzenbach, 1956; Irani, 1961; Frey and Stuehr, 1972; Smith and Martell, 1975). The binding constant of Mg^{+2} to HPP_i^{-3} (in a solution with an ionic strength of ~100 mM) is only 1.1–1.5 × 10^3 M^{-1} (Lambert and Watters, 1957; Frey and Stuehr, 1972). For reasons discussed later, the $MgPP_i$ concentration was calculated using the lowest of the literature values quoted.

The interaction between nucleotide analogue, cross-bridge head, and actin, to a first approximation, is

[1]This observation, which we reported previously (Schoenberg and Eisenberg, 1985), is surprising in light of the report of Clarke and Tregear (1982) that, after addition of millimolar amounts of the ATP analogue MgAMP-PNP to a rigor fiber, substantial tension, ~20 dyn/fiber, can persist. We have been unable to confirm their finding. We find only a small remaining tension subsequent to mM MgAMP-PNP addition (<6 dyn for a fiber with P_0 > 60 dyn) and even less remaining tension upon addition of mM $MgPP_i$ (<3 dyn, P_0 > 60 dyn).

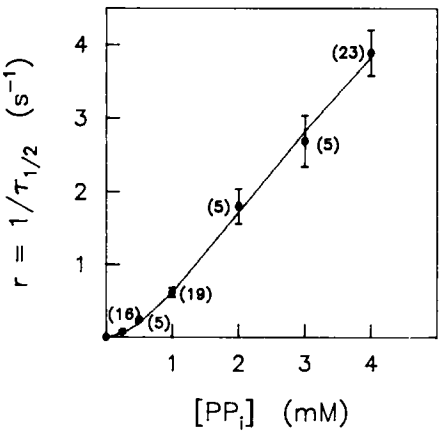

FIGURE 2 The rate of force decay after stretch as a function of total PP_i concentration. The rate of force decay is expressed as $1/\tau_{1/2}$, $\tau_{1/2}$ being the time for an individual force decay curve (Fig. 1) to decay by 50%. The numbers in parentheses give the number of different curves analyzed for each point. The vertical bars show ± the standard deviation of the mean. The solid line drawn is the best fit to the data of Eq. 3 and gave a value of $n = 1.6 \pm 0.2$. The data were not analyzed further since the rate of force decay was shown to be a function of $[MgPP_i]$, not $[PP_i]$. All fitting was done using a nonlinear least-squares procedure based upon Marquardt's compromise (Marquardt, 1963). The actual fitting was not to the displayed variable, $r = 1/\tau_{1/2}$, but to the experimentally obtained and presumably Gaussian distributed variable, $\log_{10} \tau_{1/2}$. The data, fits, and SEMs were then transformed for display as rates.

described as

$$AM + L \underset{k_{-1}}{\overset{k_1 L}{\rightleftharpoons}} AML \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A + ML. \qquad (1)$$

In Eq. 1, A stands for actin, M, the myosin head, and L, ligand. It is possible to show that if the crossbridge heads all act independently, and k_{-1} is fast compared with k_2, the rate of force decay, r, will be proportional to $k_2 \cdot [AML]$, where [AML] is the number of heads with bound analogue. If we assume (*a*) that the binding of analogue to attached heads is non-cooperative (Pate and Cooke, 1985; Schoenberg and Eisenberg, 1987), and (*b*) for the conditions studied, most of the crossbridge heads are attached to actin (Marston et al., 1976; Schoenberg and Eisenberg, 1985; Brenner et al., 1986), then [AML] will be a Michaelis function of ligand concentration and r, the rate of force decay, will be simply

$$r = \frac{r_{max}\,[L]}{k_d + [L]}. \qquad (2)$$

r_{max} is the maximal force decay rate, [L] is analogue concentration, and k_d is the dissociation constant for analogue binding.

The dotted curves in Fig. 3 *A* show an attempt to fit the concentration dependence data to the Michaelis relationship using a wide array of possible values for the analogue dissociation constant. The important point to note is that the data cannot be described by a Michaelis function,

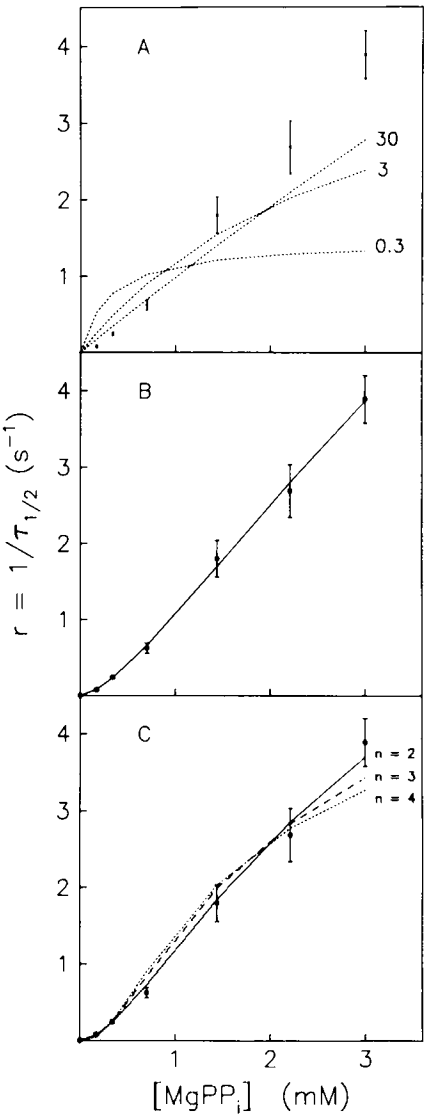

FIGURE 3 Rate of force decay after stretch as a function of $MgPP_i$ concentration. *A–C* show the same data fitted to different models. Ordinate, rate of decay expressed as $1/\tau_{1/2}$, $\tau_{1/2}$ being the time for an individual force decay curve (Fig. 1) to decay by 50%. Abscissa, concentration of $MgPP_i$ taking 1×10^3 M^{-1} as the effective binding constant of Mg to PP_i. The vertical bars show ± SEM. *A* shows an attempt to fit the data to Michaelis functions with k_d values of 0.3, 3, and 30 mM, these numbers appearing to the right of the appropriate curves. Clearly the data are not described by Michaelis functions with k_d's in this range and increasing k_d to absurdly large values improves the fit only slightly. *B* shows the best fit of the data to Eq. 3, parameter values being $n = 1.6 \pm 0.2$, $k_d = 5.3 \pm 4.6$ mM, $r_{max} = 19 \pm 14$ s^{-1}. *C* illustrates attempts to fit the data to Eq. 3 with $n = 2$, 3, and 4. For $n = 2$, $k_d = 1.9 \pm 0.2$ mM and $r_{max} = 9.7 \pm 1.4$ s^{-1}; for $n = 3$, $k_d = 0.67 \pm 0.05$ mM and $r_{max} = 6.3 \pm 0.6$ s^{-1}; for $n = 4$, $k_d = 0.39 \pm 0.02$ mM and $r_{max} = 5.3 \pm 0.4$ s^{-1}. The fit for $n = 2$ was indistinguishable at the 95% level from the best fit but those for $n = 3$ and 4 were not.

regardless of the assumed value of k_d. Instead, what we see is that small concentrations of $MgPP_i$ produce little effect on the force decay rate whereas moderate (millimolar) concentrations produce a more than proportional effect. This suggests that one or more of the assumptions made above is not valid.

One assumption that may easily not be valid in the limit is the assumption of totally independent crossbridge heads. Keeping in mind that each crossbridge has two heads, it is conceivable that both heads might need to dissociate before it would be easy for either of them to relieve the tension it supports. Another possibility, one suggested by Kuhn (1978), is that crossbridge heads might bind in cooperative clusters. In both of these cases, if n heads needed to dissociate before much tension could be relieved, it is possible to show that, under non-dissociating conditions, when k_{-1} is fast, the rate of force decay is described by

$$r = \frac{r_{max}\,[L]^n}{(k_d + [L])^n}. \quad (3)$$

Fig. 3 *B* shows that Eq. 3 gives quite a good fit to the data. The best fit to the data gives parameter values k_d = 5.3 ± 4.7 mM, r_{max} = 19 ± 13 s^{-1}, and n = 1.6 ± 0.2. r_{max} and k_d are not well determined from the data because (*a*) three parameters are being fitted simultaneously and (*b*) experimentally, it is not possible to reach high enough concentrations of $MgPP_i$ to significantly begin to saturate the effect. Previous work of Pate and Cooke (1985) and Sleep and Glyn (1986) suggests that the k_d for $MgPP_i$ binding to attached crossbridges is ~2.5 mM. The fit, k_d = 2.5 mM, r_{max} = 11.2 s^{-1}, and n = 1.8, is indistinguishable, at the 95% level, from the best fit.

Fits of Eq. 3 to the data for n = 2, 3 and 4 are shown in Fig. 3 *C*. n = 2 still produces quite a good fit but n = 3 and n = 4 give progressively worse fits.

DISCUSSION

The major finding of this paper is that the rate of force decay after stretch in the presence of $MgPP_i$ is not a Michaelis function of $MgPP_i$ concentration. What we instead find is that the rate of force decay seems to depend sigmoidally upon $MgPP_i$ concentration. Low concentrations of $MgPP_i$ produce relatively little effect and moderate (millimolar) concentrations produce a more than proportional effect. We were concerned whether systematic errors might be distorting the data to give it its sigmoidal appearance but have not found such errors. In fact, the known systematic errors have just the opposite effect. That rigor fibers can sometimes show significant force decay on the time scale of minutes will tend to make the rates of decay at low concentration of PP_i appear faster than they really are. That with high concentrations of PP_i there may be some force decay during the period of rapid stretch will tend to make the rates at high PP_i appear slower than they are. Both of these factors serve to make the data actually appear less sigmoidal than it is.

Another factor that would tend to make the data appear less sigmoidal is related to the fact that in calculating the $MgPP_i$ concentration for Fig. 3, we deliberately took the lowest literature value we found for the binding constant of Mg to PP_i. It can be shown that the weaker the binding of Mg to PP_i, the less sigmoidal Fig. 3 will be relative to Fig. 2. We chose the weakest reported value specifically to avoid making the sigmoidicity appear larger than it really is. Thus, Fig. 3 may underestimate the sigmoidicity in the concentration dependence data and the sigmoidal shape of the concentration dependence relationship is real.

Given that the sigmoidicity in the concentration dependence data is real, what might this imply? We have seen that a reasonable way of explaining the data is to assume that not all crossbridge heads act totally independently. That is, one may explain the data by postulating that both heads of a crossbridge need to bind $MgPP_i$ before either can relocate to a position of lesser strain and relax the tension it supports. This leads to a concentration dependence of the rate of force decay described by Eq. 3 with n = 2. As Fig. 3 *C* shows, this describes the data well. Note that we have not assumed that the first head of a crossbridge that binds analogue must remain forever fixed to actin until the second head also binds analogue. We only assume that when either of the two attached heads detaches, it has a high probability of reattaching back to the same actin from which it just detached, unless the other head also detaches at the same time. If, experimentally, n is identically 2, the probability of a head attaching back to the same actin needs to be 100% to explain the data. That the best fit to the data gives $n \sim 1.6$ suggests, if the above ideas are correct, that the actual probability is probably somewhat less extreme.

We also attempted to fit our data to Eq. 3 with n = 3 or n = 4 (Fig. 3 *C*). These cases correspond to models where the number of cooperatively interacting heads is >2. While these curves give somewhat less good fits to the data, and k_d values in somewhat less good agreement with previous studies (Pate and Cooke, 1985; Sleep and Glyn, 1986), the data are perhaps not so accurate as to be able to definitively rule out models of this sort.

The idea that the sigmoidicity in the concentration dependence data is due to cooperativity between the heads is an intriguing one because it could help explain, in part, another phenomenon. In rigor and in the presence of compounds such as $MgPP_i$ or MgAMP-PNP, some of the crossbridge detachment rate constants estimated from force decay are quite slow compared with the detachment rate constant of myosin subfragment 1 from actin in solution (see Kuhn, 1978; Tozeren and Schoenberg, 1986). If indeed both heads of a crossbridge need to detach simultaneously before force can decay, the force decay rate could easily be slower than the rate at which a single subfragment 1 head detaches from actin in solution.

A way in which one could attempt to explain the sigmoidicity in the concentration dependence data without

postulating interaction between heads, would be to assume that the rate of nucleotide dissociation, k_{-1}, is slow compared with k_2, the rate of crossbridge dissociation. In this case, for low concentrations of L, the rate of force decay would be dominated by k_1L, while at high concentrations, it would hyperbolically approach k_2. By judicious choice of k_{-1} and k_2, it would be possible to mimic the experimentally observed behavior. However, this explanation for the data seems much less likely than the previous one because (*a*) it would work only if the presumably unrelated rate constants k_2 and k_{-1} have just the right relationship to one another, and (*b*) in solution at least, the rate constant for analogue dissociation from acto-subfragment 1 is fast, not slow, relative to the rate constant for the dissociation of subfragment 1 from actin (Trybus and Taylor, 1982; Konrad and Goody, 1982).

Finally, further support for the idea that the two heads of a crossbridge may influence each other comes from work of Hackney and Clark (1984). They compared the ATPase rate of the two-headed myosin fragment, heavy meromyosin, with that of the single-headed myosin fragment, subfragment 1. They found that, per head, at high ATP concentrations heavy meromyosin hydrolyzes ATP at the same rate as subfragment 1, but at low ATP concentrations, heavy meromyosin hydrolyzes ATP at least twice as fast as subfragment 1. A good fit to the data was obtained only by assuming that at low ATP concentrations, heads without ATP bound will keep heads with ATP bound closer to the actin filament. While this situation is not entirely analogous with that presented here, it does suggest that it is not always correct to think of the double-headed myosin molecule as simply two subfragment ones.

In summary then, we have shown that the concentration dependence of the rate of force decay after stretch is not described by a Michaelis expression, but is instead, sigmoidal. We have shown that one way of explaining this is to assume that when a crossbridge is bound by two heads, the first head to detach may not be completely free to reposition itself in a position of lesser strain unless the second head also detaches at the same time. This idea that the behavior of a crossbridge is more than just the sum of the behavior of two independent heads has been useful in explaining a number of recent findings (Pate and Cooke, 1986; Brunsvold et al., 1986; Chaen et al., 1986) and more experimentation will help decide whether alternative explanations for the available data can be ruled out. Of particular interest will be experiments done at higher ionic strength where the strength of crossbridge binding is such as to cause more heads to be detached at equilibrium. However, experiments at higher ionic strength may be more complicated to interpret because tropomyosin effects may become more important (see Brenner et al., 1986).

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