Formation of ATP-insensitive weakly-binding crossbridges in single rabbit psoas fibers by treatment with phenylmaleimide or para-phenylenedimaleimide

Vincent A. Barnett, Alison Ehrlich, and Mark Schoenberg
Laboratory of Physical Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health
Bethesda, Maryland 20892

ABSTRACT Chaen et al. (1986. J. Biol. Chem. 261:13632–13636) showed that treatment of relaxed single muscle fibers with para-phenylenedimaleimide (pPDM) results in inhibition of a fiber's ability to generate active force and a diminished ATPase activity. They postulated that the inhibition of force production was due to pPDM's ability to prevent crossbridges from participating in the normal ATP hydrolysis cycle. We find that the crossbridges produced by pPDM treatment of relaxed muscle cannot bind strongly to the actin filaments in rigor, but do bind weakly to the actin filaments in the presence and also absence of ATP. After pPDM treatment, fiber stiffness, as measured using ramp stretches of varying duration, is ATP-insensitive and identical to that of untreated relaxed fibers (both at high [165 mM] and low [40 mM] ionic strength). These results suggest that the pPDM-treated crossbridges, in both the presence and absence of ATP, are locked in a state that resembles the weakly-binding myosin · ATP state of normal crossbridges. Their resemblance to the ATP-crossbridges of relaxed untreated fibers is quite strong; both bind to actin about equally tightly and have similar attachment and detachment rate constants. We also found that crossbridges are locked in a weakly-binding state after treatment with *N*-phenylmaleimide (NPM). In muscle fibers, this method of producing weakly-binding crossbridges appears preferable to pPDM treatment because, unlike treatment with pPDM, it does not increase the fiber's resting tension and stiffness and it does not disrupt the titin band seen on SDS-PAGE.

INTRODUCTION

Vertebrate skeletal muscle contains a complex architecture of interacting proteins. In muscle fibers, "thin" actin filaments interdigitate with "thick" bipolar myosin filaments in a double hexagonal lattice. Actin and myosin, the primary muscle proteins, interact in a cyclical fashion to produce physical work and force, presumably via conformational changes coupled to the energy derived from actin-activated adenosine-5′-triphosphate (ATP) hydrolysis by myosin. In pioneering work, Chaen et al. (1986) showed that treatment of fibers with the bifunctional alkylating agent, *N,N′*-1,4-phenylenedimaleimide (pPDM), results in inhibition of their ability to produce force. Although there is little other fiber work concerning the effect of pPDM, there is a considerable body of knowledge from solution studies about the effects and mechanism of action of pPDM on myosin.

Myosin, the major protein constituent of the thick filament, has a long (~140 nm) double-stranded alpha-helical "tail" and two globular "head" regions. Each of the myosin heads contains actin and nucleotide binding sites. Near the COOH-terminal region of each head there are two highly-reactive sulfhydryl groups designated SH1 and SH2. Modification of either of these groups individually with an alkylating reagent inhibits myosin's ATPase activity in the absence of divalent cations (Seidel, 1969), while modification of SH1 alone simultaneously stimulates the ATPase activity in the presence of divalent cations (Sekine and Kielley, 1964; Burke et al., 1973). Blockage of both SH groups abolishes all myosin ATPase activity (Sekine and Kielley, 1964; Yamaguchi and Sekine, 1966). While there is another class of SH groups located in the region of the myosin head-tail junction that react with alkylating agents at elevated pH (without change of ATPase activity), most of the myosin molecule's 42 cysteine residues are inaccessible to SH-reactive reagents except under conditions that lead to denaturation of the protein (Schaub et al., 1975; Schaub et al., 1979).

Alkylation of purified myosin with pPDM in solution crosslinks the SH1 and SH2 sulfhydryls (Reisler et al., 1974), resulting in a myosin that binds to actin in the absence of ATP as weakly as it does in the presence of ATP and as weakly as untreated myosin binds in the presence of ATP (Muhlrad et al., 1976). This finding has been interpreted as suggesting that the crosslinking mechanically locks the myosin in a configuration resembling that of the weakly-binding M · ATP state (Wells et al., 1979; Wells et al., 1980). In a parallel fashion, we find that pPDM treatment of muscle fibers results in creation of crossbridges locked in a state functionally similar to the weakly-binding myosin · ATP crossbridge state of

Dr. Barnett's present address is Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455.

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normal relaxed fibers. In addition, we find that the monofunctional alkylating agent *N*-phenylmaleimide (NPM) similarly produces M · ATP crossbridges in fibers. Our results fit well those of Chaen et al. (1986) who found that treatment with pPDM inhibits an active muscle fiber's stiffness and ability to produce force.

METHODS

Treatment of the muscle fibers

Single skinned rabbit psoas fibers were mounted between a modified Akers 801 force transducer (Sensonor; Horten, Norway) and a displacement driver as described previously (Schoenberg, 1988a). The fibers were treated with alkylating agent in either 165 mM ionic strength relaxing or rigor solution (Table 1). Stock solutions of 30 mM pPDM or NPM (Aldrich; Milwaukee, WI) in dimethylformamide (stored at −20°C for periods of up to 1 mo) were added to the 2-ml fiber bath in the correct amount to make the final concentration of alkylating reagent 200 μM for pPDM or 100 μM for NPM. After thorough mixing, the fiber was incubated without stirring for the desired treatment period. To quench the alkylating reaction at the end of the treatment period, the fiber was rinsed thoroughly in a solution similar to the incubating one except that alkylating reagent was replaced with 0.5 mM dithiothreitol (DTT). Because this procedure involved adding a small amount of dimethylformamide, it was shown in control experiments that adding comparable amounts of dimethylformamide without alkylating reagent had no effect (data not shown).

Measuring the time course of pPDM and NPM treatment

In determining the time course of the pPDM- or NPM-induced changes in muscle fiber stiffness, the relaxed and rigor stiffnesses of the untreated fibers were first measured as a control. The fiber was then incubated with alkylating reagent for a specified period of time. At the end of the treatment period, the reaction was quenched with DTT and the relaxed and rigor stiffness again measured. This sequence was repeated several times consecutively until the accumulated exposure time to alkylating agent was 60 min. Treatment of a relaxed fiber for 60 min with pPDM or NPM reduces its rigor stiffness to the level of the relaxed stiffness (see Results). Therefore, in experiments where it was desirable that all the crossbridges be reacted, the fibers were incubated with alkylating reagent for 60 min.

Mechanical characterization of the fibers

In measuring the response to step stretches, the fibers were stretched ~2 nm/half-sarcomere in 0.4 ms and then held isometric for measurement of the force response. The ionic strength for the step stretch experiments was 165 mM. In experiments where chord stiffness (Schoenberg, 1985) was measured, a stretch amplitude of 2 nm/half-sarcomere was used and the chord stiffness for nine different durations of the stretch ranging from 10^{-4} to 1 s was measured. The ionic strength for the chord stiffness experiments was 40 mM.

TABLE 1 Millimolar concentrations of solution constituents

Solution	KCl	EGTA	$MgCl_2$	MgATP	Imidazole
165 mM relaxing	125	4	1	4	10
165 mM rigor	136	4	4	—	10
40 mM relaxing	—	4	1	4	10
40 mM rigor	11	4	4	—	10

All solutions were pH 7.0 ± 0.1 at 5°C and contained 0.5 mM DTT. Solution name gives total ionic strength.

Preparation of gel samples

For gel studies, small muscle bundles 1–2 mm in diameter and 1 cm in length were attached with a small amount of cyanoacrylic glue between two fixed stainless steel posts. The length-fixed bundles were then incubated for 1 h in 0°C relaxing buffer containing either 200 μM pPDM or 100 μM NPM. After the appropriate incubation period, treatment of the fibers was terminated by bathing the fibers in relaxing solution containing 0.5 mM DTT. SDS-PAGE was done using a protocol based on that of Somerville and Wang (1981). The fiber bundles were transferred into 200 μl of cold (~4°C) homogenization buffer and then homogenized in an ice-cold ground glass homogenizer. The homogenization buffer contained 2 mM EDTA, and 20 mM tris-(hydroxy-methyl)-aminomethane (Tris), pH 8. Protein concentrations of the homogenates were determined using the Lowry assay and the final concentration adjusted to 2 mg/ml. To denature the proteins before application to the gel, they were mixed 1:1 with 2× electrophoresis sample buffer (140 mM β-mercaptoethanol, 2 mM EDTA, 2% (vol/vol) SDS, 20% (vol/vol) glycerol, 0.02% (wt/vol) bromphenol blue, 20 mM Tris, pH 8) and then heated for 2 min in a boiling water bath.

SDS-PAGE

10–40 μg samples of homogenized fibers were applied via a Hamilton syringe to a 2–12% continuous linear gradient polyacrylamide (Fairbanks et al., 1971) gel as modified by Somerville and Wang (1981). The gels were run in a Hoeffer SE600 chamber (Hoeffer Scientific, San Francisco, CA) at 250 V for approximately 2.5 h. Upper and lower running buffers were identical and contained 20 mM NaAcetate, 2 mM EDTA, 0.1% (vol/vol) SDS, 40 mM Tris pH 7.4. The gels were stained either for 1 h or overnight while gently shaking in a solution of 0.063% (wt/vol) Coomassie Blue R-250 (Bio-Rad, Richmond, CA), 10% acetic acid, and 50% methanol. They were destained with a 1 h soak in 10% acetic acid/50% methanol, followed by 3–4 one hour soaks in 7.5% acetic acid/15% methanol.

Site of pPDM and NPM binding

In order to determine the site of pPDM and NPM binding, fibers were treated as described except ^{14}C-pPDM (a gift of Dr. Lois Greene of NHLBI) or ^{14}C-NPM (Synthesized by Amersham Corp., Arlington Heights, IL) was used. The dried gels were scanned for ^{14}C using an AMBIS Radioanalytic Imaging System (AMBIS Systems Inc., San Diego, CA). The location of radioactivity was compared with the known migration of the muscle proteins on SDS-PA gels.

Western blotting

Western blots of SDS-PA gels were performed by transferring the proteins to PVDF paper using a current of 1.0 A for 3.5 h. The transfer buffer contained 2.5 mM Tris, 19.2 mM glycine, 0.1% SDS, 20% (vol/vol) methanol, pH 7.5. After the transfer, the nitrocellulose paper was incubated overnight, at 4°C, in a solution of 150 mM NaCl, 0.5% bovine serum albumin, and 20 mM Tris, pH 7.5. After overnight incubation, anti-myosin IgG was added to the solution and the

nitrocellulose paper was allowed to equilibrate for 2 h at room temperature. After reaction with the anti-myosin IgG, the nitrocellulose filter was thoroughly washed and then incubated for 30 min at room temperature with mouse anti-rabbit IgG conjugated to alkaline phosphatase (Promega Inc., Madison, WI). The myosin present was visualized using the alkaline phosphatase to catalyze a color producing reaction involving nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The anti-myosin IgG (donated by Drs. J. Sellers and Trudy Cornwell of NHLBI) was made from serum of rabbits innoculated with bovine tracheal muscle myosin and showed good reactivity to rabbit skeletal muscle myosin.

RESULTS

Treatment of relaxed fibers with pPDM

When purified myosin subfragment-1 (S1) is labeled with pPDM in the presence of nucleotide, the fast reacting sulfhydryls, SH1 and SH2, are crosslinked to one another (Reisler et al., 1974). This covalent modification results in a reduction in myosin's affinity for actin in the absence of ATP to the level of untreated myosin in the presence of ATP (Muhlrad et al., 1976; Greene et al., 1986; Katoh and Morita, 1984; Katoh and Morita, 1984). In fibers, treatment with pPDM under relaxing conditions results in a decrease of the stiffness of the fibers during activation (Chaen et al., 1986). In light of the biochemical results, it seems quite possible that the reduction in stiffness of activated fibers may be due to pPDM's ability to crosslink SH1 and SH2 and reduce the affinity of myosin for actin. If this is the case, treatment of a relaxed fiber with pPDM should decrease a fiber's rigor stiffness as well.

In order to examine the changes in rigor stiffness that accompany pPDM treatment of a resting (μ = 165 mM) skinned rabbit psoas fiber, we measured the resting and rigor stiffness after incremental exposures of fibers to 200 μM pPDM. The total exposure time was 1 h. Fig. 1 *A* shows the time course of the change of both the resting and rigor stiffness with increasing duration of pPDM-treatment in relaxing solution. To be certain that the incremental nature of the exposure was not affecting the results, we also exposed several fibers to 60 min of continuous pPDM treatment. Those fibers were found to be identical to ones treated intermittently for 60 min (data not shown). As the length of the exposure of the muscle fiber to pPDM increases, there is a large decrease in the rigor stiffness and also a gradual small increase in the resting stiffness. As discussed above, the decrease in rigor stiffness with increased exposure to pPDM is consistent with the observations of Chaen et al. (1986) on the stiffness of activated fibers.

Treatment of rigor fibers with pPDM

In solution, the rigor attachment of S1 to actin decreases the rate of pPDM crosslinking of myosin's essential sulfhydryls to less than 1/20th the rate in the absence of actin (Katoh and Morita, 1984). Thus, if crossbridges are in rigor when treated with pPDM this should inhibit the ability of pPDM to reduce rigor stiffness. As Fig. 1 *B* shows, this is indeed the case. However two unexpected results also are seen. When pPDM is used to treat skinned rabbit psoas fibers in rigor, the rigor stiffness increases early on to ~119% of control value (and therefore remains relatively constant as incubation time

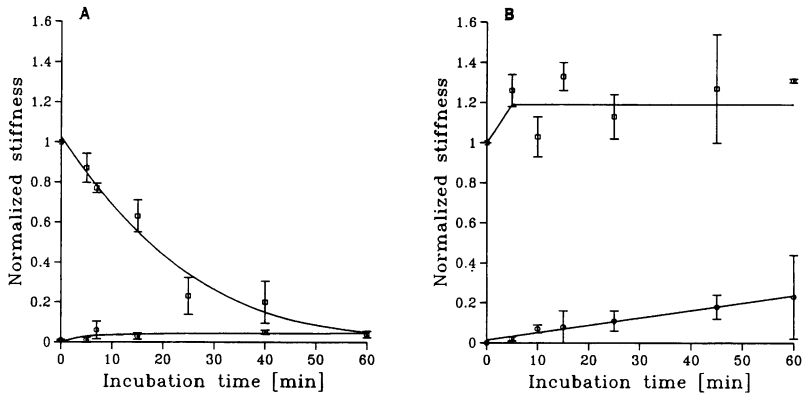

FIGURE 1 Time course of *N,N'*-para-phenylene dimaleimide (pPDM) treatment of skinned rabbit psoas fibers. pPDM concentration = 200 μM; temperature, 5°C. (*A*) Treatment in relaxing solution. As the cumulative time of pPDM exposure increases, the rigor stiffness decreases to the level of the resting stiffness. The resting stiffness shows an ~5% increase over the 60-min treatment period. Rigor stiffness after pPDM treatment while relaxed (□). Relaxed stiffness after pPDM treatment while relaxed (○). (*B*) Treatment in rigor solution. In contrast to the results for treatment of relaxed fibers, rigor protects against pPDM-induced loss of rigor stiffness. There is, however, an unexplained increase in the resting stiffness. Rigor stiffness after pPDM treatment while in rigor (□). Relaxed stiffness after pPDM treatment while in rigor (○). Data points are average values; the error bars show ±SEM; the total number of experiments is 14 for relaxed fiber treatment (n = 3 − 6/point) and 6 for rigor fiber treatment (n = 2 − 6/point). When n = 2, the error bar gives the range.

is extended out even to 60 min). Another change that occurs with pPDM treatment of rigor fibers is that the resting stiffness shows an approximately linear increase with increasing duration of treatment, reaching as high as 20% after 60 min incubation. The fact that the increase in rigor stiffness occurs quickly and then remains relatively constant, while the increase in resting stiffness occurs more gradually, suggests that these changes are likely the result of separate labeling events.

Mechanical properties of pPDM-treated fibers

Step stretch measurements at 165 mM ionic strength

Fig. 2 shows the mechanical response of a fiber treated with 200 μM pPDM in relaxing solution for 60 min. After the treatment, 2 nm/half-sarcomere step stretches were applied to the fiber in both 165 mM ionic strength rigor and relaxing solutions. As seen from Fig. 1, this amount of treatment totally eliminates the additional crossbridge stiffness induced by rigor. Fig. 2 shows that after 60 min of pPDM treatment the stretch response of the fiber in both rigor and relaxing solution is largely indistinguishable from the stretch response of a normal relaxed fiber. The characteristic large response of a normal fiber to stretch in rigor solution is gone in the treated fiber.

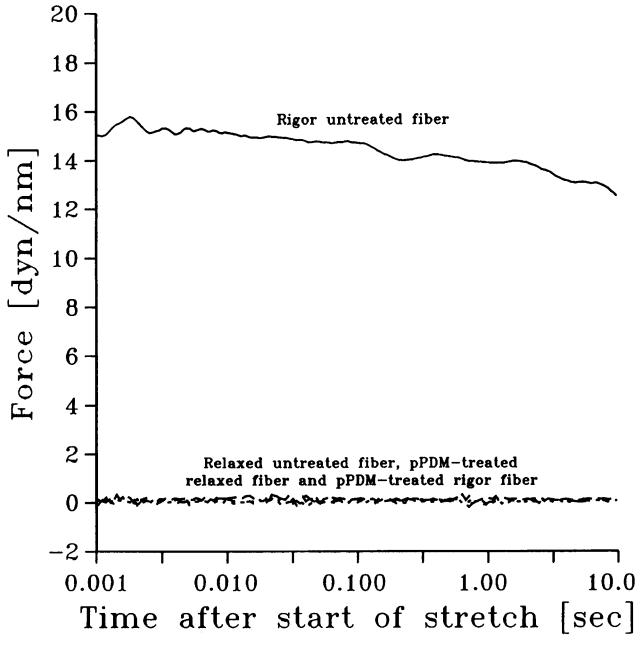

FIGURE 2 Original records of response to stretch in 165 mM rigor and relaxing solution, before and after pPDM-treatment of a resting fiber. Incubation time was 60 min. The untreated fiber shows a substantial resistance to stretch in rigor. When relaxed it no longer resists the stretch. After pPDM-treatment, the fiber is unable to resist the step length change (~2 nm/half-sarcomere) either while relaxed or in rigor. Experiment 031589.

Chord stiffness-duration of stretch relationship at 40 mM ionic strength

Although at 165 mM ionic strength the stiffness of the fiber treated for 60 min in relaxing solution with pPDM is too small to easily study quantitatively, by lowering ionic strength to 40 mM, it was hoped that the strength of binding of the pPDM-treated crossbridges might increase enough to allow an examination of their kinetics. Using a procedure similar to that in Schoenberg (1988a) the chord stiffness-duration of stretch (S_c-log t_d) relationship was measured at an ionic strength of 40 mM. As seen in Fig. 3, the chord stiffness-duration of stretch relationship for the modified fiber in either relaxing or rigor solutions, is the same as that of a control relaxed fiber at the same ionic strength. Because the shape and amplitude of the chord stiffness-log duration of stretch relationship is determined by the

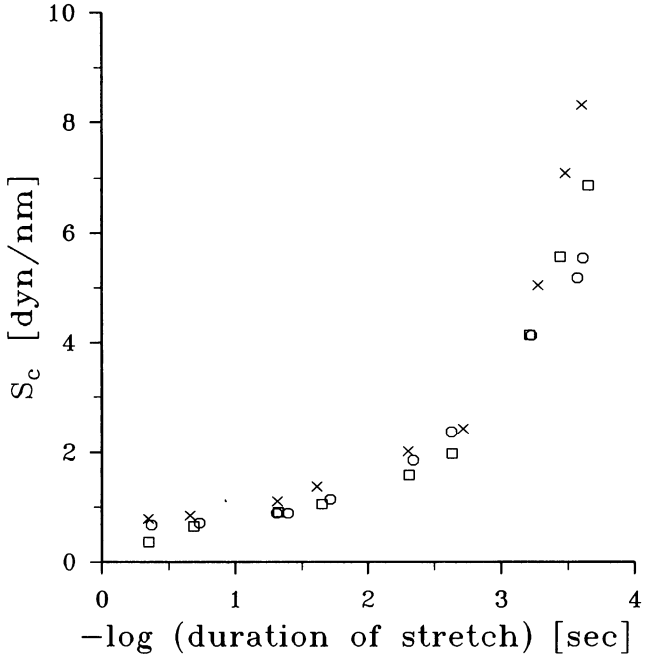

FIGURE 3 S_C − log(t_d) relationship at μ = 40 mM. Comparison of unmodified fiber in ATP with pPDM-modified fibers ± ATP. Treated fiber treated with 200 μM pPDM for 60 min. For stretches of relatively long duration, the stiffness remains low as the crossbridges are able to detach and reattach in positions of lesser strain. However, as the duration of stretch decreases, the crossbridges can no longer release the strain before completion of the stretch and the chord stiffness rises. The fiber after pPDM treatment, whether in rigor or relaxing solution, gives the same response it gave before modification in the presence of ATP. Relaxed untreated fiber (□). Relaxed pPDM-treated fiber (○). Rigor pPDM-treated fiber (X). Experiment 011789.

crossbridge attachment and detachment rate constants (Schoenberg, 1985; Schoenberg, 1988a, b), it is clear that the attachment and detachment rate constants of the pPDM-modified crossbridge, both in the presence and absence of ATP, are identical to those of the relaxed M · ATP crossbridge of normal fibers. In agreement with previous work (Schoenberg, 1988a), the data of Fig. 3 suggest crossbridge detachment rate constants on the order of $5 \times 10^3\ s^{-1}$.

Site of pPDM reactivity in fibers

In order to examine where pPDM reacts in fibers, several fibers were treated with ^{14}C-labeled pPDM after which SDS-PAGE was performed. Some typical results are shown in Fig. 4. Lanes *A* and *B*, respectively, show SDS-PAGE of both an untreated fiber and a fiber treated with ^{14}C-pPDM. From the labels on the side of the picture, it is clear that the control in lane A shows bands corresponding to nearly all the major muscle proteins: titin (also referred to as connectin), nebulin, myosin heavy chain, actin, α-actinin, troponin, and the myosin light chains. The pattern of the pPDM-treated fiber in lane *B* is quite similar except that two new bands appear: one at the level of myosin dimer (Wang, 1982), the other somewhat higher. Two other differences between the control and pPDM-treated gels is that in the latter the titin bands are significantly diminished and there is a fair amount of material remaining at the top of the gel. As lanes *E* and *F*, from a Western blot of duplicates of lanes *A* and *B* stained with anti-myosin IgG/alkaline phosphatase-conjugated anti-IgG show, both of the new bands that appear after pPDM treatment react with myosin antibody. The lower molecular weight band likely represents a myosin dimer caused by crosslinking of two myosin heavy chains and the higher molecular weight band may well represent a polymer of several crosslinked myosin heavy chains. Lanes *C* and *D*, autoradiograms of lanes *E* and *F*, show that after ^{14}C-pPDM treatment, most of the radioactivity in the bands entering the gel is found in the myosin and myosin dimer bands with a very small amount in the troponin-I, actin, and α-actinin bands. With further exposure of the autoradiogram, not only does the lower molecular weight new band show radioactivity, but the higher molecular weight one does as well. The material remaining at the top of the treated gel also shows a fair amount of radioactivity. This presumably high molecular weight material at the very top of the gel does not react with myosin antibody and hence is clearly not heavily crosslinked myosin. The reduction in intensity of the normal titin bands in the gel of the pPDM-treated fiber suggests the possibility it may be crosslinked titin.

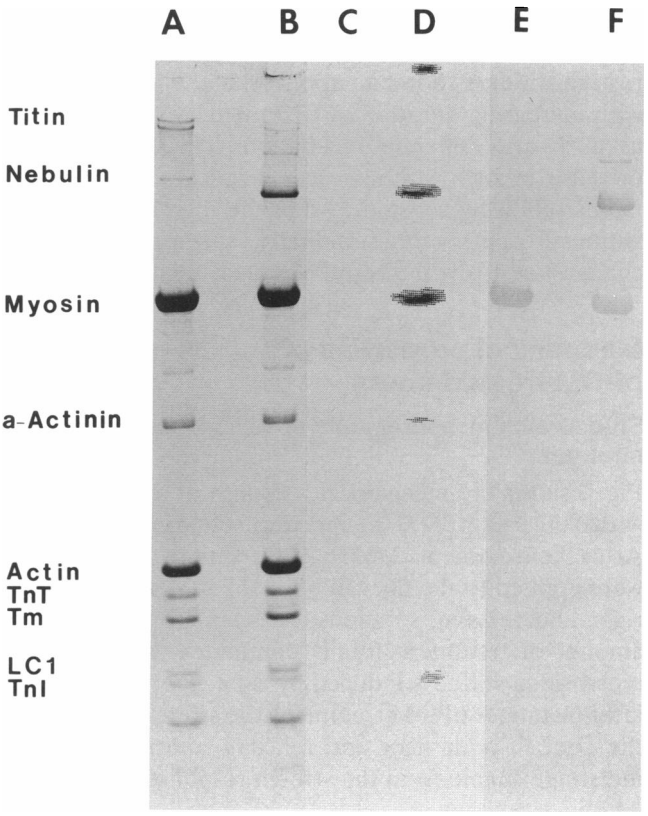

FIGURE 4 2–12% gradient gel of myofibrils from control fibers and fibers labeled with ^{14}C-pPDM. (*A*) Control fiber. (*B*) ^{14}C-pPDM-treated. (*C, D*) Scan of β-emission from Western blot of duplicates of lanes *A* and *B* (lanes *E* and *F*) (*E, F*) Western blot analysis of lanes *A* and *B*. Of the three bands that stain with Western blot analysis using anti-myosin, the lowest molecular weight band is clearly myosin heavy chain, the next highest is likely a dimer of myosin heavy chain, and the highest molecular weight band is possibly a higher polymer of crosslinked myosin heavy chains. The distribution of radioactive β-emission in lane *D*, averaged with one other identically-loaded lane (not illustrated) is: top of gel, 60%; myosin dimer, 12%; myosin, 19%; α-actinin, trace; actin, trace; troponin I subunit, 8%. The radioactive material at the top of the gel is believed to be crosslinked titin (see text).

Source of the increase in resting stiffness with pPDM-treatment

The effect of pPDM treatment on the titin band of SDS-PA gels of muscle fibers was unexpected to us, as was the small linear increase in resting stiffness with duration of treatment. Even though the increase in resting stiffness with pPDM treatment is <5% of the initial rigor stiffness, even after 60 min of treatment, the increase is real and readily detectable. The conjecture of Horowitz and Podolsky (1987) that the resting tension at long sarcomere length is related to titin raises the possibility that our two unexpected findings are related. Fig. 5 shows the resting tension of a control fiber compared

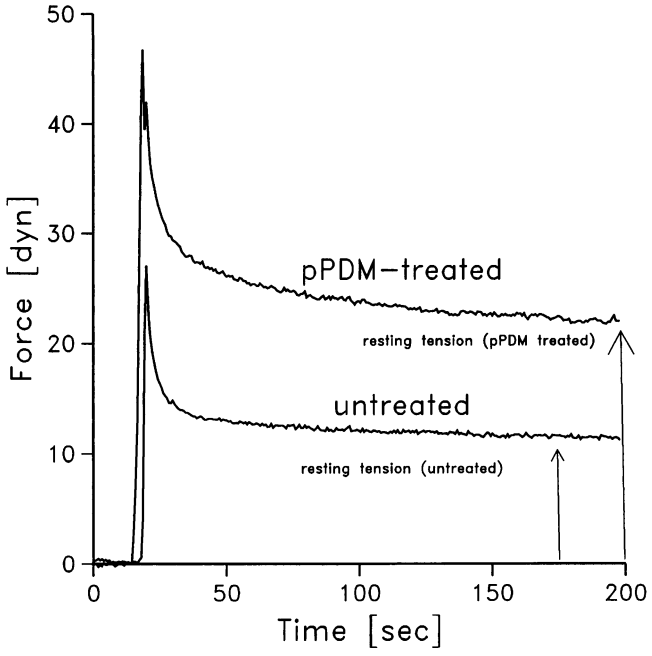

FIGURE 5 Resting tension in control and pPDM-treated fibers. Figure shows tension response to changing a fiber's resting length from 2.5 μm, where resting tension is zero, to 3.0 μm. Arrows show resting tension of a control fiber at 3.0 μm and the resting tension of the same fiber at 3.0 μm after 60 min of treatment with 200 μm pPDM. As illustrated, that amount of treatment approximately doubles the resting tension. Experiment 032690; fiber segment length = 5.3 mm; fiber diameter, 140 μm.

with that of a pPDM-treated fiber. It is clear that treatment with 200 μM pPDM for 60 min approximately doubles the fiber's resting tension. This finding not only supports the conjecture of Horowits and Podolsky about the origin of resting tension at long sarcomere length, it also reveals the origin of the small increase in resting chord stiffness at normal sarcomere length and supports the calculation of Schoenberg (1988a) that the chord stiffness at normal sarcomere length is largely related to crossbridges and not the resting tension structures.

Treatment of relaxed fibers with NPM

The use of pPDM as an agent to reduce crossbridge affinity for actin is based on solution determinations that pPDM crosslinking of SH1 to SH2 results in the stabilization of myosin in an M · ATP-like conformation. Because there is solution evidence that monofunctional reagents such as *N*-phenylmaleimide (NPM) produce similar effects (Duong and Reisler, 1989), we also examined the response of skinned psoas fibers to treatment with NPM.

Fig. 6 *A* shows the time course for the change in relaxed and rigor stiffness after NPM treatment (100 μM, 5°C) of a relaxed fiber. As with pPDM (Fig. 1), the rigor stiffness decreases as the length of exposure to NPM increases and approaches the value of a relaxed fiber after 40–50 min. In contrast to the pPDM results there is not a concomitant increase in relaxed stiffness. The relaxed stiffness is essentially unchanged throughout the treatment procedure.

Treatment of rigor fibers with NPM

It was of interest to determine if having the crossbridges in rigor during NPM treatment would also protect against the decrease in rigor stiffness. Fig. 6 shows that this is partially the case. As with pPDM, the rigor

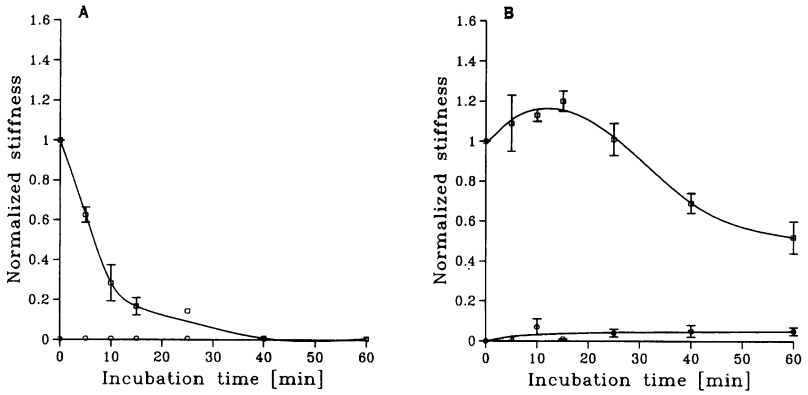

FIGURE 6 Time course of *N*-phenylmaleimide (NPM) treatment of skinned rabbit psoas fibers. NPM concentration = 100 μM; temperature, 5°C. (*A*) Treatment in resting solution. Rigor stiffness after NPM treatment while relaxed (□). Relaxed stiffness after NPM treatment while relaxed (○). (*B*) Treatment in rigor solution. Relaxed stiffness after NPM treatment while in rigor (○). Rigor stiffness after NPM treatment while in rigor (□). Data points give the average values, with the error bars showing ± SEM. The total number of experiments is 10 for relaxed fiber treatment (with n = 2 − 10/point), and 6 for rigor fiber treatment (n = 2 − 6/point). When n = 2 the error bar gives the range.

stiffness after PM treatment in rigor initially increases, but then, instead of remaining elevated, it decreases so that it is ~55–65% of the rigor stiffness of an untreated fiber after 60 min (Fig. 6 *B*). It is seen then, that while rigor offers some protection against the action of NPM, it is not as complete as the protection against pPDM action. This seems compatible with the idea that NPM is actually more potent an inhibitor of rigor stiffness than pPDM, causing a more rapid decrease in rigor stiffness at 100 μM concentration than pPDM causes at 200 μM (cf. Figs. 1 and 6).

It should also be noted that during NPM treatment of the rigor fiber, the stiffness of the fiber while relaxed increases to ~5% of the original rigor stiffness within the first 10–25 min and then remains at that level for the remainder of the labeling time.

Mechanical properties of NPM-treated fibers

The mechanical properties of NPM-treated fibers at 165 mM ionic strength was examined using step stretches. As was the case for pPDM-treated fibers, the stiffness of the NPM-treated fibers, as seen in Fig. 7, was near zero. To examine the kinetics of the NPM-treated crossbridges, it was necessary to lower the ionic strength to 40 mM and obtain a chord stiffness-duration of stretch relationship. Fig. 8 shows that, like the pPDM-treated fiber, the NPM-treated fiber has approximately the same response, either in the presence or absence of ATP, as an untreated relaxed fiber. The detachment rate constants for NPM-treated fibers, thus, are similar to those of pPDM-treated fibers and normal relaxed fibers, and are on the order of 10^3 to 10^4 s^{-1}.

Site of reactivity of NPM in muscle fibers

To determine the site of reactivity of NPM in muscle fibers, a small bundle of muscle fibers was treated with 100 μM ^{14}C-NPM for 60 min. SDS-PAGE was then done on 10–40 μg samples of homogenized fiber. Lanes *A* and *B* of Fig. 9 show the Coomassie Blue stain of both untreated (lane *A*) and NPM-treated (lane *B*) fibers.

Lanes *C* and *D* in Fig. 9 show a ^{14}C scan of lanes *A* and *B*, respectively. Although myosin heavy chain is the most heavily labeled band, containing 28% of the total counts in peaks, it is seen that 100 μM NPM reacts considerably more promiscuously than 200 μM pPDM, the titin band also showing heavy labeling (20%), with α-actinin, actin,

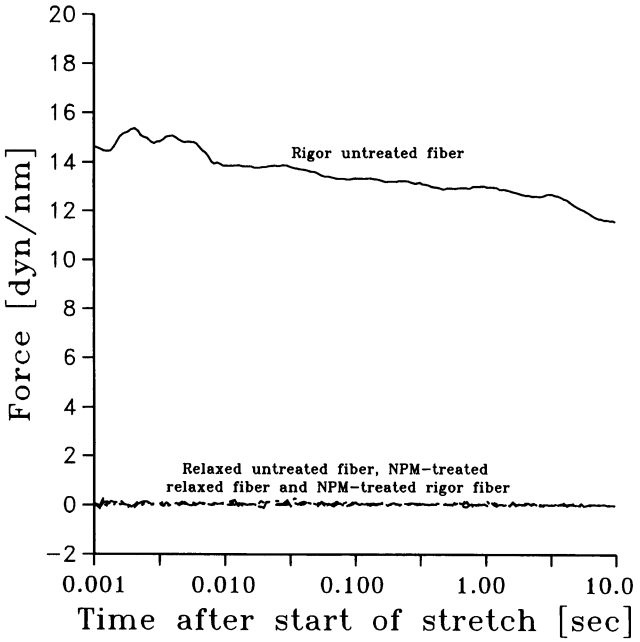

FIGURE 7 Original records of response to stretch, in rigor and resting solutions, before and after NPM treatment of a resting skinned rabbit psoas fiber. Treatment conditions were 100 μM NPM for 60 min. When $n = 2$ the error bar gives the range. Just as was shown for pPDM treatment in Fig. 3, NPM treatment causes the modified fiber to behave as if it were relaxed both in the presence and absence of ATP. Experiment 031389.

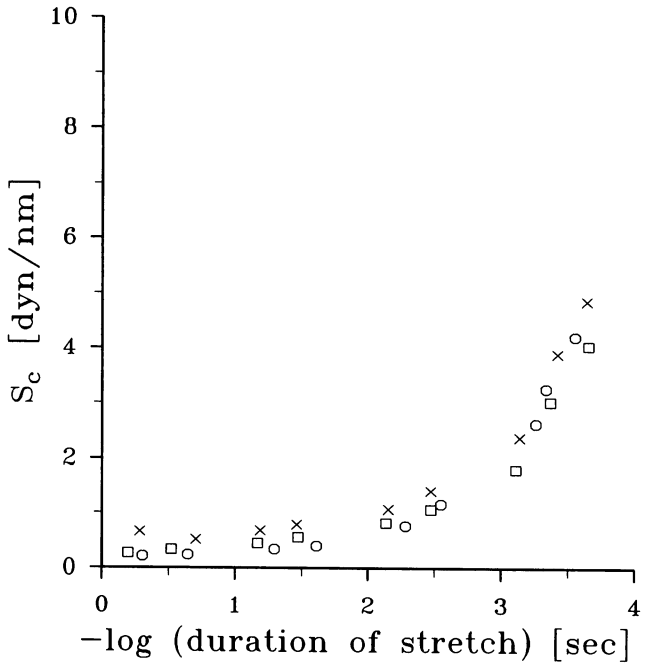

FIGURE 8 $S_c - \log(t_d)$ relationship at 40 mM ionic strength. Comparison between unmodified fiber in ATP with NPM-modified fibers ± ATP. Incubation time = 60 min. The NPM-modified fiber ±ATP is identical in its response to the untreated relaxed fiber. Relaxed untreated fiber (□). Relaxed NPM-treated fiber (○). Rigor NPM-treated fiber (X). Experiment 020189.

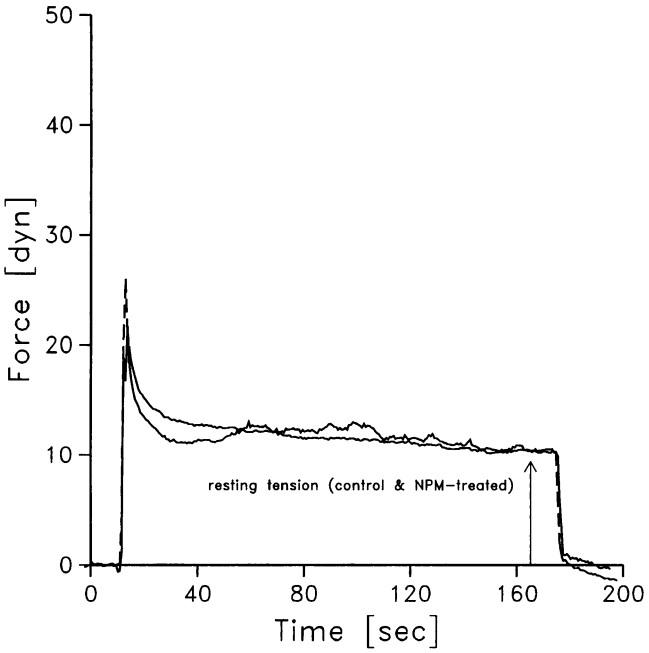

FIGURE 9 2–12% gradient gel of myofibrils from control fibers and fibers treated with ^{14}C-NPM. (*A*) Control fiber. (*B*) ^{14}C-NPM-treated. (*C*, *D*) Scan of β-emission from lanes *A* and *B*. The distribution of radioactive β-emission in lane *D* due to binding of ^{14}C-NPM to protein is: top of gel, 14%; titin, 20%; myosin heavy chain, 28%; first small peak below myosin, 4%; second small peak below myosin, 2%; α-actinin, 14%; actin, 7%; troponin T subunit, 4%; troponin I subunit, 7%.

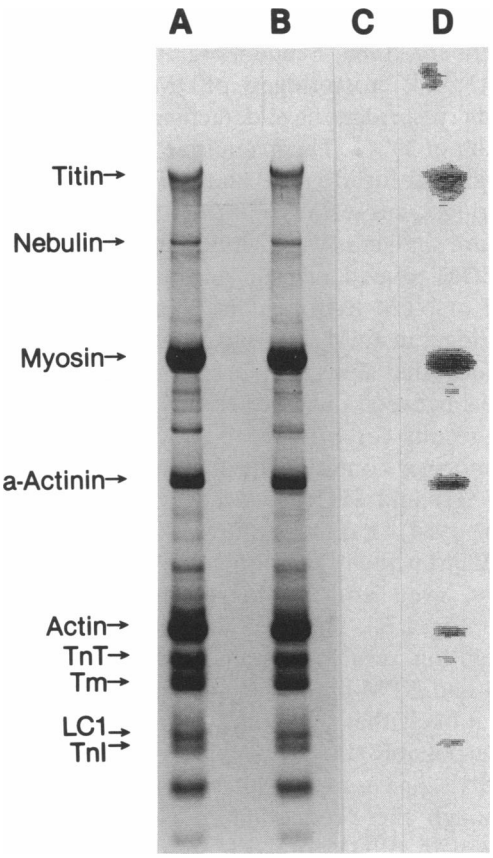

FIGURE 10 Resting tension in control and NPM-treated fibers. Figure shows tension response to changing a fiber's resting length from 2.5 μm, where resting tension is zero, to 3.0 μm. Arrows show resting tension of a control fiber at 3.0 μm and the resting tension of the same fiber at 3.0 μm after 60 min of treatment with 100 μM NPM. As illustrated, the fiber resting tension is unaffected by the NPM treatment.

and troponin I, showing modest labeling (7–14%). A small amount of radioactivity (~ 14%) also is seen at the top of the gel.

Effect of NPM treatment on fiber resting tension

Fig. 5 revealed that, in our hands, treatment of muscle fibers with pPDM increases the fiber's resting tension. It is not clear whether treatment with NPM has a similar effect, particularly because, as Fig. 9 shows, NPM binds with titin in the fiber. Fig. 10 reveals that NPM treatment does not increase fiber resting tension. As seen, treatment of a msucle fiber with 100 μM NPM for 60 min, which totally eliminates a fiber's rigor stiffness, has no effect upon the fiber's resting tension.

DISCUSSION

Chaen et al. (1986) showed that treatment of muscle fibers with pPDM inhibits their ability to produce active force. They reasoned that this effect of pPDM on muscle fibers was due to pPDM's ability to reduce the myosin head's ability to bind to actin, similar to what occurs when myosin is treated with pPDM in solution. Our findings support this interpretation. We find that in the presence or absence of ATP, pPDM-treated crossbridges bind to actin with only about the same strength of binding as normal (untreated) crossbridges bind to actin in the presence of ATP. This is true both at 165 and 40 mM ionic strength. Thus, pPDM treatment of fibers indeed reduced crossbridge binding affinity. NPM treatment produces a similar effect.

It is interesting how closely pPDM- or NPM-treated crossbridges resemble the M · ATP crossbridges of normal fibers. At 165 mM ionic strength, only a small fraction of pPDM- or NPM-treated crossbridges are attached, but at 40 mM ionic strength, sufficient numbers are attached to make it possible to compare the kinetics of the treated crossbridges with those of normal

M · ATP crossbridges. By generating S_c-log(t_d) plots (Schoenberg, 1985; Schoenberg, 1988a), we find that, like M · ATP crossbridges, pPDM-treated and NPM-treated crossbridges have detachment rate constants on the order of 10^4 s^{-1}. The magnitude of the chord stiffness is also similar for M · ATP and pPDM- or NPM-treated crossbridges, suggesting that their attachment rate constants are similar as well. Thus, paralleling the situation for pPDM-treated myosin subfragment-1 in solution, pPDM or NPM treatment in fibers seems to lock the crossbridges in an M · ATP-like state.

Besides the above, there are a number of other parallels between our fiber results and previously published results for pPDM and myosin in solution. In solution, rigor conditions protect against the crosslinking of SH1 and SH2 produced by pPDM (Katoh and Morita, 1984). Paralleling this, skinned fibers treated with pPDM while in rigor do not exhibit a reduced rigor stiffness, even after prolonged treatment (see *filled squares,* Fig. 1 *B*). The close similarity between the fiber and solution results, combined with our finding that pPDM and NPM bind to myosin heavy chain in fibers, makes it likely that the reduction in acto-myosin affinity is quite possibly due to reaction of the SH1 and SH2 sulfhydryls, just as in solution.

Although the production of weakly-binding crossbridges with pPDM or NPM is relatively straightforward, there are a number of unwanted side effects in both cases. The major effect of treatment of relaxed fibers with pPDM or NPM is inhibition of rigor stiffness and active force production. Having the fibers in rigor during treatment completely (pPDM) or partially (NPM) prevents this. One of the complications of treatment with pPDM or NPM is seen when the fibers are treated in rigor.

If the only effect of pPDM and NPM treatment is creation of weakly-binding crossbridges, and rigor conditions protect against this, then the stiffness of a rigor fiber treated with either of these compounds should remain unchanged. Unfortunately, as Figs. 1 *B* and 6 *B* show, treatment with these compounds seems to very rapidly cause a small, but nonetheless significant, increase in fiber stiffness. We do not, at present know whether this increase in stiffness is due to an increase in the stiffness per bridge or an increase in the stiffness of the structures responsible for the fiber's resting tension. In either case, if this same effect occurs with treatment of the relaxed fiber, it makes it difficult to estimate, using stiffness, the number of crossbridges attached in the treated fiber in a given condition.

A second complication we saw regarding treatment with these compounds in the fiber occurred with pPDM treatment but not with NPM treatment. In our hands, upon treatment of a relaxed fiber with pPDM, there is a small, slowly developing, increase in resting stiffness (see Fig. 1 *A*). Treatment of a relaxed fiber with NPM does not increase fiber resting stiffness. Our finding that pPDM treatment causes an increase in resting tension, while NPM treatment does not, suggests that the increase in resting stiffness is due to an increase in the stiffness of the structures responsible for the fiber's resting tension, rather than to a change in the properties of the crossbridges.

A final complication with regard to pPDM and NPM treatment is that the latter compound, under the conditions used in this work, reacts rather promiscuously. Although the results are entirely consistent with the hypothesis that NPM exerts its effect in muscle fibers because it reacts with SH1 or SH2 on the myosin heavy chain, the promiscuity of the agent makes more work necessary before this can be proven conclusively.

In summary, following the lead of Chaen, Shimada, and Sugi (1986), we have developed two similar but different techniques for creating weakly-binding crossbridges in muscle fibers. The weakly-binding crossbridges have kinetics extremely similar to those of normal M · ATP crossbridges. The similarity is much more marked than in the case of the weakly-binding crossbridges produced by ATP-γ-S binding (Dantzig et al., 1988). Because the crossbridges produced by treatment with pPDM or NPM are locked in the weakly-binding configuration regardless of the presence or absence of nucleotide, it is hoped that these crossbridges will be useful in providing additional information about weakly-binding crossbridges, particularly under conditions (ATP plus Ca^{2+}), where M · ATP crossbridges undergo hydrolysis, release product, and go into a strongly binding (M · ADP) configuration.

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