Behavior of *N*-Phenylmaleimide-Reacted Muscle Fibers in Magnesium-Free Rigor Solution

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ABSTRACT Using x-ray diffraction and mechanical stiffness, the response of *N*-phenylmaleimide (NPM)-reacted cross-bridges to solutions containing different amounts of ATP and Mg^{2+} has been studied. In relaxing solution containing greater than millimolar amounts of ATP and Mg^{2+}, NPM-treated muscle fibers give x-ray diffraction patterns and stiffness records, which are nearly indistinguishable from those of untreated relaxed fibers. In a solution devoid of added ATP, but with Mg^{2+} (rigor(+Mg) solution), the muscle fibers still give x-ray diffraction patterns and mechanical responses characteristic of relaxed muscle. The new finding reported here is that in a solution devoid of both ATP and Mg^{2+} (rigor(−Mg) solution containing EDTA with no added ATP), NPM-reacted cross-bridges do give rigor-like behavior. This is the first report that NPM-reacted cross-bridges, at least in the presence of EDTA, are capable of going into a strongly binding conformation.

INTRODUCTION

Chaen et al. (1986) reported that muscle fibers reacted with para-phenylenedimaleimide (pPDM) do not produce force. This was confirmed by Barnett et al. (1992) who also found, in agreement with solution studies on pPDM-treated myosin subfragment-1 (Greene et al., 1986), that pPDM-reacted cross-bridges are locked in a weakly binding conformation and do not go into a strongly binding conformation either in the presence of MgATP and calcium or in the absence of ATP. This led to the conclusion that pPDM reaction prevented force production by locking cross-bridges in a weakly binding conformation.

Barnett et al. (1992) also reported that a muscle fiber treated in relaxing solution for 1 h with 100 μM *N*-phenylmaleimide (NPM) similarly produces zero force in response to calcium stimulation and does not go into a strongly binding or rigor-like conformation in the presence of calcium and ATP or in the absence of ATP. Thus, it was postulated that NPM modification, which, like pPDM-modification, links maleimide groups to SH1 and SH2 (Burke and Knight, 1980; Huston et al., 1988; Ehrlich et al., 1995), also inhibits force by locking the cross-bridges in a weakly binding conformation.

Although the previous data suggest that the mechanism by which NPM and pPDM inhibit force is similar, here we show that cross-bridges in NPM-reacted muscle fibers indeed go into a strongly binding conformation in magnesium-free rigor solution.

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METHODS

Muscle preparation and solutions

All experiments were performed on chemically skinned muscle of the rabbit psoas major. Small bundles, ~0.3 × 0.6 mm in cross section and 30 mm in length, were used for the x-ray diffraction experiments (Xu et al., 1997), and single fiber segments, ~0.1 mm in diameter and 10 mm long, were used for the stiffness measurements (Schoenberg, 1988). The sarcomere length of both the bundles and fibers were determined by light diffraction and adjusted to 2.5 μm. The specimen chambers were cooled to 4 ± 1°C, and for the bundles, the solution in the chamber was continuously stirred by a syringe pump at the rate of 1 ml/s to minimize any gradients along the length of the fibers.

Relaxing solution contained 2 mM MgATP, 2 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol (DTT), 48 mM creatine phosphate, and 10 mM imidazole. Magnesium-free rigor solution, rigor(−Mg), contained 2.5 mM EGTA, 2.5 mM EDTA, 150 mM KPropionate, and 10 mM imidazole. Magnesium-containing rigor solution, rigor(+Mg), contained 2.5 mM EGTA, 2 mM $MgCl_2$, 152 mM KPropionate, and 10 mM imidazole. Quick-rinse solution, used when changing from relaxing to rigor solution, contained 5 mM EGTA, 15 mM EDTA, and 20 mM imidazole. NPM-treatment solution contained 0.1 mM *N*-phenylmaleimide, 4 mM EGTA, 1 mM $MgCl_2$, 4 mM MgATP, 125 mM KCl, and 10 mM imidazole. The pH of all the solutions was 7.0 ± 0.05. The ionic strength of all the solutions was ~170 mM except for the quick-rinse solution that had an ionic strength of ~70 mM. All solutions except NPM-treatment solution contained 0.5 mM dithiothreitol.

X-ray camera and imaging plate detector system

X-ray experiments were performed using a rotating anode x-ray generator (Elliot GX-6) at power 35 kV × 35 mA. The x-ray beam was focused by two gold-coated mirrors. Specimen to detector distance was 89 cm. The two-dimensional x-ray patterns were recorded on an imaging plate detector system (MAR Research, Hamburg, Germany) with 50 × 50-μm pixels. The scanned area was 10 cm in diameter. To compare directly the intensities obtained under different conditions with minimum error, diffraction patterns always were recorded from the same bundle for all the conditions of interest.

Experimental protocols

Treatment

Bundles or single fibers were treated with 0.1 mM NPM for 1 h; the reaction was terminated by replacing the NPM-treatment solution with

relaxing solution that contained DTT. Before going into rigor(+Mg) solution or rigor(−Mg) solution, the bundles or fibers first were incubated in quick-rinse solution for 5 min.

X-ray diffraction

The exposure time for each pattern was 1 h. The data were displayed and analyzed on a Silicon Graphics Indigo workstation (Mountain View, CA) using a program, Profida, originally written by M. Lorenz (Max Plank Institute, Heidelberg, Germany) and modified by D. Gilroy (National Institutes of Health, Bethesda, MD). The patterns of the original data were first rotated and translated. Then the data in the four quadrants were added (folded) and averaged. Measuring the integrated intensities of interest, two kinds of slices on the patterns were made: a horizontal slice along the equator with a width covering the whole equatorial reflection area and a vertical slice crossing the first actin layer line at the peak of the reflection (width shown by dashed lines in Fig. 4 *C*). All values of integrated intensities were normalized by the equatorial 11 reflection, setting I_{11} for the muscle in rigor(−Mg) solution at 100.

RESULTS

Fig. 1 shows the effect of NPM treatment on the behavior of rabbit psoas fibers in relaxing solution. NPM treatment has very little effect on either the structure or mechanical properties of a muscle fiber in relaxing solution. The whole x-ray diffraction patterns of the muscle bundle after NPM treatment are nearly identical to those before (compare Fig. 1, *A* and *B*). The equatorial I_{11}/I_{10} ratio increases by only ~10%, and more importantly, there are no differences in the intensity and spacings of the first order layer lines (see Fig. 3 *A*). The apparent stiffness in response to rapid stretch similarly remains relaxed-like after treatment (Fig. 1, *C*), as reported previously (Barnett et al., 1992).

In rigor solution containing no ATP but having 2 mM Mg (rigor(+Mg) solution), NPM-treated fibers retain the characteristics of relaxed muscle (Fig. 2). The relaxed x-ray pattern persists for as much as 11 h after treatment (Fig. 3 *A*). Only a very slight drift toward rigor is seen in the I_{11}/I_{10} ratio and the 2-D pattern. Although the drift in the I_{11}/I_{10} ratio and the 2-D pattern is small, it is none the less somewhat larger than that seen in a treated or untreated fiber exposed to the same dose of x-rays in relaxing solution. It should be pointed out that the mechanical response also, although slightly increased, remains relaxed-like for many hours after treatment (Fig. 3 *B*).

In contrast to the above behavior, in a solution in which ATP and Mg both are absent (rigor(−Mg) solution), fibers in a muscle bundle go into a rigor-like state within just a few hours (Fig. 4 *A*). The single fiber preparation develops full rigor stiffness even more quickly, within 30 min, as seen in Fig. 4 *B*. The time course of rigor development in a muscle bundle presumably is limited by slower diffusion of molecules into and out of the thicker preparation. Fig. 4 *C* shows the 2-D x-ray pattern 3 h after removal of Mg^{2+}. After 3 h in rigor(−Mg) solution, the pattern shows changes that are typical of the transition to rigor. The I_{11}/I_{10} ratio increases from 1.39 ± 0.05 to 2.46 ± 0.08. In parallel, the intensity of the first order layer line increases from 0.40 ± 0.03 to 2.47 ± 0.49. Finally, the spacing of the first layer line changes from close to 43 nm (myosin-based) to close to 36.5 nm (actin-based).

The rigor condition induced in the NPM-treated fibers by removal of Mg^{2+} and ATP is rapidly reversed upon the addition of MgATP as shown in Fig. 5 *A*, and the mechanical stiffness returns within minutes to the same value as before removal (Fig. 5 *B*). Just as initially, removal of Mg^{2+} and ATP a second time again leads to development of a rigor-like state, and removal of ATP alone results in the cross-bridges remaining weakly binding (data not shown). The data suggest that the cross-bridges that go into a rigor-like state upon Mg^{2+} removal are not in a denatured irreversible state but are in a state that is capable of binding MgATP when it is reintroduced to the solution.

DISCUSSION

Although elucidation of the molecular structure of actin and myosin has shed much light on the mechanism of force production by muscle, many questions remain concerning the role of myosin's two essential sulfhydryls, SH1 (Cys-707) and SH2 (Cys-697). These two sulfhydryls, which can be alkylated by a variety of compounds, have been revealed to be situated close to the bottom of the nucleotide binding pocket and also the bottom of the cleft thought important for actin binding (Rayment et al., 1993). Whereas there is good agreement that reaction of both sulfhydryls by a variety of compounds results in the loss of the ability of myosin to hydrolyze ATP and make force (Yamaguchi and Sekine, 1966; Reisler et al., 1974; Chaen et al., 1986; Ehrlich et al., 1995), there is surprisingly considerable disagreement whether inhibition of force production occurs with reaction solely at SH1 or solely at SH2 (Crowder and Cooke, 1984; Root et al., 1991).

Two of the many compounds that react with SH1 and SH2 are pPDM and NPM. Both inhibit force production, and both, for the treatment conditions generally used, alkylate both SH1 and SH2 (Barnett et al., 1992; Ehrlich et al., 1995). pPDM is a bifunctional reagent with two reactive maleimide rings, and it would likely prove difficult to limit its reaction to just one of the sulfhydryls. However, NPM has but a single reactive ring and should with a slight modification of the standard reacting protocol be capable of reacting with either solely SH1 (Xie et al., 1997) or solely SH2 (Reisler et al., 1974). This, in turn, should provide information on whether the effects attributable to alkylation of myosin's sulfhydryls are because of alkylation of just one or the other or whether the effects require alkylation of both sulfhydryls.

It is known that treatment of muscle fibers with paraphenylenedimaleimide locks cross-bridges in a weakly binding state, a finding that in and of itself is sufficient to explain the ability of pPDM-treatment to inhibit force production in muscle fibers (Chaen et al., 1986; Barnett et al., 1992). Previous evidence suggested that *N*-phenylmaleimide acts similarly. Like pPDM, it also has been reported to inhibit force production and lock the cross-bridges in a

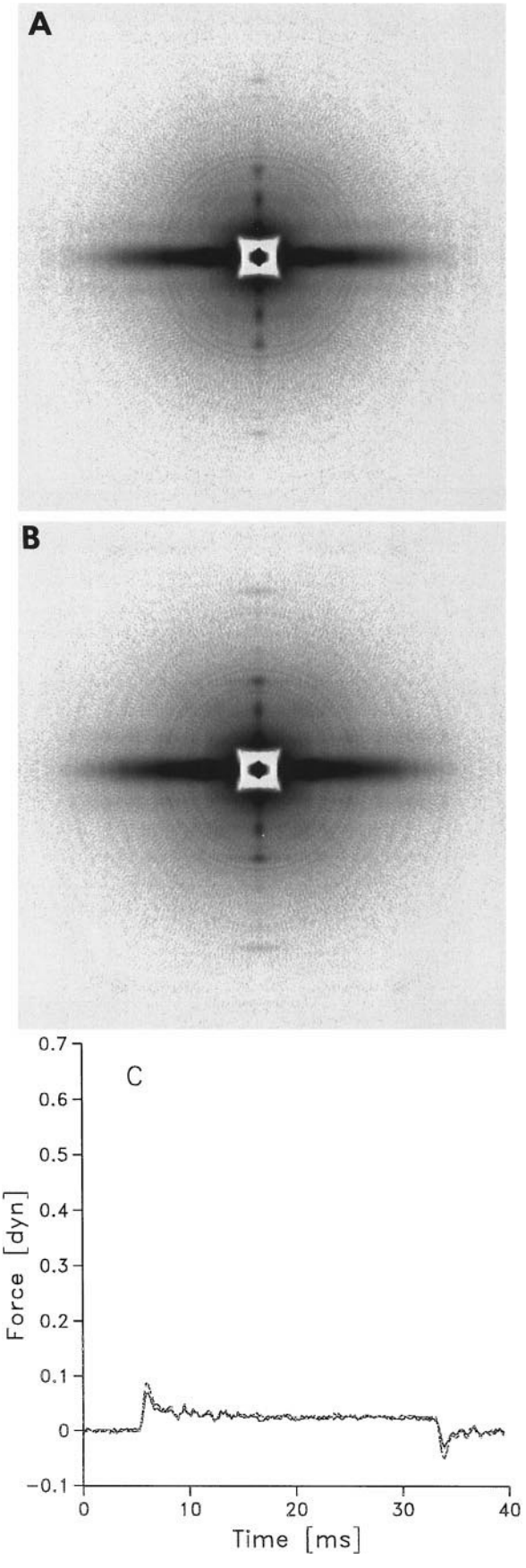

FIGURE 1 X-ray diffraction patterns (*A* and *B*) and stiffness measurements (*C*) of NPM-treated muscle. (*A*) ATP-relaxed bundle before NPM treatment; (*B*) same bundle in the same ATP solution immediately after 1 h of NPM treatment; (*C*) effect of NPM treatment on the stiffness of a single fiber in ATP-relaxing solution. Curves before (*solid line*) and after (*dotted line*) are nearly indistinguishable.

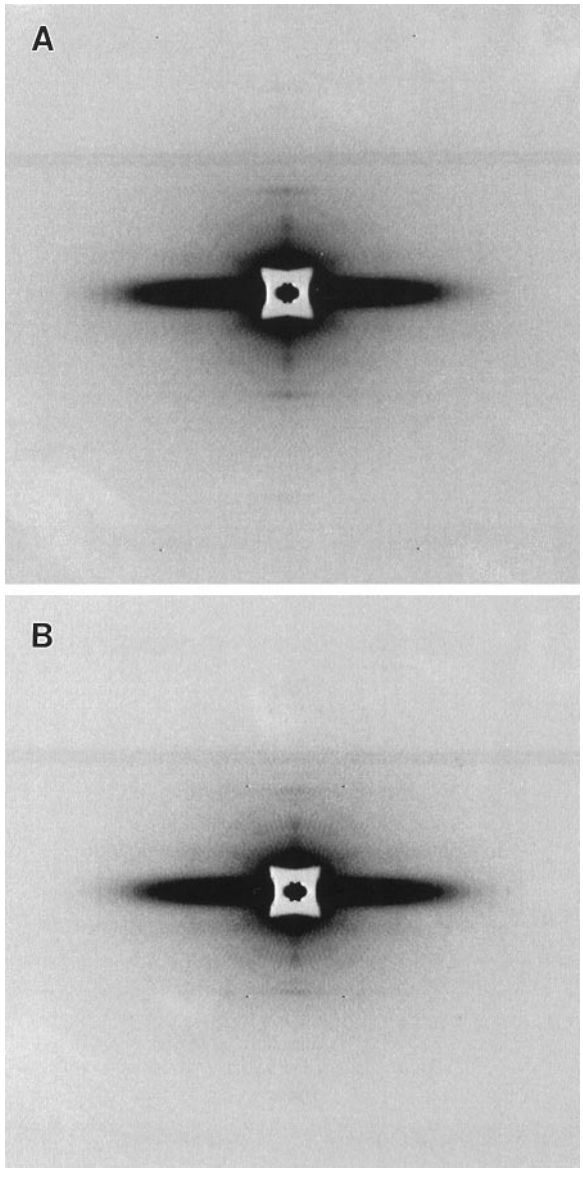

FIGURE 2 (*A*) X-ray diffraction pattern from NPM-treated fiber bundle in relaxing solution. (*B*) Diffraction pattern of same fiber in rigor(+Mg) solution. This fiber bundle is different from the one shown in Fig. 1. Classic relaxed features are present in both.

weakly binding state (Barnett et al., 1992). The data in this paper, however, suggest that in Mg-free rigor solution NPM-treated cross-bridges clearly go into a strongly binding rigor-like state characterized by a rigor-like 2-D x-ray diffraction pattern (Fig. 4 *C*) and a stiffness the same as that of a rigor fiber. The data here present the first evidence that NPM-treated cross-bridges are not always locked in a weakly binding state and further suggest that magnesium is

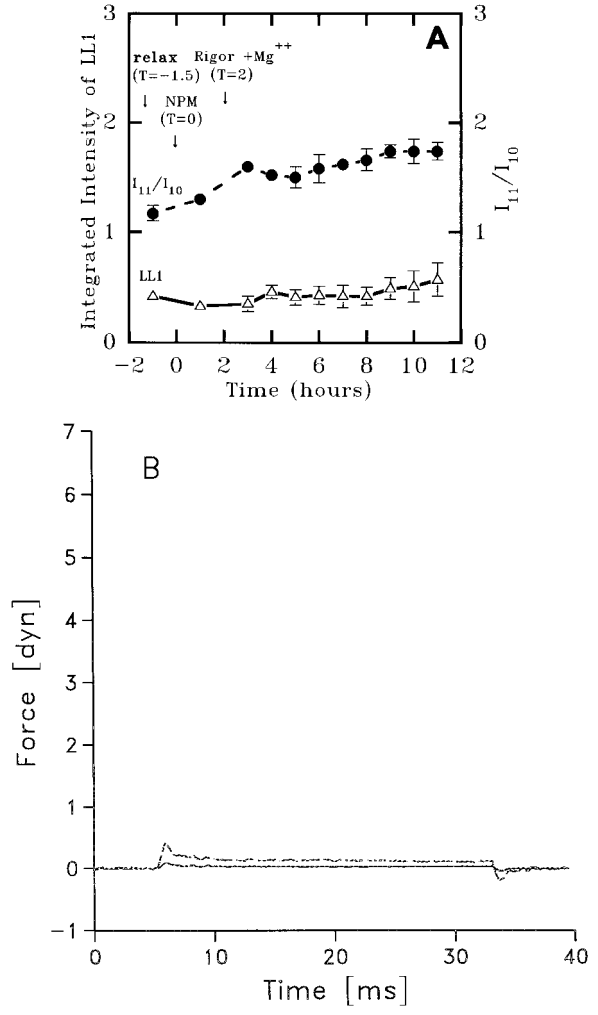

FIGURE 3 (*A*) Quantitation of x-ray patterns of small NPM-reacted muscle bundles in relaxing and in rigor(+Mg) solution; (*B*) typical stiffness measurements from a single fiber in ATP-relaxing and rigor(+Mg) solution. The mechanical response in rigor(+Mg) solution (*dotted line*) is only slightly larger than the response in relaxing solution (*solid line*).

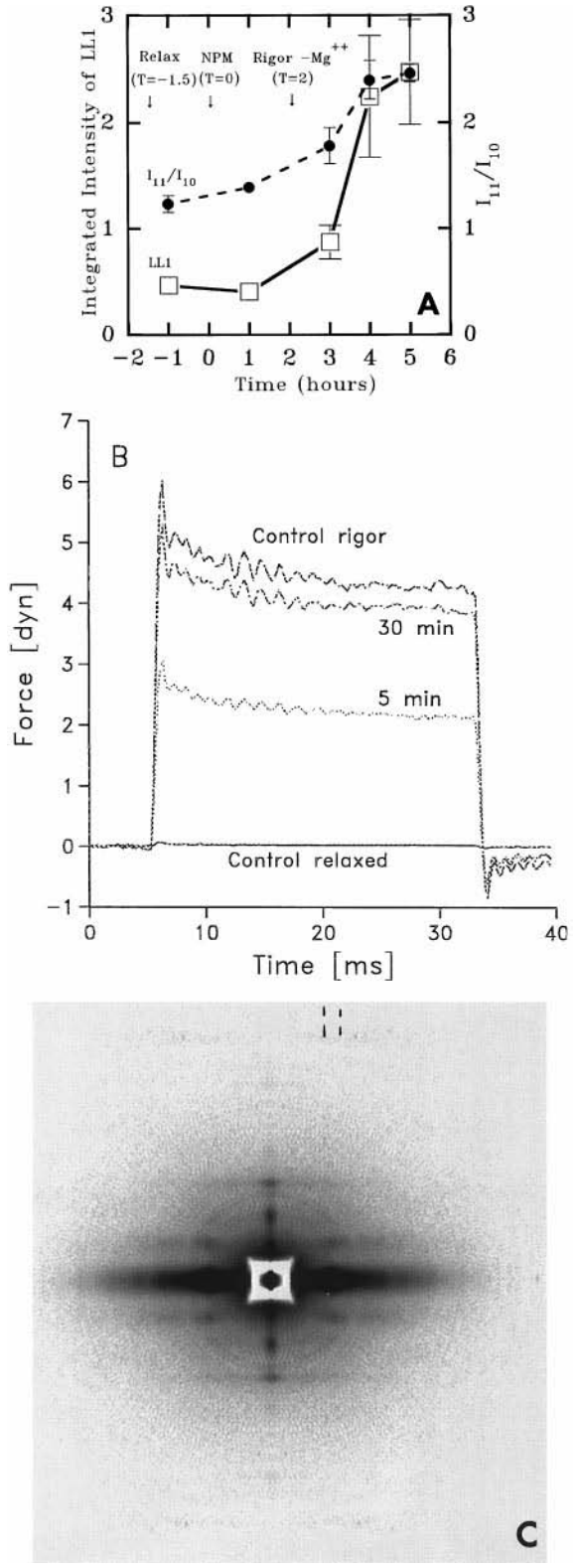

FIGURE 4 Quantitation and time course of rigor development in the absence of Mg. (*A*) Integrated intensity of the first layer line (*left coordinate*) and the I_{11}/I_{10} (*right coordinate*) as a function of time. Value of integrated intensity is normalized by I_{11} of the muscle in rigor(−Mg). NPM treatment started at time = 0. Rigor (−Mg) solution was introduced at time = 2 hours. (*B*) Time course of stiffness development in a single fiber upon removing Mg. (*C*) 2-D X-ray diffraction pattern after 3 hours in rigor (−Mg) solution (same fiber as in Fig. 1).

an important ion in determining if the cross-bridge can go into a strongly binding conformation. It is not clear from these studies how magnesium is preventing cross-bridges from going into a strongly binding conformation in rigor solution or even where magnesium is acting. There are several possibilities with regard to this latter question.

One site at which magnesium has a direct effect is the high-affinity site of troponin. Magnesium binding to the high-affinity site in the absence of calcium is important for maintaining the structural integrity of troponin. In the absence of calcium or magnesium, the troponin C subunit tends to dissociate from the troponin complex, particularly at lower ionic strength (Zot and Potter, 1982). Although it is not at all clear that such a phenomenon would occur under the ionic strength conditions of our experiment, this finding does raise the possibility that the magnesium effect is because of magnesium binding to the high-affinity Ca^{2+}/Mg^{2+} site of troponin.

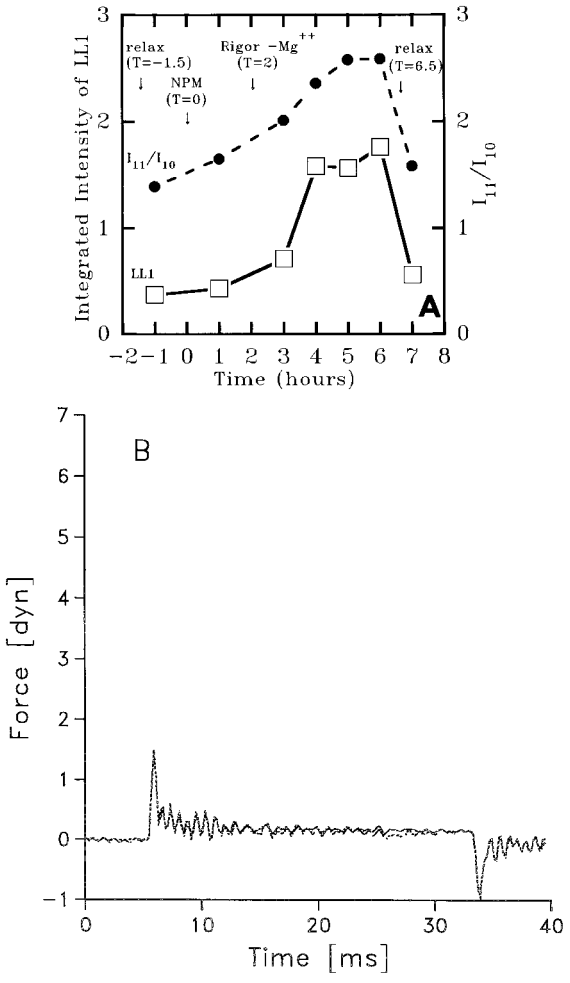

FIGURE 5 Addition of MgATP after development of rigor brings the muscle back to its initial relaxed state. (*A*) X-ray diffraction data; (*B*) stiffness measurement.

A second site that magnesium binds to directly is the regulatory light chain of myosin. Divalent cation binding to the regulatory light chain is thought to affect the rate constants of force development in skeletal muscle fibers (Diffee et al., 1996). It is difficult to understand, however, how this would account for the results reported here.

The most important role for magnesium is as the divalent cation bound to ATP. Free ATP without magnesium is not a good substrate for force production and does not relax skeletal muscle (Rizzino et al., 1970; Cooke and Franks, 1980). This raises the possibility that the relaxing effect of Mg^{2+} reported here is not because of Mg^{2+} binding directly to a protein, but because of binding to cation-free ATP trapped at the nucleotide binding site of myosin.

Regardless of the site of action of magnesium, it is interesting that, contrary to previously published results (Barnett et al., 1992), NPM-reacted cross-bridges can go into a strongly binding conformation, at least under the condition of 0 Mg^{2+}. Whether elimination of Mg^{2+} from all the sites discussed above is necessary for this to occur, remains to be seen.

The data suggest that NPM-reacted cross-bridges can undergo the weakly to strongly binding conformational change and that the nucleotide driven weakly to strongly binding conformational change in the contractile cycle is a step distinct from the hydrolysis step. For NPM-reacted cross-bridges, hydrolysis is greatly reduced but the weakly to strongly binding transition can still occur.

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