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CALCIUM AND pH-INDUCED STRUCTURAL CHANGES IN SKINNED MUSCLE FIBERS: PREVENTION BY N-ETHYLMALEIMIDE

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SUMMARY

Optical diffractometry was used to investigate structural changes in contractile proteins from chemically skinned muscle fibers. The intensity of the first order diffraction line decreased in response to calcium and alkaline pH. Fibers pre-treated with sulfhydryl blocking agents did not exhibit the calcium- or pH- induced intensity decreases. We suggest that the decreases in intensity result from structural changes in the thick filament. Optical diffractometry may be a useful technique for the investigation of conformational changes in myosin.

INTRODUCTION

It has been well established that, in vertebrates, calcium induces conformational changes in the troponin-tropomyosin complex and that these changes are necessary for the binding of myosin heads to the thin filament (1, 2). In some invertebrates such an actin "switch" does not exist; rather, it is the myosin molecule which bears the calcium sensitivity and which regulates the actomyosin interaction (3-5). This myosin switch is activated by calcium and requires the presence of light chains associated with the myosin molecule (3, 6).

A calcium-sensitive myosin switch as seen in molluscs may not exist in vertebrate skeletal muscle. However, calcium has been shown to activate the ATPase of rabbit myosin (4, 7). Furthermore, numerous investigators have observed or inferred calcium dependent structural changes in myosin which are independent of the presence of actin (8-14).

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We have recently reported additional evidence for the existence of a calcium-dependent, actin-independent change in the structure of the thick filament by analysis of the light diffracted by single chemically skinned (glycerinated, detergent-treated) skeletal fibers (15). We found that the first order diffraction line intensity decreased as the amount of free calcium present in the bathing medium was increased. Furthermore, myosin extracted fibers did not exhibit calcium-dependent decreases in intensity suggesting that thick filament structural changes were responsible for the observed calcium dependence of non-extracted fibers. However, it is possible that the extraction process adversely affected the thin filament regulatory proteins.

This study provides further evidence which indicates that the calcium-dependent changes in first order diffraction line intensity are a result of structural changes in the thick filament.

MATERIALS AND METHODS

Single semitendinosus fibers from Rana pipiens were chemically skinned following the method detailed by Julian (10). Fibers were then suspended in relaxing medium containing 100mM KCl, 1mM $MgCl_2$, 2mM EGTA, 5mM ATP, 10mM imidazole or MOPS[1] buffer, pH 7.0. Activating solutions consisted of 100mM KCl, 1mM $MgCl_2$, 4mM EGTA, 5mM ATP, 10mM MOPS buffer, pH 7.0 (Sigma Chemical Co., reagent grade), and appropriate amounts of $CaCl_2$ to bring the pCa of the solution to the desired level. Stock calcium solutions were standardized using a Radiometer calcium-sensitive electrode. In some preparations, NEM[2] or DTNB[3] was added to the relaxing medium to a final concentration of 100 uM under conditions described by Reisler et al. (20), to block either one or both of the essential sulfhydryl groups on the myosin heads. 100 uM NEM was chosen because Barany et al.(16-19) have shown that, at this concentration, NEM easily penetrates whole muscle and binds to myosin identically to the way it binds to purified myosin.

The computerised acquisition and analysis of optical diffraction data have been detailed previously (15).

RESULTS

Figure 1a illustrates the first order diffraction line profile from a single muscle fiber in relaxing medium. The sarcomere length was 4.95 um.

1. Morpholinopropane sulfonic acid
2. N-ethylmaleimide.
3. 5-5'-dithiobis(2-nitrobenzoic acid).

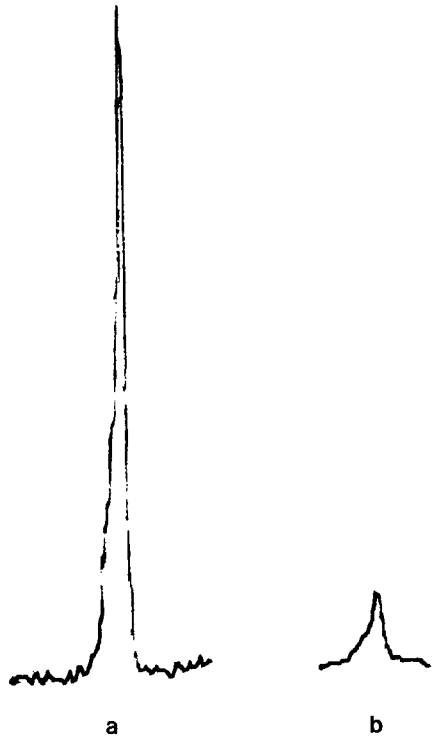

Figure 1.
Calcium dependence of the first order line intensity of a fiber at zero filament overlap (4.95 um). (a) Line profile of the fiber in relaxing solution and (b) in activating solution with pCa 5.49.

When the relaxing solution was replaced with an activating solution at a pCa of 5.49, the first order line intensity decreased by 86.5% (Fig. 1b). Intensity decreases were found to be dependent upon the pCa in the physiological range known to activate myofibrillar proteins (15).

A summary of the calcium-dependent decrease in intensity for five fibers is shown in Table I. These data show that fibers treated for 20-30 minutes with Guba-Straub solutions (100mM KCl, 10mM potassium phosphate buffer, pH 6.4, 1mM $MgCl_2$, 5mM pyrophosphate) exhibited 40-50% decreases in intensity. Moreover, myosin extraction abolished the ability of calcium to produce any change in diffraction line intensity.

The intensity of the first order line produced by fibers at zero filament overlap was also dependent on the pH of the calcium-free relaxing medium (Fig. 2). No changes in intensity were observed from pH 6.4 to 7.3. From

Table I
Average Sarcomere Lengths and First Order Line Intensities of Skinned Muscle Fibers

	n	Sarcomere length (μM ± 2 SEM)	Intensity of First Order Line (± 2 SEM) relative to Control Value
Control Relaxing soln., pH 7.0	5	4.95 ± .108	1.00
Activating soln. pCa 5.49	5	4.98 ± .068	0.32 ± .18
Guba-Straub extracted, relaxing medium	6	4.65 ± .63	0.53 ± .06
Guba-Straub, pCa 5.49	6	4.46 ± .93	1.03 ± .12*
Relaxing soln. + NEM, pH 7	7	4.31 ± .46	1.12 ± .27
Relaxing soln. + NEM, pH 7	2	2.46	1.08
NEM treated, pCa 5.49	7	4.47 ± .55	1.07 ± .17
NEM treated, pCa 5.49	2	2.46	0.99
NEM treated, pH 8.0	7	4.76 ± .39	1.08 ± .18

*Relative to the values (Line 3) which were re-standardized to 1.00 prior to the addition of calcium

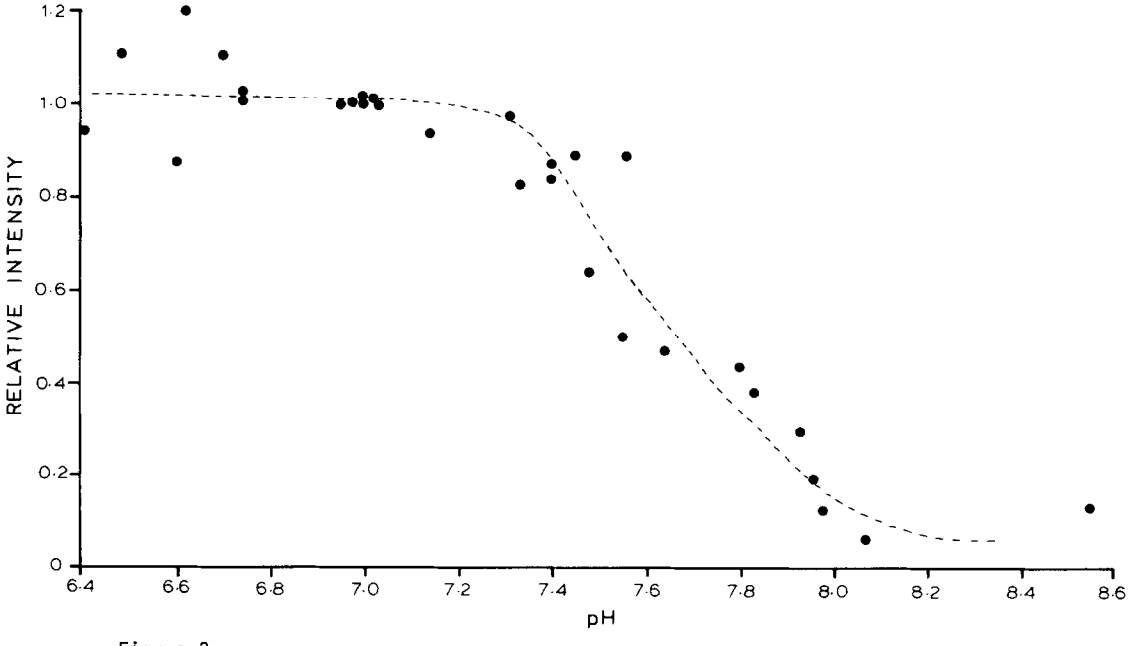

Figure 2.
Intensity (relative to values at pH 7.0) of the first order diffraction line from skinned fibers as a function of the pH of the relaxing medium.

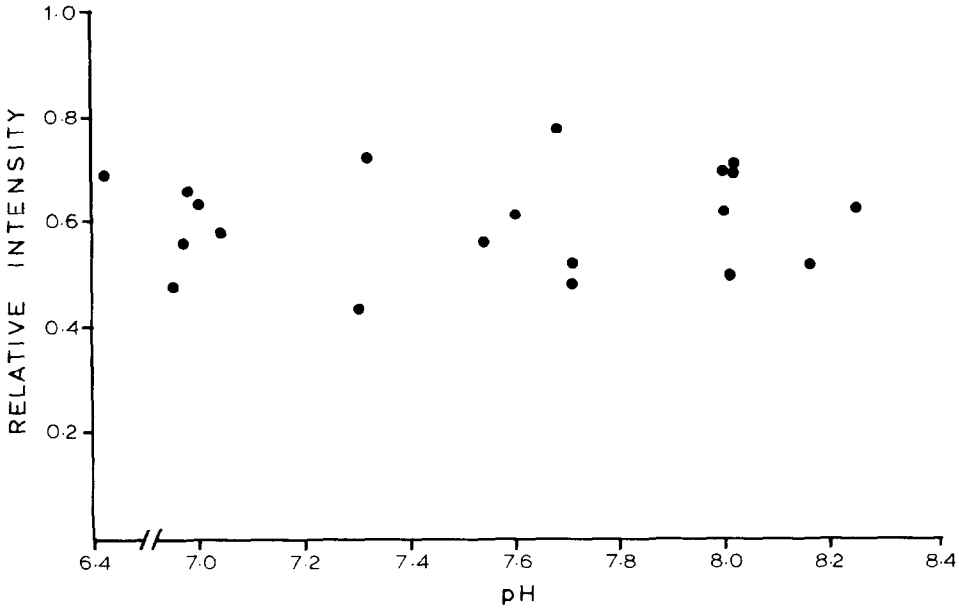

Figure 3.
Intensity (relative to values for non-extracted fibers at pH 7.0) of the first order line as a function of pH for myosin-extracted fibers.

pH 7.3 to 8.2, the intensity decreased sharply as the pH was increased and remained reduced by 90% from pH 8.2 to 8.6. At high pH values (8.0-8.6) no further reductions in first order line intensity were produced when cells were exposed to calcium (pCa 5.49).

The pH dependence of the diffraction line intensity was examined in fibers treated with Guba-Straub solutions. Unlike the behavior seen in non-extracted fibers, first order line intensity was unaffected by pH changes in the range 6.4 to 8.3 (Fig. 3).

However, it might be argued that Guba-Straub treatment interferes with the normal calcium-induced conformational changes in the thin filaments. Therefore, attempts were made to selectively alter myosin's ability to undergo structural changes without altering the calcium-dependent responses of the actin filaments. This selective myosin perturbation was accomplished with the use of the sulfhydryl blocking agents NEM and DTNB. When fibers were exposed to relaxing solutions containing 100uM NEM, no significant changes in first order line intensity were observed (Table I) whether or not

the fibers were stretched beyond filament overlap. Activating solutions (pCa 5.49) were unable to produce the characteristic intensity decreases from stretched fibers treated with NEM. The ability of NEM to block these changes was not dependent on the presence of ATP in the NEM-relaxing solution. In addition, no evidence of sarcomere shortening or decrease in intensity was observed in NEM treated fibers at 2.46 um. Furthermore, when fibers pretreated with NEM were exposed to relaxing media in the pH range 6.4 to 8.4, no changes in first order line intensity were detected. DTNB (100 uM) treatment also prevented both the calcium and the pH-dependent structural changes in the thick filament.

DISCUSSION

The data presented here provide additional evidence for the existence of calcium-dependent structural changes in thick filament proteins which are independent of the presence of actin. The evidence can be summarized as follows:

1) Calcium produced substantial decreases in the intensity of the first order diffraction line from highly stretched fibers; this effect was abolished by removal of myosin with Guba-Straub solutions (15).

2) Treatment of skinned fibers with sulfhydryl blocking agents at or beyond filament overlap completely abolished the calcium effect. The conditions used in these experiments to block the essential sulfhydryl groups of myosin substantially reduce myosin's calcium and potassium stimulated ATPase activities (20, 21). However, NEM-treated myosin has been shown to retain its ability to bind to actin (23, 24, 27-29) whether or not the thin filament proteins were also labelled with NEM (24). In addition, the presence of unreacted sulfhydryl groups on troponin and tropomyosin are not essential for the Ca^{++}-dependent response of actomyosin (22, 23, 30). Therefore, in our skinned fiber preparation, it is likely that all contractile proteins react with NEM but that only myosin is functionally affected by such treatment.

3) Variations of pH in the range known to produce large conformational changes in myosin (25) also produced large decreases in the intensity of the first order diffraction line. Moreover, the intensity decreases produced by alkaline pH were completely blocked by NEM treatment and were not seen after myosin extraction. Furthermore, the pH dependence of the intensity decrease is similar to the pH dependence of chemical cross-linking of native and synthetic myosin filaments (25, 26). This suggests that both diffractometry and chemical cross-linking techniques may be useful tools for the investigation of myosin structural changes. While both techniques are sensitive to pH-induced structural changes in thick filament proteins, calcium-induced conformational changes are not detected by the cross-linking technique (14). This implies that calcium and alkaline pH may induce different types of structural changes in the thick filament.

4) Both the calcium and pH-induced structural changes and the ability of sulfhydryl reagents to block these effects were not dependent on filament overlap. This suggests that the structural changes in the thick filaments are independent of the presence of thin filament proteins.

The possibility of calcium-dependent, actin-independent structural changes in the myosin filament is corroborated by X-ray diffraction (8, 9) and hydrodynamic studies (13). Furthermore, the DTNB light chains of myosin have been shown to bind calcium strongly (13) and the initial velocity of sarcomere shortening is calcium dependent (10-12). The calcium-induced conformational change in myosin is probably not a regulatory "switch" as is observed in some invertebrates (3, 4, 6), but may be part of a more subtle regulatory process in vertebrate striated muscle.

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