The Role of Calcium in the Adenosine Triphosphatase Activity of Myofibrils and in the Mechanism of the Relaxing Factor System of Muscle*

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Several factors tend to create the impression that the removal of Ca from the myofibrils plays an important role in the mechanism of relaxation. Among these factors are the inhibition of the activity of the relaxing factor system of muscle by Ca (2); the similarity between the effect of ethylenediaminetetraacetate and the relaxing factor system in producing inhibition of myofibrillar adenosine triphosphatase (3) and reversal of the adenosine triphosphate-induced contraction of glycerinated muscle fiber bundles (4, 5); the binding of Ca to the so-called granules (a constituent of the relaxing factor system), which, as recently shown, requires the presence of ATP (cf. (1)); and the dependence of the myofibrillar ATPase on the presence of small amounts of Ca (6). Although Bozler has shown that about half of the Ca bound to muscle fibers is removed by EDTA when relaxation occurs, after washing, even in the absence of Ca++, relaxed fibers contract again upon the addition of ATP (4, 7). He therefore interpreted relaxation in terms of the combination of relaxing agents with a site associated with firmly bound Ca.

Our studies, involving the comparison of the effect of Ca on both the complete relaxing factor system and the recently described soluble relaxing substance (8), suggest that it is the latter which is inhibited by Ca, or Ca-ATP, and that granules, in addition to producing the relaxing substance, combine with Ca in maintaining conditions favorable for the activity of the relaxing substance.

In the present report, direct evidence is presented to show that treatment with granules or EDTA does not lead to the removal of myofibrils of a Ca fraction essential to their ATPase activity, and that the mechanism of relaxing activity is most likely to depend on the binding of the relaxing substance or EDTA to the contractile proteins. The experiments show the presence of a readily removable and exchangeable, but apparently functionally unimportant, Ca fraction in myofibrils and contractile proteins.

EXPERIMENTAL PROCEDURE

Myofibrils, natural actomyosin, reconstituted actomyosin, myosin, F-actin, and muscle granules were prepared from rabbit muscle as described previously (8). Before use, natural and reconstituted actomyosin and myosin were precipitated with 10 volumes of water, and the precipitate was suspended in 0.065 M succinate buffer at pH 7.5.

Protein bound Ca was determined colorimetrically. To a 2.0-ml sample, 0.2 ml of 1 M HClO₄ was added; the precipitate was removed by centrifugation, and a 1.0-ml aliquot was neutralized with a predetermined amount of KOH and cooled to 0°C. The blank and standards were carried through the same procedure. Cold murexide solution (2 ml), prepared according to Ames and Nesbitt (9), was added to the neutralized aliquot at 0°C; the mixture was brought to room temperature and read immediately at 500 mp.

Radioactivity measurements of Ca⁴⁵ were carried out by drying protein-free samples (0.05 ml) on filter paper disks in steel planchets and counting them in a gas flow counter.

Myofibril ATPase activity was determined as described previously (10), and protein was determined by the biuret method (11).

Ca⁴⁵Cl₂ was obtained from the Union Carbide Nuclear Company, Oak Ridge, Tennessee. Crystalline disodium ATP was obtained from the Fabs laboratory, Milwaukee, Wisconsin. Spec-pure MgO (1 g of Ca per 10⁶ g of MgO) was purchased from the Jarrel-Ash Company, Newton, Massachusetts.

RESULTS

Myofibrils—The Ca found in well washed myofibrils before and after incubation with 5 × 10⁻⁵ M Ca is shown in Table I. It will be seen that essentially no net uptake of Ca takes place under these conditions.

When myofibrils were added to a solution containing Ca⁴⁵ (total Ca, 6 × 10⁻⁴ M), a decrease in the radioactivity was noted in the supernatant after removal of the myofibrils by centrifugation (Fig. 1A). Since no net Ca uptake took place under these conditions, this decrease in radioactivity must be due to an exchange with bound Ca. The exchangeable Ca, as the per-
of the bound Ca, was calculated according to the following equation:

\[
\text{Percentage of exchange} = 100 \left( \frac{A_0}{A} - 1 \right) \left( \frac{C_b}{C_b} \right)
\]

where \(A_0\) is radioactivity in c.p.m. before addition of the protein suspension; \(A\) is the radioactivity of the supernatant after addition of protein; \(C_b\), the concentration of the added Ca; \(C_b\), the concentration of bound Ca. The exchange in the experiment shown in Fig. 1A amounted to 58%; it varied between 30 and 70% with different myofibril preparations. Addition of 5 mM Mg++ and 5 mM ATP to the system resulted in the reappearance of the radioactivity in the supernatant, suggesting a displacement of bound Ca to the extent of about half of the total Ca in the myofibrils.

The displacement of bound Ca by Mg++ has been confirmed by direct Ca analysis (Table II). It can be seen that myofibrils washed with either Mg++, Mg++ and ATP, Mg++ and ATP and granules, or EDTA lose about 50% of their total Ca. The loss of about half of the bound Ca after washing with EDTA is in good agreement with the findings of Bozler (7). Treatment with either Mg++ or EDTA, in the latter case, followed by washing out of EDTA, had no effect on the ATPase activity. This strongly suggests that the Mg-displaceable and EDTA-extractable Ca is not involved in ATP cleavage.

The identity of the Ca fraction displaced by Mg and that removed by EDTA is supported by the data shown in Fig. 1B. The radioactivity incorporated into the myofibrils during exchange with Ca45 is completely removed by EDTA. Subsequent addition of Mg++ and ATP causes no further increase in radioactivity of the supernatant.

According to the above results, granules or EDTA do not remove more Ca than is displaced by Mg++. It might be argued, however, that Ca++ and/or the granules do remove additional Ca from the myofibrils, but that this Ca fraction, not displaced by Mg++, is again restored by Ca++ which is present as a contamination in the washing solution used to remove the granules or EDTA before Ca analysis or in the buffer mixture used for ATPase determination. According to this view, part of the bound Ca that is not displaceable by Mg++ but is removable by granules or EDTA would be essential for full ATPase activity.

To shed further light on this point, myofibrils were washed as outlined in Fig. 2, with and without granules or EDTA in the presence of Mg++ and ATP, and isolated by centrifugation. They were then suspended in buffer containing 6 x 10^-7 mol Ca45 (6 x 10^4 c.p.m. per ml), centrifuged, and, after being washed twice with buffer containing 5 mM Mg++, they were resuspended in buffer containing 5 mM Mg and ATP, with or without granules or EDTA, and the radioactivities of the supernatants measured. If, after the first granule or EDTA treatment, the radioactivities of the supernatants after addition of the protein.

Table II.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment</th>
<th>Bound Ca</th>
<th>ATPase activity (μmol Pi liberated/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>2.7</td>
<td>0.16</td>
</tr>
<tr>
<td>2</td>
<td>EDTA, 1 mM</td>
<td>1.6</td>
<td>0.18</td>
</tr>
<tr>
<td>3</td>
<td>Mg++, 5 mM, ATP, 5 mM</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>Mg++, 5 mM, ATP, 5 mM, granules, 330 μg protein/ml</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>3.0</td>
<td>0.54</td>
</tr>
<tr>
<td>6</td>
<td>Mg++, 5 mM</td>
<td>1.6</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Fig. 1. Exchange and displacement of myofibrillar Ca. Myofibrils were suspended in 20 mM histidine, 50 mM KCl pH 7.0; total volume, 7 ml. Ca was added as indicated in the table. The myofibrils were separated by centrifugation and resuspended in 65 mM succinate at pH 7.5; final volume, 2 ml. Ca was determined as described under "Experimental Procedure."
Treatment of myofibrils for study of tightly bound Ca

Myofibrils in 4 ml of buffer* + 5 mM ATP with or without granules or EDTA

\[
\begin{align*}
\text{Centrifuged} & \quad \rightarrow \quad \text{Supernatant} \\
\text{ppt} & \quad \rightarrow \quad \text{Suspended in 15 ml of buffer* containing } 6 \times 10^{-7} \text{ M Ca}^{4+} \\
\text{Centrifuged} & \quad \rightarrow \quad \text{Supernatant (S)} \\
\text{ppt} P_1 & \quad \rightarrow \quad \text{Suspended in 15 ml of buffer*} \\
\text{Centrifuged} & \quad \rightarrow \quad \text{Supernatant (S)} \\
\text{ppt} P_2 & \quad \rightarrow \quad \text{Suspended in 15 ml of buffer*} \\
\text{Centrifuged} & \quad \rightarrow \quad \text{Supernatant (S)} \\
\text{ppt} P_3 & \quad \rightarrow \quad \text{Suspended in 15 ml of buffer*} \\
\text{Centrifuged} & \quad \rightarrow \quad \text{Supernatant (S)} \\
\text{ppt} P_4 & \quad \rightarrow \quad \text{Suspended in 15 ml of buffer*} \\
\text{Centrifuged} & \quad \rightarrow \quad \text{Supernatant (S)} \\
\text{ppt} P_5 & \quad \rightarrow \quad \text{Suspended in 15 ml of buffer*} \\
\end{align*}
\]

* Buffer: 20 mM histidine, 50 mM KCl, 2.5 mM K-oxalate, and 5 mM Mg++.  

**TABLE III**

Effect of granules and EDTA on uptake of Ca by myofibrils

Myofibrils (8.8 mg of protein) were carried through the scheme shown in Fig. 2 with combinations as shown in the table. Bound Ca, 24 pmol; added Ca, 6 \times 10^{-6} M Ca^{4+}; was obtained by extrapolation of the data in Fig. 3. Under these conditions, the Ca uptake is 0.15 \mu mole per g of protein; this would account for a 5% exchange, i.e. 10% of that observed.

The contribution of actin to the observed exchange is negligible, since the apparent Ca exchange with actin is only 5 to 10% of the Ca bound to actin, and it is not reversed by Mg++, in contrast to the effect of Mg++ on myofibrils and actomyosin.

Experiments similar to those described in Fig. 2 and Table III

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**TABLE IV**

Calcium bound to muscle proteins

The results given in the table represent determinations on two deproteinized preparations of each protein. Ca was determined as described under “Experimental Procedure”; actomyosin and myosin suspensions, 65 mM succinate pH 7.5; and F-actin solutions, 0.1 M KCl.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Bound Ca</th>
<th>Bound Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu)mol/g protein</td>
<td>(\mu)mol/g protein</td>
</tr>
<tr>
<td>Natural actomyosin</td>
<td>4.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Reconstituted actomyosin</td>
<td>5.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Myosin</td>
<td>3.2</td>
<td>3.7</td>
</tr>
<tr>
<td>F-actin</td>
<td>10.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

**TABLE V**

Exchange and displacement of Ca bound to actomyosin, myosin, and F-actin

Conditions: 20 mM histidine, 2.5 mM oxalate, and 0.006 mM Ca^{4+}; protein concentration as indicated in table. Aliquots (2 ml) were centrifuged* before and after the addition of 5 mM Mg++ and 5 mM ATP and the radioactivities determined.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein concentration</th>
<th>Bound Ca</th>
<th>Radioactivity</th>
<th>Calculated exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>(\mu)mol/g</td>
<td>c.p.m./ml</td>
<td>%</td>
</tr>
<tr>
<td>Natural actomyosin</td>
<td>2.1</td>
<td>4.9</td>
<td>10,600</td>
<td>6,320</td>
</tr>
<tr>
<td>Recrystallized actomyosin</td>
<td>1.9</td>
<td>5.5</td>
<td>9,860</td>
<td>5,840</td>
</tr>
<tr>
<td>Myosin</td>
<td>2.4</td>
<td>3.2</td>
<td>9,200</td>
<td>5,860</td>
</tr>
<tr>
<td>F-actin</td>
<td>0.99</td>
<td>16.0</td>
<td>8,800</td>
<td>8,320</td>
</tr>
</tbody>
</table>

* Myosin and actomyosin are present in a suspension under the conditions of this experiment; they were centrifuged in a clinical centrifuge for 2 minutes. Actin was removed by centrifuging at 90,000 \times g for 5 hours.
**DISCUSSION**

The findings of Bozler (7) and of Hasselbach (12) clearly show that about half of the Ca bound to muscle fibers or actomyosin can be removed by EDTA. The results presented here confirm this and show that the EDTA-extractable Ca fraction of myofibrils is freely exchangeable with added Ca and can be displaced by Mg.

Our values for the Ca content of washed myofibrils agree well with the earlier results for glycerol-extracted muscle fibers (7). Our values reported for Ca in myosin, actin, and natural actomyosin, however, are somewhat lower than those found by Hasselbach (12).

Assuming a weight ratio of myosin to actin of 4 to 1 (13) in natural actomyosin and in the myofibrils, it follows from the values given for bound Ca in Table VI that half of the bound Ca in actomyosin is associated with myosin and half with actin. About the same percentage of the Ca bound to myosin is exchangeable or displaceable by Mg in myosin and actomyosin. None of the Ca bound to actin has this property. It follows, therefore, that the Mg-reversible exchange of about 40 to 50% of the total myofibril or actomyosin Ca cannot be accounted for in terms of the behavior of the Ca bound to the two proteins, myosin and actin, and it would seem that some of the nonexchangeable Ca of myosin and/or actin becomes exchangeable when the two proteins interact.

The correlation between relaxing factor-like activity and the ability to form chelates with Ca, in the case of EDTA and a number of EDTA analogues, has prompted Ebashi (14) to suggest that the relaxing factor system of muscle acts by removing Ca, essential for maximal ATPase activity and contraction, from the myofibril. This view gained support from the fact that muscle granules bind Ca in the presence of ATP. A similar view was expressed by Weber (6). From the findings reported herein, it is apparent that although half of the bound Ca is removed from the myofibrils by EDTA or granules, this removable Ca is not essential for maximal ATPase activity, since the Mg present in the usual ATPase assay system displaces the same Ca fraction. The possibility has been examined that a portion of the bound Ca, necessary for maximal ATP cleavage and contraction but removed by EDTA or granules, is restored by traces of Ca present in the reagents used for the suspension of the myofibrils before ATPase assay or Ca analysis. Experiments with myofibrils pretreated with granules or EDTA and then exposed to Ca show that no significant amounts of Ca are taken up that would subsequently appear as displaceable by granules or EDTA but not by Mg.

These results, taken in conjunction with those presented in the preceding paper (8), suggest that the mechanism of the relaxing factor system depends on a soluble relaxing substance which is inactivated by Ca or Ca-ATP. The relaxing substance could conceivably act on the myofibrils or on actomyosin by attaching itself to the firmly bound Ca, an idea expressed in different terms some years ago by Bozler (4). The granules, according to our view, not only produce a relaxing substance but, in view of their ability to bind Ca, also maintain conditions favorable for its activity.

**SUMMARY**

1. About 50% of the Ca bound to myofibrils, actomyosin, and myosin is displaceable by Mg++ or removable by ethylenediaminetetraacetate and muscle granules (relaxing factor).
2. Experiments with Ca++ show that the above Ca fraction is freely exchangeable with added Ca.
3. Removal of the exchangeable Ca fraction has no effect on the adenosine triphosphatase activity of the myofibrils. The existence of a Ca fraction which is extractable with ethylenediaminetetraacetate or granules, but not displaceable by Mg++, and is essential for adenosine triphosphatase activity has been excluded.
4. These results have been discussed from the point of view of...
the participation of Ca in the mechanism of action of the relaxing factor system of muscle.

REFERENCES

CORRECTIONS

In the paper by W. H. Pearlman, on page 700, Vol. 236, No. 3, March 1961, beginning line 9 of first paragraph should read as follows:

sufficiently high specific activity to be useful for metabolism experiments in man. The 16-H₃-steroid hormones, although suitable for studying their intermediary metabolism, are not appropriate for studying metabolic transformations entailing loss of the C-17 side chain because loss of the isotope might possibly ensue. A method was therefore sought for introducing tritium at an appropriate site in the steroid molecule, for example, at C-7.

(Please note that this correction has been set so that it can be cut out and pasted over the incorrectly set lines.)


In the paper by S. Sano and S. Granick, on page 1177, Vol. 236, No. 4, April 1961, Table VI, the heading "Incubation with thioglycollate and diethyldithiocarbamate" refers to the last three columns of the table only.
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