The Role of Calcium in the Superprecipitation of Actomyosin*

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In living muscle, calcium appears to be intimately involved in the reactions leading to contraction (1-4). In isolated actomyosin systems (e.g. glycerol-extracted fibers and myofibrils) the addition of calcium will result in contraction under certain conditions: when adenosine triphosphate, magnesium, and one of several relaxing factors are present, e.g. ethylenediaminetetraacetic acid, adenosine triphosphate in excess over magnesium, or the physiological relaxing factor (5-10). All of these compounds strongly bind calcium (11). This fact has recently been demonstrated for the physiological relaxing factor by Ebashi and confirmed and extended by Hasselbach and Makinose (12).

When the addition of calcium to an actomyosin system containing magnesium and adenosine triphosphate in the presence of one of the relaxing factors results in contraction, at least two different interpretations of the mode of action of calcium are possible. Calcium might combine with actomyosin, a complex between actomyosin and calcium being necessary for contraction. The inhibition of contraction before the addition of calcium would then have been due to the removal of calcium from actomyosin by the relaxing factor (13). This possibility has been considered also by Ebashi (14) and Baird and Perry (15). Or it might be considered that calcium interferes with a reaction between one of the relaxing factors and actomyosin (16-18). On this assumption, contraction is inhibited by relaxing factors because they combine with actomyosin.

For further clarification of this question we studied the effect of the removal of calcium on the superprecipitation and adenosine triphosphatase activity of actomyosin. We consider superprecipitation and high adenosine triphosphatase activity to be comparable to contraction, and inhibition of superprecipitation together with a low adenosine triphosphatase activity to be comparable to relaxation.

We used for our investigation "synthetic" actomyosin prepared from purified actin and myosin in order to be reasonably sure that the system was free from endoplasmic reticulum, the physiological relaxing factor (19). It has been found by inspection with the electron microscope that glycerol-extracted fiber bundles, well washed myofibrils, and "natural" actomyosin, but not "synthetic" actomyosin, still contain remnants of endoplasmic reticulum. Such a contamination should be avoided to exclude the possibility that the effects of calcium and calcium-chelating agents on actomyosin are indirect, i.e. mediated through their influence on the physiological relaxing factor.

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1 Personal communication.

2 W. Hasselbach, personal communication.

Experimental Procedure

Reagents

We tried to avoid contamination by extraneous Ca as much as possible. Therefore, only acid-washed Pyrex or Kimax glassware and polyethylene or polypropylene laboratory ware were used. Distilled water was treated with mixed bed resin which was changed every 2 weeks. Solid KCl was washed with methanol-EDTA followed by methanol, a treatment which reduced the EDTA-titratable metal contaminations from about 0.08 μmole per g to 0.04 μmole per g.

The iminodiacetate resin, Chelex 100, 200 to 400 mesh, was obtained from the California Corporation for Biochemical Research. It was thoroughly washed with EDTA after neutralization with HCl, followed by exhaustive washing with water. Before use in the assay, the resin was washed exhaustively with a solution of the same composition of cations as were present in the assay. Equilibration was checked by titration of Mg or Ca in the efflux solution.

EGTA was brought to pH 7.2 with KOH and titrated with a standard CaCl2 solution in presence of HHSNN4 indicator (Fisher Scientific Company) (20). A solution of Ca EGTA was obtained by adding an equivalent amount of CaCl2 (analytical reagent, titrated against EGTA) to EGTA, followed by neutralization to pH 7.0. Crystalline disodium ATP (Fisher Scientific Company) (20). A solution of MgATP was obtained by adding an equivalent amount of MgCl2 (analytical grade) to ATP (concentration by weight), followed by neutralization to the pH desired in the assay. Solutions of MgCl2 (analytical grade) were titrated against a standard solution of EDTA in presence of Eriochrome Black T (21).

Imidazole was obtained from Eastman Organic Chemicals Department and was adjusted to the desired pH with HCl.

Protein preparations

Myosin, prepared according to Portzehl et al. (22) (three precipitations), was centrifuged in 0.6 M KCl for 2 hours at 78,000 X g to remove any contaminating relaxing factor and was stored in 50% glycerol. Before use, it was reprecipitated by dilution, redissolved in 0.6 M KCl, and centrifuged for 30 minutes at 78,000 X g.

For preparation of actin, acetone powders were prepared.
according to Straub or Bárány and Bárány (23, 24). Actins from different acetone powders varied (see "Results"), but independently of known variations in preparation. All acetone powder extracts were centrifuged at 78,000 × g for 30 minutes, followed by polymerization in 0.1 M KCl, 1.0 mM MgCl₂, and centrifugation for 2 hours at 78,000 × g (25). The differences in the actin preparations from various acetone powders were not obliterated by treating G-actin with the anion exchange resin Amberlite 400 (26), EGTA, or Chelex 100. Solutions of actin prepared from the first pellet or after an additional cycle of depolymerization and repolymerization behaved in a similar manner.

However, after several purification steps, sometimes the capacity of the "synthetic" actomyosin to superprecipitate and its rate of ATP hydrolysis at Ψ/2 0.11 were decreased. We found that older preparations of actin in combination with myosin could lose the capacity to superprecipitate at Ψ/2 0.11 and 2.0 mM ATP, whereas superprecipitation and ATPase activity at Ψ/2 0.06 or with 0.2 mM ATP remained unchanged.

**Superprecipitation and ATP hydrolysis**

These processes were studied as follows. First, 2.0 ml of myosin (1 mg per ml, Ψ/2 approximately 0.15) and 1.0 ml of actin (0.5 mg per ml, 0.1 M KCl) were mixed; KCl and imidazole buffer, to obtain the final ionic strength and pH, and additional reagents were added (as mentioned in text) in a total volume of either 5 or 10 ml. The reaction was initiated by adding 1.0 ml of ATP or MgATP with vigorous magnetic stirring. After 2 minutes, either trichloroacetic acid was added, followed by the determination of inorganic phosphate (27), or the mixture was transferred to graduated tubes and centrifuged for 2 minutes in a clinical centrifuge. The volume of the precipitate was read, and the protein concentration in the supernatant was determined by the procedure of Lowry et al. (28).

**RESULTS**

We found that a number of "synthetic" actomyosin preparations at Ψ/2 0.11 and with 2.0 mM Mg hydrolyzed ATP at a low rate unless Ca++ was added (Table I). In the absence of Ca++ very little or no actomyosin precipitate could be collected after centrifugation in a clinical centrifuge (duration usually 2 minutes); actomyosin remained in the supernatant. This was in contrast to the behavior of both actomyosin to which no ATP had been added and actomyosin to which, in addition to MgATP, Ca had been added. In these two cases actomyosin was precipitated. The precipitate of 2.5 mg of actomyosin was about 0.3 ml in the absence of ATP and 0.1 ml after the addition of MgATP and Ca. Myosin alone, in the presence of MgATP either with or without Ca, remained in the supernatant.

The actomyosin preparation used in Table II hydrolyzed ATP at nearly maximal rate and superprecipitated without previous addition of Ca. However, EGTA and Chelex 100 caused an inhibition which was reversed by Ca. We used EGTA instead of EDTA because corrections for binding of Mg by EGTA may be neglected, whereas binding of Ca by the two chelating agents under our experimental conditions is quite similar. The imino-

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### Table I

**ATPase activity and superprecipitation before and after addition of CaCl₂**

<table>
<thead>
<tr>
<th>Actomyosin preparation No.</th>
<th>ATPase activity*</th>
<th>Total protein precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole/min/mg myosin</td>
<td>%</td>
</tr>
<tr>
<td>CaCl₂, 0.1 mM</td>
<td>0.16</td>
<td>0.06†</td>
</tr>
<tr>
<td>CaEGTA, 1.0 mM + CaCl₂, 0.04 mM</td>
<td>80.0</td>
<td>0.29</td>
</tr>
<tr>
<td>Chelex 100* + 0.1 mM CaCl₂ added</td>
<td>79.0</td>
<td>0.35</td>
</tr>
<tr>
<td>No CaCl₂ added</td>
<td>71.0</td>
<td>0.20</td>
</tr>
<tr>
<td>EGTA, 1.0 mM</td>
<td>22.0</td>
<td>0.06</td>
</tr>
<tr>
<td>CaCl₂, 0.1 mM + Chelex 100*</td>
<td>23.0</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* At 24.5°.
† Imidazole, 0.02 M, pH 6.6.
‡ Imidazole, 0.02 M, pH 7.0.

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### Table II

**ATPase activity and superprecipitation in presence of chelating agents with and without additional CaCl₂**

<table>
<thead>
<tr>
<th>Conditions as in Table I, pH 7.0.</th>
<th>Total protein precipitated</th>
<th>ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂, 0.1 mM</td>
<td>80.0</td>
<td>0.30</td>
</tr>
<tr>
<td>CaEGTA, 1.0 mM + CaCl₂, 0.04 mM</td>
<td>80.0</td>
<td>0.29</td>
</tr>
<tr>
<td>Chelex 100* + 0.1 mM CaCl₂ added</td>
<td>79.0</td>
<td>0.35</td>
</tr>
<tr>
<td>No CaCl₂ added</td>
<td>71.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Chelex 100*</td>
<td>22.0</td>
<td>0.06</td>
</tr>
<tr>
<td>EGTA, 1.0 mM</td>
<td>23.0</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Equilibrated with cations of assay.

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diacate resin binds both Ca and Mg. Before use, therefore, it had been equilibrated with Mg, or Ca and Mg, as described in "Experimental Procedure." The inhibition by Chelex 100 was not confined to the period when the resin was mixed with actomyosin and MgATP; after the resin had been separated from the protein by centrifugation, actomyosin still remained in the supernatant and did not superprecipitate. The amounts of resin needed to reduce activity to values similar to those produced by 1.0 mM EGTA varied between 0.2 to 0.8 ml with different preparations of actomyosin (capacity = 0.33 mmoles of Cu (NH₃)₄⁺⁺ per ml of resin).

On the preliminary evidence of two experiments, it appeared as if Chelex 100 might remove some Ca from myosin and actin also in the absence of ATP. Pretreatment with Chelex 100 of myosin in one experiment and of actin in the other resulted in a decrease in the rate of ATP hydrolysis (when no Ca had been added) from 85 to 65% and from 76 to 55% of the maximal rate.

Table III shows that CaEGTA reversed the inhibition due to EGTA, although the concentration of free EGTA remained unchanged. The addition of CaEGTA (accurately titrated so as not to contain an excess of calcium) to a solution containing...
TABLE III
Inhibition of activity by EGTA with and without additional CaEGTA
Conditions are the same as in Table I, pH 6.6.

<table>
<thead>
<tr>
<th></th>
<th>ATPase</th>
<th>Superprecipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% maxima l rate</td>
<td>% maximal amount</td>
</tr>
<tr>
<td>EchTA, 0.1 mm</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>EchTA, 0.1 mm + 0.90 mm</td>
<td>70</td>
<td>74</td>
</tr>
<tr>
<td>CaEGTA</td>
<td>17</td>
<td>74</td>
</tr>
<tr>
<td>EGTA, 0.05 mm</td>
<td>87</td>
<td>74</td>
</tr>
<tr>
<td>EGTA, 0.05 mm + 0.95 mm</td>
<td>87</td>
<td>74</td>
</tr>
</tbody>
</table>

either 0.1 mM or 0.05 mM EGTA resulted in a more than 4-fold increase of activity. By varying CaEGTA:EGTA, the concentration of Ca** at pH 6.6 is varied according to the expression

\[
\frac{[Ca**][EGTA]}{[CaEGTA]} + \frac{[H+EGTA]}{[EGTA]} + \frac{[H+EGTA]}{[EGTA]} = 1.2 \times 10^{-6}
\]

and can be calculated with the use of the appropriate equilibrium constants. When the rate of hydrolysis of ATP was plotted against the concentration of Ca**, an S-shaped curve was obtained (Fig. 1).

At the lower ionic strength of 0.05, the half-maximal activity shifted to lower concentrations of Ca** (Fig. 2). In correlation with this result at \( \Gamma / 2 \) 0.05, when neither EGTA nor Ca had been added, ATP was hydrolyzed at a much higher rate (0.34 mole per minute per mg = 80% of the maximal rate) than at \( \Gamma / 2 \) 0.11 (0.12 mole per minute per mg = 42% of the maximal rate). A comparison of these two rates with those in Figs. 1 and 2 suggests, in good agreement between the two, a contamination with Ca++ of about 1 to 2 \times 10^{-6} M. Furthermore, at \( \Gamma / 2 \) 0.05 superprecipitation occurred in the absence of added Ca even with those preparations which at \( \Gamma / 2 \) 0.11 superprecipitated only after the addition of Ca, although 1.0 mM EGTA was sufficient to prevent superprecipitation at \( \Gamma / 2 \) 0.05 (Table IV). However, actomyosin did not remain in the supernatant, but precipitated. The precipitate occupied a volume similar to that of actomyosin in the absence of ATP, about 3 times larger than that of the superprecipitated protein. The effect of the reduction of the ionic strength to 0.05 on superprecipitation was similar to that of the reduction of the concentration of ATP to about 0.01 mM (Table IV).

In contrast, a reduction in the concentration of Mg++ to about 0.01 mM resulted in superprecipitation of actomyosin even in the presence of 1.0 mM EGTA (Table V).

On a number of occasions we obtained actomyosin preparations, which not only were fully active without the addition of Ca, but which also, even in the presence of 1.0 mM EGTA, superprecipitated nearly maximally and hydrolyzed ATP at rates up to 88% of maximal. These findings agree with an earlier report by Perry and Grey (29) that “synthetic” actomyosin free Ca++

\[ \text{Fig. 1. Rate of hydrolysis of ATP at } \Gamma / 2 0.059 \text{ as a function of concentration of Ca++ as calculated from the ratio } \frac{[EGTA]}{[CaEGTA]}. \text{[EGTA] + [CaEGTA]} = 1.0 \text{ mM, 2.0 mM MgATP, 2.0 mg of myosin, 0.5 mg of actin; total volume, 5.0 ml at 23.8°, pH 6.6.} \]

\[ \text{Fig. 2. Rate of hydrolysis of ATP as a function of concentration of Ca++ at } \Gamma / 2 0.06 \text{. Conditions are the same as in Fig. 1.} \]

TABLE IV
EGTA inhibition under conditions of low ionic strength or low ATP concentration
Myosin, 2.0 mg; actin, 0.5 mg; imidazole, 0.02 mM, pH 7.0, 24°, 5 minutes of centrifugation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume of precipitate</th>
<th>Total protein precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA, 1.0 mm</td>
<td>0.30</td>
<td>86.0</td>
</tr>
<tr>
<td>CaCl₂, 0.1 mm</td>
<td>0.30</td>
<td>86.0</td>
</tr>
<tr>
<td>Control, no ATP</td>
<td>0.30</td>
<td>86.0</td>
</tr>
</tbody>
</table>

* MgCl₂, 2.0 mM; total volume, 5.0 ml.
† MgCl₂, 0.2 mM; total volume, 10.0 ml.
solutions added after pretreatment with Chelex 100 had been but followed a change in the ratio [EGTA]/[CaEGTA] (Table VI), i.e. on the acetone powder from which the actin was extracted. By treating G-actin with EGTA or Chelex 100, we were not able to convert an actin preparation which formed an actomyosin complex insensitive to EGTA to an actin which could form an actomyosin sensitive to EGTA.

**DISCUSSION**

In the experiments described in Table I, Ca or CaATP apparently reacted with actomyosin, and in the presence of Mg and ATP induced superprecipitation and a 2-to-4-fold increase in the rate of ATP hydrolysis. Before the addition of Ca, in the presence of 2.0 mM MgATP at p/2 0.11, not only was superprecipitation absent, but the behavior of actomyosin also suggested that dissociation of the complex into actin and myosin might have occurred. As with myosin, but not actomyosin in the absence of ATP, no precipitate settled during 2 minutes of centrifugation in a clinical centrifuge. The state of actomyosin under these conditions corresponded to the "sol" state in the presence of high concentrations of ATP, as described by Hasselbach et al. (30), to the "clearing phase" as described by Spicer (31) and recently by Maruyama and Gergely (32), or to the "solvabilization" of actomyosin as described by Mueller (33). Maruyama and Gergely obtained results pointing towards dissociation as the cause of the "clearing."

Such an increase in the solubility of actomyosin, possibly caused by dissociation, might be considered analogous to relaxation in a fiber system. In our experiments, this increase in solubility apparently was due to a lack of Ca in a system containing Mg and ATP at the sufficiently elevated ionic strength of 0.11.

In preparations that were fully active before addition of Ca, we assume that sufficient bound Ca was present to obtain maximal activity. This assumption is supported by the fact that superprecipitation and ATP hydrolysis were suppressed by Chelex 100. The inhibition by Chelex 100 cannot be due to a combination between protein and resin, because the inhibition remained after the resin was separated from the protein by centrifugation. Furthermore, when the resin had been equilibrated with 0.1 mM Ca, full superprecipitation occurred. Therefore, we assume that Chelex 100 inhibited superprecipitation because it removed calcium from actomyosin.

Chelex 100 reduced the ATPase activity of actomyosin only to about 60% maximal activity when actin or myosin were pre-treated with Chelex 100 in the absence of MgATP, whereas it reduced the ATPase activity to 20% when it was present together with MgATP in the final assay for ATPase activity. This finding suggests that either Chelex 100 removed Ca from actomyosin more completely in the presence of MgATP or that the solutions added after pretreatment with Chelex 100 had been contaminated by Ca.

The reversal of the EGTA inhibition by Ca was not caused by removal of free EGTA, which could combine with the protein, but followed a change in the ratio [EGTA]/[CaEGTA] (Table III, Figs. 1 and 2). The experiment lends itself easily to the interpretation that activity depended on the concentration of Ca++. On this assumption, the affinity of actomyosin for Ca++ would increase with decreasing ionic strength.

Although 1.0 mM EGTA inhibited superprecipitation and ATPase activity also at p/2 0.05, there were the following indications that dissociation did not occur. The concentration of protein remaining in the supernatant was equal in the presence of and absence of ATP and sometimes equalled only one-third of the actin concentration. In this respect the EGTA inhibition at low ionic strength differed from the polyethylene sulfonate inhibition, described by Bárány and Jaisle (34), who found dissociation to occur under similar conditions.

The removal of Ca was inhibitory for superprecipitation and ATP hydrolysis only when the concentration of total Mg was greater than 0.01 mM. Therefore it appears that the inhibition was caused by Mg and that Mg could exert its inhibitory effect only in the absence of Ca. In other words, we also assume that Ca prevents the interaction of a relaxing agent with actomyosin, but we consider Mg to be the primary relaxing agent. The usual relaxing factors would then exert their effect indirectly by removing Ca and thus making it possible for Mg to inhibit superprecipitation. In such a scheme, Ca is not considered necessary for superprecipitation unless conditions are such that an inhibition of superprecipitation by Mg has to be suppressed.

We do not yet know why some actin preparations form actomyosin complexes which are inhibited only poorly by EGTA and Chelex 100. These findings, however, demonstrate that the effect of the removal of Ca depends not only on the presence of inhibitory concentrations of Mg, but also on certain undefined properties of actin. Evidently, the inhibition of superprecipitation and ATPase activity of actomyosin in the presence of ATP needs further study. This fact is emphasized by the experiments of Parker and Gergely (17) and Maruyama and Gergely (35) who found, respectively, that EDTA did not remove calcium from myofibrils, nor was EDTA bound by myofibrils.

<table>
<thead>
<tr>
<th>MgCl₂ concentration</th>
<th>Volume of precipitate</th>
<th>Total protein precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 mM</td>
<td>0.0</td>
<td>6.0</td>
</tr>
<tr>
<td>0.20 mM</td>
<td>0.03</td>
<td>0.0</td>
</tr>
<tr>
<td>0.008 mM</td>
<td>0.10</td>
<td>88.0</td>
</tr>
<tr>
<td>0.008 mM + 0.10 mM Ca</td>
<td>0.10</td>
<td>88.0</td>
</tr>
<tr>
<td>Control, no ATP</td>
<td>0.35</td>
<td>92.0</td>
</tr>
</tbody>
</table>

* EGTA, 1.0 mM.

**TABLE VII**

*Influence of MgCl₂ concentration on EGTA inhibition*

Myosin, 2.0 mg; actin, 0.5 mg; ATP, 2.0 mM; ionic strength, 0.109; imidazole, 0.02 M, pH 7.0; total volume, 5 ml.

---

<table>
<thead>
<tr>
<th>MgCl₂ concentration</th>
<th>Volume of precipitate</th>
<th>Total protein precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 mM</td>
<td>0.0</td>
<td>6.0</td>
</tr>
<tr>
<td>0.20 mM</td>
<td>0.03</td>
<td>0.0</td>
</tr>
<tr>
<td>0.008 mM</td>
<td>0.10</td>
<td>88.0</td>
</tr>
<tr>
<td>0.008 mM + 0.10 mM Ca</td>
<td>0.10</td>
<td>88.0</td>
</tr>
<tr>
<td>Control, no ATP</td>
<td>0.35</td>
<td>92.0</td>
</tr>
</tbody>
</table>

* EGTA, 1.0 mM.
SUMMARY

1. Some actomyosin preparations superprecipitate very little or not at all and hydrolyze adenosine triphosphate at one-quarter or one-half of the maximal rate obtained on addition of CaCl₂ to give a concentration of 0.1 mM.

2. A number of preparations which show maximal activity without addition of Ca are fully inhibited by iminodiacetate resin (Chelex 100) or 1.0 mM ethylene glycol bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA). The experiments suggest that both reagents act by removing calcium from actomyosin.

3. There are indications that in the absence of Ca at T/2 0.11 and with 2 mM Mg-adenosine triphosphate dissociation of actomyosin into myosin and actin may have occurred. The evidence suggests that at T/2 0.06 and with 1.0 mM EGTA superprecipitation is inhibited without concomitant dissociation.

4. Lack of Ca inhibits superprecipitation only if the concentration of total Mg is higher than 0.01 mM. This suggests that Ca is not necessary for the reactions involved in superprecipitation but that it blocks the inhibitory effect of Mg.

5. Some actomyosin preparations are inhibited only to a small extent, even by as much as 1.0 mM EGTA. This is shown to be a property of the actin moiety.

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