Studies on Myofibrillar Adenosine Triphosphatase with Calcium-free Adenosine Triphosphate

II. CONCERNING THE MECHANISM OF INHIBITION BY THE FRAGMENTED SARCOPLASMIC RETICULUM*

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It has been reported by several groups of workers that incubation of the relaxing factor grana of muscle with magnesium and adenosine triphosphate results in the formation of a soluble relaxing substance capable of inhibiting the magnesium-activated adenosine triphosphatase activity of myofibrils or actomyosin (3-6) and the contraction of single glycerinated muscle fibers (7). In addition, Briggs and Fuchs have reported that a soluble relaxing substance can be extracted directly from muscle (8) and have suggested that this substance may be a phospholipid (9).

The physiological significance of a soluble relaxing substance has been brought into question by experiments (10-14) which have shown that the inhibition by grana or chelating agents of ATPase activity and superprecipitation of actomyosin are accompanied by the removal of bound exchangeable calcium from actomyosin.

We have recently demonstrated a calcium requirement of the magnesium-activated myofibrillar ATPase in the absence of calcium-binding agents (15, 16). Calcium contamination present in commercially available ATP could account for earlier results which suggested that calcium was not required for maximal ATPase activity in the presence of magnesium (17). In view of these facts, we have reinvestigated the problem of the soluble relaxing substance and found that the removal of calcium which contaminates commercially available ATP can account for effects previously attributed to a soluble relaxing substance released from relaxing factor grana.

EXPERIMENTAL PROCEDURE

Glycerinated myofibrils from rabbit skeletal muscle were prepared as described previously (16). For the preparation of grana, muscle was homogenized for 1 minute with 3 volumes of 0.1 M KCl and 0.005 M histidine, pH 7.3. Myofibrils were removed by centrifugation at 1,000 × g for 20 minutes, and a mitochondrial fraction was removed by centrifugation at 8,000 × g for 20 minutes; grana were obtained by centrifugation at 25,000 × g for 1 hour (18).

In order to obtain a grana-free relaxing system, grana, 0.005 to 0.05 mg of protein per ml, were incubated for 10 minutes at pH 7.5 in 0.08 or 0.09 M Tris-HCl buffer, 5 mM MgCl₂, 5 mM ATP, 5 mM potassium oxalate, and 0.0625 M KCl. The grana were removed by filtration through a Millipore filter (pore size, 0.45 μ) (18); the filtrate was passed through a second filter of the same pore size and stored at −10°C. This filtrate was used for testing myofibrillar ATPase activity.

Removal of calcium from solution by grana was determined under the conditions described above in the presence of 10⁻⁴ M CaCl₂, containing 0.5 μc of ⁴⁵Ca, by measuring the decrease in radioactivity in the filtrate after removal of grana by Millipore filtration. Dowex 50-treated ATP was used in these experiments. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter according to the method of Loftfield and Eigner (19). All grana preparations, when tested in a concentration of 0.1 mg of protein per ml, reduced the calcium concentration in the filtrate to less than 10⁻⁴ M.

For measurements of myofibrillar ATPase activity in the presence of Dowex 50-treated ATP, 0.4 ml of a suspension of myofibrils in 0.1 M Tris, pH 7.5, containing 1.0 to 1.25 mg of protein, was added to 1.6 ml of a solution containing 0.08 or 0.09 M Tris (pH 7.5), 5 mM MgCl₂, 5 mM Dowex 50-treated ATP, 5 mM potassium oxalate, 0.0625 M KCl, and varying concentrations of calcium. Incubation was carried out for 5 minutes at 20-23°C, and the reaction was stopped by adding 2 ml of 10% trichloroacetic acid. The inorganic phosphate liberated was determined according to Fiske and SubbaRow (20).

To study the effect of the filtrate on myofibrillar ATPase activity, 0.4 ml of a suspension of myofibrils containing 1.0 to 1.25 mg of protein and varying amounts of calcium was added to 1.6 ml of the filtrate, and the ATPase activity was determined as described above. Since less than 4% of the ATP was hydrolyzed during incubation with grana, no additional ATP was added to the filtrate.

† The term "filtrate" throughout this paper will be used to denote the grana-free relaxing system after two filtration steps.
Table I

<table>
<thead>
<tr>
<th>Treatment of myofibrils</th>
<th>Treatment of ATP or assay system</th>
<th>ATPase activity</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>μmole Pi/mg protein/min</td>
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<td>No CaCl\textsuperscript{2}</td>
<td>10\textsuperscript{-4} M CaCl\textsuperscript{2}</td>
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<tr>
<td>DOC</td>
<td>Grana</td>
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<tr>
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<td>Dowex 50-H\textsuperscript{+}</td>
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<tr>
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<td>Dowex 50-H\textsuperscript{+}</td>
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</tr>
<tr>
<td>none</td>
<td>Grana</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* The bound exchangeable calcium of the myofibrils contributes about 10\textsuperscript{-4} m calcium to the system.

Fig. 1. Effect of pH on myofibrillar ATPase activity. Conditions of ATPase assay: 0.06 m Tris-HCl, 4 mM MgCl\textsubscript{2}, 4 mM ATP, 4 mM potassium oxalate, and 0.05 m KCl, pH 7.5. Incubation with grana, 0.05 mg of protein per ml, was carried out at pH 7.5, as described in "Experimental Procedure." Grana were removed by Millipore filtration, and the pH of the filtrate was adjusted before addition of myofibrils. The pH was measured after addition of myofibrils. ATPase activity in the presence of 10\textsuperscript{-4} m CaCl\textsubscript{2} was taken as 100%. This activity remained constant throughout the pH range tested. Myofibrils contributed about 10\textsuperscript{-4} m calcium to the system. Protein was determined by the biuret method, standardized against crystalline human serum albumin.

ATP was obtained from Pabst Laboratories and purified with Dowex 50 in the H\textsuperscript{+} form as described previously (16). All preparations of purified ATP, 0.04 to 0.08 m, were checked for the removal of calcium contamination by titration with EGTA\textsuperscript{3} with calcium as the indicator (21, 22). By this method, the presence of 10\textsuperscript{-4} m calcium could be detected in 0.04 to 0.08 m ATP. ATP used in incubation with grana was not treated with Dowex 50.

The abbreviation used were: EGTA, 1, 2-bis-(2-dicarboxy-methylaminoethoxy)ethane, or ethylene glycol bis-(β-aminoethyl ether)-N\textsubscript{2}, N\textsubscript{4}-tetracetic acid; DOC, sodium deoxycholate.

RESULTS

The ATPase activity of myofibrils suspended in the grana-treated filtrate was about 30% of that observed with untreated ATP. The addition of 10\textsuperscript{-4} m calcium restored the original activity (Table I). The use of ATP purified with Dowex 50 in a reaction mixture not exposed to grana had essentially the same inhibitory effect as the filtrate. The same results were obtained with myofibrils that had been treated with 1 mM sodium deoxycholate (see Paper I (16)) to destroy any residual fragments of the sarcoplasmic reticulum that may have been present (14, 23). The ATPase activity in the presence of 10\textsuperscript{-4} m CaCl\textsubscript{2} was somewhat lower with DOC-treated myofibrils.

Fig. 1 illustrates the effect of pH on ATPase activity of myofibrils suspended in the filtrate or in a medium containing Dowex 50-treated ATP. The ATPase activity in the presence of 10\textsuperscript{-4} m calcium, which was unaffected by pH in the range studied, was considered to be 100%. The use of Dowex 50-treated ATP resulted in 60% inhibition throughout the pH range studied, while with the filtrate that had been subjected to a single filtration the inhibition was greater at lower pH values than at pH 8.2 or 8.5. After the filtrate was passed through a Millipore filter for a second time, the results were identical with those obtained with Dowex 50-treated ATP; i.e., there was no dependence on pH. Solutions which had been treated with grana, filtered once, and stored at -10\textsuperscript{o} for 2 weeks produced no inhibition of ATPase activity, while twice filtered solutions retained their inhibitory effects after storage at -10\textsuperscript{o} for at least 4 weeks. Addition of EGTA increased the inhibition by the filtrate throughout the pH range tested.

These observations clearly indicated that two filtrations were necessary to obtain a grana-free system. This was brought out further by the fact that after one filtration up to 50% of the ATP present was hydrolyzed during storage overnight at -10\textsuperscript{o} and subsequent thawing, while after two filtrations no hydrolysis of ATP was found under these conditions. All experiments reported below were done with the use of two filtration steps.

As shown in Fig. 2, at pH 7.5, the dependence of the myofibrillar ATPase activity on calcium concentration was the same whether Dowex 50-treated ATP or the filtrate was used. This dependence on calcium was also the same for myofibrils which had been treated with DOC, although the maximal activity was lower.

Since, according to Briggs and Portzehl (24), the relaxing factor is more effective at pH 6.2 than at 7.2, and since, according to Briggs, pH 6.5 to 6.7 is optimal for obtaining preparations of soluble relaxing substance, the effects on myofibrillar ATPase activity of the filtrate obtained at pH 6.5 and of Dowex 50-treated ATP were compared. As shown in Fig. 3, the inhibition of ATPase activity and the dependence of activity on calcium concentration again were similar for both systems, although at pH 6.5 the absolute value of the inhibition was less than at pH 7.5.

The calcium concentration in the filtrate and the ATPase activity of myofibrils suspended in the filtrate are shown in Fig. 2. A reaction mixture not exposed to grana had essentially the same inhibitory effect as the filtrate. The same results were obtained with myofibrils that had been treated with 1 mM sodium deoxycholate (see Paper I (16)) to destroy any residual fragments of the sarcoplasmic reticulum that may have been present (14, 23). The ATPase activity in the presence of 10\textsuperscript{-4} m CaCl\textsubscript{2} was somewhat lower with DOC-treated myofibrils.

* The ATPase activity in the presence of 10\textsuperscript{-4} m CaCl\textsubscript{2} varied within the range of 0.3 to 0.6 μmole of Pi per mg of protein per minute, depending on the preparation of myofibrils used.

5 F. N. Briggs, personal communication.
FIG. 2 (left). Effect of calcium on myofibrillar ATPase activity with grana-treated system or Dowex 50-treated ATP at pH 7.5. Conditions of ATPase assay: 0.06 M Tris-HCl, 4 mM MgCl₂, 4 mM ATP, 4 mM potassium oxalate, and 0.05 M KCl, pH 7.5. Incubation with grana was carried out at pH 7.5 as described in "Experimental Procedure." •, Dowex 50-treated ATP; □, filtrate prepared with 0.02 mg of grana protein per ml; Δ, filtrate, myofibrils treated with DOC and EDTA.

FIG. 3 (right). Effect of calcium on myofibrillar ATPase with the grana-treated system or Dowex 50-treated ATP at pH 6.5. Conditions of ATPase assay: 4 mM MgCl₂, 4 mM ATP, 4 mM potassium oxalate, 0.06 M imidazole, and 0.05 M KCl, pH 6.5. Incubation with grana was carried out at pH 6.5 as described in "Experimental Procedure." •, Dowex 50-treated ATP; □, filtrate prepared with 0.05 mg of grana protein per ml.

DISCUSSION

Two views have been put forward concerning the mechanism by which relaxing factor grana inhibit the magnesium-activated ATPase activity and contractile responses of actomyosin systems. According to one, upon incubation with magnesium and ATP the grana produce a soluble relaxing substance which inhibits the ATPase activity of actomyosin and the contraction of muscle fibers (3–7). According to the other view, the binding of calcium to actomyosin is required for maximal ATPase activity of actomyosin systems, and inhibition occurs when the grana remove this calcium from actomyosin (10–14). The ability of grana to bind calcium has been well documented (13, 14, 18, 25, 26), and Weber, Hera, and Reiss (12) have shown that bound calcium of actomyosin is removed by grana in the presence of magnesium and ATP. Since the calcium requirement for magnesium-activated ATPase activity of actomyosin suggested by Weber (27) now appears to be well established (10, 11, 13, 16), it appears that the second mechanism of action could account for the inhibition of the ATPase activity of actomyosin by grana.

It is seen from the present report that the same degree of inhibition of myofibrillar ATPase activity produced by preincubation of the assay system with grana can be produced upon treatment of commercially available ATP with Dowex 50. Furthermore, the dependence of the myofibrillar ATPase activity on calcium concentration is identical after either treatment. ATPase activity with 2 × 10⁻⁴ M added calcium is the same as that observed with untreated ATP.

When the concentration of grana protein is varied during the pretreatment of the ATPase assay system, the inhibition of myofibrillar ATPase activity parallels the removal of calcium from the system by grana. The inhibition reaches a maximum when the total calcium concentration in the filtrate is reduced to 2 × 10⁻⁵ M, and further increases in the grana concentration do not produce further inhibition. However, even at the high grana concentrations, the inhibition of ATPase activity is not complete as seen by the fact that the addition of EGTA to the filtrate did produce further inhibition. The greater activity

Addition of EGTA to the filtrate increased the inhibition to about 90%.

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in the absence of EGTA appears to be due to the 2 to 4 mmoles of calcium originating in the myofibrils. If a soluble relaxing substance had been released from the grana during preincubation, one would have expected the myofibrillar ATPase activity to be dependent on the grana concentration, even in the range of maximal calcium uptake, owing to the presence of increasing amounts of the postulated relaxing substance.

To obtain a grana-free filtrate, it is necessary to filter the ATP-grana system twice since some grana apparently pass through the filters during the first filtration, as indicated by the hydrolysis of the ATP in solutions which are filtered only once. At pH 8.2 to 8.5, the release of calcium from grana which leak through the filter during the first filtration probably accounts for the smaller inhibitions by the once filtered system at pH 8.2 and 8.5. The myofibrillar ATPase activity with Dowex 50-treated ATP, with the filtrate after two filtrations, or in the presence of 10^{-4} M CaCl_2 or 1 mM EGTA showed no pH dependence in the pH range from 7.0 to 8.5. There was also no difference between the effects of the filtrate or of Dowex 50-treated ATP. The pH dependence in grana-treated solutions that have been filtered only once is similar to that found by Parker and Gergely for the so-called “soluble relaxing substance” (3). This similarity suggests that some grana may have been present in the preparations of Parker and Gergely (3).

It appears from the above discussion that under conditions used in the present experiments, the inhibition of myofibrillar ATPase activity produced by preincubation of grana with magnesium and ATP, which was previously attributed to the presence of a soluble relaxing substance, is the result of the removal of calcium contaminating the system. This contamination present in commercially available ATP (15, 16, 28) was not taken into account in previous reports on the formation by grana of a soluble relaxing substance (3, 4, 7, 29-32). However, the “soluble relaxing substance” present in the supernatant fraction of a muscle homogenate reported by Fuchs and Briggs (8, 33) cannot be explained on the basis of a lack of calcium. Thus the present results do not preclude the existence of a soluble relaxing substance under any circumstances. However, it appears to be unnecessary to postulate a role for such a substance in the regulation of ATPase activity of actomyosin and contraction of muscle models under the present conditions in vitro, since the removal of calcium from actomyosin systems is sufficient to inhibit the ATPase activity and contractile response (12, 13, 16, 34).

Nagai, Makinose, and Hasselbach (35) reported that the inhibition of myofibrillar ATPase activity by grana was reversed on separation of the myofibrils from the grana. In their experiments, myofibrils incubated in a solution containing grana, magnesium, and ATP were removed by centrifugation and resuspended either in fresh solutions containing magnesium and ATP or in the original solution, from which grana had also been removed. In the former case, their results can be explained by the calcium added as a contaminant with ATP. The observations in the second case are at variance with the results reported in this paper.

The reported inhibition by caffeine of the relaxing effects of grana without an effect on the calcium binding (36) has been considered as an argument against attributing relaxation to the accumulation of calcium by grana. Weber, Herz, and Reiss (12) found, however, that caffeine does not prevent the inhibition by grana of superprecipitation of actomyosin. We have found that neither the calcium binding of grana nor the inhibition by grana of myofibrillar ATPase activity is influenced by caffeine. A recent report that a relaxing effect of a so-called light microsomal fraction of muscle can be observed without calcium binding (37) will have to be evaluated on the basis of further evidence.

Uchida and Mommaerts (38) suggested that 3',5'-cyclic adenylic acid might be the soluble relaxing substance. Their observations, however, have not been confirmed by other workers (39), including ourselves, or in Mommaert's laboratory (40). Evidence for the production of a soluble relaxing substance by grana prepared from cardiac muscle has been presented by Honig et al. (5, 6), but the inhibition of myofibrillar ATPase activity by this system is not reversed by calcium (5, 6).

However, the use of the ATPase activity of cardiac myofibrils as a measure of the activity of actomyosin is complicated by the fact that the ATPase activity of cardiac myofibrils is due in part to the activity of a non-actomyosin ATPase which is not sensitive to EGTA or calcium (41). The sensitivity of the ATPase activity of actomyosin to EGTA is the same for actomyosin from cardiac or skeletal muscle (41). Weber, Herz, and Reiss (42) and Fanburg, Finkel, and Martonosi (41) have found that cardiac grana bind calcium and inhibit the syneresis of myofibrils at grana concentrations below those used by Honig et al. (5, 6), suggesting that the ATPase activity of cardiac actomyosin also may be regulated primarily by the concentration of free calcium.

**Summary**

The inhibition of magnesium-activated adenosine triphosphatase activity of myofibrils from rabbit skeletal muscle produced after preliminary incubation of the assay system with fragments of the sarcoplasmic reticulum (grana) has been shown to be the result of the removal of contaminating calcium from the system. This calcium contamination is present in commercially available adenosine triphosphatase in sufficient amounts to activate the myofibrillar enzyme fully. The effect of purification of adenosine triphosphatase with Dowex 50 on myofibrillar adenosine triphosphatase activity is identical with the effect of preincubation with grana. Under these conditions there is no evidence for the formation or release of a soluble relaxing substance by grana. Some earlier reports on the release of such a substance by grana can be explained by the removal of contaminating calcium from the assay system.

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**References**


*J. C. Seidel and J. Gergely, unpublished observations.*
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