The Role of the Calcium-Troponin-Tropomyosin Complex in the Activation of Contraction*  

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SUMMARY

This paper considers the function of troponin-tropomyosin in the control of actomyosin contraction. The findings indicate that: (a) mild heat treatment, known to desensitize native actomyosin to calcium and to inhibit relaxation through the oxidation of certain —SH groups, acts by the oxidation of —SH groups on actomyosin per se, not on troponin-tropomyosin, and these —SH groups appear to play no essential role in the attachment of troponin-tropomyosin to actomyosin (shown by a new turbidimetric method for measuring the combination of these proteins); (b) removal of troponin-tropomyosin from native actomyosin by washing, which is another procedure known to desensitize the system to calcium, prevents the rapid contraction that normally occurs in the presence of calcium and high levels of substrate, MgATP. The system recovered its ability to contract under these conditions only when troponin-tropomyosin was returned to it.

These observations support the hypothesis that relaxation of actomyosin is caused by the binding of MgATP to an inhibitory (relaxing) site, and that muscular contraction is controlled by the modification of this inhibitory interaction. Thus, it is proposed that the troponin-tropomyosin complex without calcium favors relaxation by facilitating MgATP binding to this site, whereas the formation of a calcium-troponin-tropomyosin complex has the opposite effect, i.e., it favors contraction by interfering with the binding of MgATP at the relaxing site.

Muscular contraction is controlled by changing the level of calcium available to the contractile filaments. When there is virtually no calcium (less than 0.1 µM) in the sarcoplasm, the muscle is relaxed because the high physiological level of substrate, MgATP, inhibits ATP hydrolysis and contraction. With calcium (between 1 and 10 µM) in the sarcoplasm, the muscle contracts because calcium overcomes this substrate inhibition (1-5). This control by calcium depends on the presence of the troponin-tropomyosin complex, which is often called the relaxing protein (6-15). In this paper we present evidence to support the view that the physiological role of these control proteins is not only to facilitate relaxation in the absence of calcium, as their name implies, but also to potentiate contraction in the presence of calcium. In this work we made use of a new turbidimetric method for directly measuring the combination of actomyosin with the troponin-tropomyosin complex.

METHODS

Preparation of Natural Actomyosin Gel—Natural actomyosin was prepared from rabbit skeletal muscle as previously described (10). The gel, suspended in 0.1 M KCl, was kept on ice in the cold room and used within 7 days of its preparation.

Preparation of Troponin-Tropomyosin Solution, Whole Extract—A muscle extract containing the troponin-tropomyosin complex was prepared by the method of Ebashi and Ebashi (8). After myosin was extracted from 100 g of rabbit back muscle, the residue was washed two times at 0° with 300 ml of a solution containing 0.02 M KCl and 0.2 M NaHCO₃, and two more times at 0° with 300 ml of water. The washed residue was suspended in 100 ml of water at room temperature and allowed to stand for 4 hours. It was then filtered by gravity through Whatman filter paper No. 40 for 4 hours at room temperature and for another 24 hours in the cold room. About 125 ml of filtrate were obtained containing approximately 6 mg of protein per ml. The solution was stored on ice in the cold room and used within 3 weeks of its preparation; during this time it lost practically none of its activity.

Partially Purified Extract—To remove α-actinin from the whole extract, 25% ammonium sulfate was added to the filtrate. The precipitate was removed by centrifugation for 10 min at 5000 rpm, and the supernatant was dialyzed overnight against 0.1 M KHC₀₃ at 3°. This partially purified extract, considered to be free of α-actinin (17), gave exactly the same results as the whole extract in all experiments.

Preparation of Heated Gel; Heat Inactivation of Control System—The method for selectively desensitizing the native gel to calcium was essentially the same as one previously described (18) except for small changes in heating time and temperature. The gel was heated for 10 min at 45° under the standard conditions used for measuring superprecipitation (given below). To start, a small volume (4 ml) of gel was added dropwise to a large volume (150

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ml) of reaction solution already at 45°. The suspension was stirred continuously during the entire procedure. At the end of the 10-min heating period, the mixture was rapidly cooled to 20° and allowed to stand at this temperature (or lower) for at least 10 min before returning it to 25° where all the measurements were made.

**Preparation of Washed Gel; Removal of Troponin-Tropomyosin Complex**—To remove the troponin-tropomyosin complex from the gel, we used a method similar to that described by Komins and Yoshioka (10). Stock actomyosin suspension (5 ml) was stirred thoroughly into 30 ml of an alkali washing solution containing equal volumes of 2 mM Na₂CO₃ and 2 mM NaHCO₃. The gel was then separated by low speed centrifugation and washed two more times with 30 ml of the same solution. During the second and third wash, the gel swelled considerably and, with time, became increasingly more difficult to centrifuge down. Therefore, in each of these washes, after the protein was allowed to swell for 15 to 20 min, 0.3 ml of 2.4 M KCl was added before centrifugation. This salt caused the gel particles to become more discrete and easily centrifuged. It should be emphasized that the washing procedure was ineffective when the salt was added to the washing solution at the start; apparently some swelling must occur.

**Measurement of Superprecipitation**—Superprecipitation of the gel suspension was measured by recording the increase in turbidity at 545 μm under the standard conditions given below. The suspension was stirred continuously with a magnetic stirrer and maintained at 25° in the sample cell (2-cm light path) of a Zeiss PMQ II spectrophotometer. The reaction was started by adding a small volume (e.g. 0.1 ml) of an ATP solution to 12 ml of gel suspension. The method, routinely used in this laboratory, has been described in detail (16). Under standard conditions, at 0.1 mM ATP and with calcium, the native gel superprecipitated at the rate of 5 to 7 absorbance units per mg per sec (2-cm light path).

**Measurement of Clearing**—Clearing of the gel suspension was measured by recording the decrease in turbidity at 545 μm under the standard conditions given below. The clearing reaction was started by adding a small volume (usually 0.1 ml) of 0.3 mM sodium PPᵢ to the reaction solution. When this PPᵢ was added to the gel suspension, the turbidity fell sharply and then settled at a new steady value. The new level was set by the concentration of PPᵢ in the reaction mixture; the higher this concentration, the lower the turbidity, with the lowest value corresponding to complete dissolution of the gel. At each concentration of PPᵢ, the steady turbidity reading was the same no matter how the PPᵢ was added; when it was added all at once, the record showed one large step down; when it was added successively in parts, as, for example in Fig. 1, the record showed a number of smaller steps down to the same final value.

The response of the gel to PPᵢ was highly reproducible. With conditions carefully controlled, nearly identical records of the kind shown in Fig. 1 were obtained from one day to another and from one preparation to another. In such stepwise records, each steady turbidity reading can be used to determine the optical density changes caused by a given concentration of PPᵢ in the reaction solution. A plot of these optical density changes as a function of PPᵢ concentration gives a sigmoid curve of the kind shown in Fig. 2. For comparing such curves, we define a clearing constant, C₁/₂, as the concentration of PPᵢ causing one-half the maximum fall in optical density; the higher this constant, the greater the resistance of the gel to clearing. A measure of calcium sensitivity is the effect of this ion to increase the C₁/₂, i.e. to shift the clearing curve to the right, as shown in Fig. 2.

**Measurement of Combining Reaction**—The increase in optical density that occurs on the addition of a troponin-tropomyosin solution to washed actomyosin gel was measured under the stand-
The increase in optical density caused by the addition of troponin-tropomyosin to a suspension of washed gel. One unit of troponin-tropomyosin equals 0.15 mg added to the reaction mixture. In A the total amount of gel in the reaction mixture was 4.8 mg; in B, 2.4 mg; and in C, 1.2 mg. One mg of troponin-tropomyosin saturated 8 mg of washed gel. The symbols represent different days on which the experiments were done.

ard conditions given below. To determine the maximum change, small aliquots (e.g. 0.1 ml) of troponin-tropomyosin were added successively until the gel was saturated, i.e. until no further increase in optical density occurred. The same reaction was seen with the whole extract and the partially purified extract (see Fig. 5).

**Standard Conditions for Measuring Superprecipitation, Clearing, and Combining Reaction**—For measuring superprecipitation, clearing, and the combining reaction, the conditions were: 25°; 0.06 M Tris, pH 7.4; 5 mM MgCl₂; 0.1 M KCl. The solutions “without calcium” contained 2 mM EGTA. The solutions “with calcium” contained 2 mM EGTA and 2 mM CaCl₂, giving a free calcium ion concentration of approximately 10 μM. The concentration of protein was 0.1 to 0.4 mg of actomyosin per ml in a total volume of 12 ml.

**Measurement of Protein Concentration**—The concentration of protein in the stock gel suspensions and in the troponin-tropomyosin solutions was determined by the biuret reaction, using bovine serum albumin as the standard.

**Materials**—ATP was obtained from Sigma; dithiothreitol from Nutritional Biochemicals; and sodium pyrophosphate from Fisher. All other chemicals were reagent grade. All water was glass distilled and passed through a deionizing column.

RESULTS

**Increase in Turbidity Associated with Combination of Actomyosin and Troponin-Tropomyosin Complex**—In the course of our work with actomyosin gel suspensions, we noticed that the addition of a muscle extract containing the troponin-tropomyosin complex (see “Methods”) to a suspension of washed gel always caused an increase in optical density; the size of the turbidity change was constant for a given set of conditions, and the change was observed only when the extract was added to washed gel. A systematic study of this effect indicates that it reflects the extent of a combining reaction between actomyosin, which is free of the troponin-tropomyosin complex, and the troponin-tropomyosin present in the muscle extract. The turbidity increase was directly proportional to the amount of protein solution added, up to some point which apparently exceeded the combining capacity of the gel; the maximum increase that could be induced this way was directly proportional to the amount of gel.

The stoichiometry of this combining reaction is shown in Fig. 3. Curve C is for 1 unit of gel, which is an arbitrary unit chosen for convenience and which equals 1.2 mg of actomyosin. The magnitude of the turbidity change increased linearly until 1 unit of whole extract (0.15 mg of protein) was added, extract in excess of this amount had no further effect. Curve B is for 2 units of gel; here the size of the turbidity change increased linearly along the same slope until 2 units of extract were added. Curve A is for 4 units of gel.

Typical records of the turbidity change are shown in Fig. 4. The effect was specific for washed gel; there was no apparent reaction between the extract and native (Fig. 4E) or heated (Fig. 4D) gel. Fig. 4 also shows the lack of any further response to the extract once the gel was saturated. Fig. 5 shows that a partially purified preparation of troponin-tropomyosin (see “Methods”) had exactly the same effect as the whole extract.

The turbidity change, then, can be used to determine whether
Fig. 5. Comparative effects of the whole and partially purified muscle extract on the turbidity of washed gel. The general conditions were the same as those described in Fig. 4. In A, 0.2 ml of whole extract (2.84 mg of protein per ml) was added to the 12-ml reaction solution; in B, 0.2 ml of partially purified extract (0.62 mg of protein per ml) was added. The addition of another 0.2 ml of extract, in each case, had no further effect.

Fig. 6. The effect of troponin-tropomyosin (TN-TM) to potentiate contraction in the presence of calcium. At each arrow ATP was added to give a final concentration of 50 μM. The measurements were made by the standard procedure and under the standard conditions given in the text with 0.1 mg of washed actomyosin gel per ml. In a and b, the washed gel was first saturated with 0.2 ml of troponin-tropomyosin solution (6 mg per ml) and showed the typical combining reaction (see Fig. 4). With calcium, 2 mM EGTA and 2 mM CaCl₂ giving an estimated free calcium ion concentration of 10 μM; without calcium, 2 mM EGTA (less than 0.1 μM free calcium).

Fig. 7. The effect of calcium and troponin-tropomyosin on the clearing response of washed gel. The conditions and procedure were the same as for Fig. 2, except that the protein was washed to remove the troponin-tropomyosin as described in the text. O, 2 mM EGTA (less than 0.1 μM calcium); ●, 2 mM EGTA and 2 mM CaCl₂ (approximately 10 μM calcium). In B the suspension contained 0.10 mg of troponin-tropomyosin per ml. The washed protein showed no sensitivity to calcium (A); the troponin-tropomyosin restored most of this sensitivity (B).

Fig. 8. The superprecipitation of native, washed, and heated gel as a function of ATP concentration. The methods for preparing the different proteins and the standard conditions for measuring superprecipitation are described in the text. The rate with and without calcium for heated and washed gels showed no significant difference. Note that the washed gel did show a slow rate of contraction even at the highest level of ATP. However, as shown in Fig. 6, some washed gel preparations were even more severely inhibited by substrate and actually cleared at 50 μM.

or not, and to what extent, actomyosin combines with troponin-tropomyosin under different experimental conditions. We have used this indication of a combining reaction together with two functional tests of calcium sensitivity to answer certain questions about the control system.

Two Different Tests for Calcium Sensitivity—One of the tests
TABLE I

<table>
<thead>
<tr>
<th>Type of protein</th>
<th>Combining reaction</th>
<th>Calcium sensitivity of clearing response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>+ Troponin-Tropomyosin complex</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Washed</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>+ Troponin-Tropomyosin complex</td>
<td></td>
<td>0</td>
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<tr>
<td>Heated</td>
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<td>+</td>
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<tr>
<td>+ Troponin-Tropomyosin complex</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Washed then heated</td>
<td>+</td>
<td>0</td>
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<tr>
<td>+ Troponin-Tropomyosin complex</td>
<td></td>
<td>0</td>
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<tr>
<td>Heated then washed</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>+ Troponin-Tropomyosin complex</td>
<td></td>
<td>0</td>
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* The methods for preparing, combining, and testing the clearing response of these proteins are described in the text.

1 Positive combining reaction indicates that the appropriate increase in optical density occurred for the amount of washed gel used (0.15 to 0.2 mg per ml). No combining reaction indicates that no significant optical density change occurred even when a large excess of troponin-tropomyosin was added.

2 Positive calcium sensitivity indicates that calcium caused a significant increase in the C4 for clearing (see Fig. 2 and Fig. 6b). No calcium sensitivity indicates that the C4 for clearing was the same with and without calcium (see Fig. 6a).

The results may be summarized as follows. (a) Native gel, even when heated, never combined with the added troponin-tropomyosin; (b) washed gel, even when heated, always combined with the added troponin-tropomyosin; (c) although the washed gel, when heated before or after washing, could still combine with the added troponin-tropomyosin, it did not regain its calcium sensitivity by this reaction; (d) heating the troponin-tropomyosin (under the same conditions used to desensitize the gel) did not affect its ability to combine with washed gel or to confer calcium sensitivity.

From these observations we conclude that heating the gel desensitizes the protein to calcium by oxidizing —SH groups on actomyosin per se, not on the troponin-tropomyosin complex, and that these —SH groups do not play an essential role in linking the troponin-tropomyosin complex to actomyosin.

Table II summarizes another typical experiment that demonstrates a number of features of the control system. In Group A we see that all forms of the protein, with and without added troponin-tropomyosin, were able to contract at a low level of ATP (10 µM) (cf. Fig. 8); no significant calcium sensitivity was apparent. At this low substrate concentration, little or no ATP binds to the inhibitory site. Therefore, calcium, which normally activates contraction by blocking the inhibitory site, can have no effect.

Group B of Table II shows the typical effect of calcium on the response of three different calcium-sensitive gels to high levels of ATP. For each gel without calcium, there was substrate inhibition and clearing, but with calcium there was rapid contraction.

Note that the control system of the washed gel was reconstituted by the readdition of the troponin-tropomyosin, and that the con-

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troponin-tropomyosin system of the heated gel was reconstituted by treatment with dithiothreitol. The final protein concentration in each case was 0.1 mg per ml.

In Group B, we see three experimental situations in which there was no calcium sensitivity even at high levels of ATP. The one common feature of these insensitive proteins is not the nature of their response to ATP, but rather, the indifference of their response to calcium. This is demonstrated by comparing the response to ATP, but rather, the indifference of their response to calcium. This is demonstrated by comparing the rate of superprecipitation in the presence of calcium (see Fig. 6).

In Group C, we see three experimental situations in which there was no calcium sensitivity even at high levels of ATP. The one common feature of these insensitive proteins is not the nature of their response to ATP, but rather, the indifference of their response to calcium. This is demonstrated by comparing the rate of superprecipitation in the presence of calcium (see Fig. 6).

The general conditions were the same as those described in Fig. 6.

**DISCUSSION**

**Two Effects of Troponin-Tropomyosin Complex**—The idea that the primary or major action of the troponin-tropomyosin complex is to directly facilitate clearing, or relaxation, stems from experiments in which the washed gel contracted, although often at a relatively slow rate, and was relaxed by the addition of troponin-tropomyosin (7, 8, 10, 14, 15, 19, 25-27). From these past observations, it seemed reasonable to assume that the only function of these proteins was to facilitate relaxation, and that calcium activated contraction by biasing troponin toward some way, inactivating the relaxing system. In other words, calcium would simply negate or offset the relaxing action of troponin-tropomyosin. Thus, when all elements of the control system were absent (washed or trypsin-treated gel without calcium) the actomyosin would be expected to contract just as it did when calcium and the relaxing proteins were present.

However, the results reported in this paper indicate a more direct and positive control function for the calcium-troponin-tropomyosin complex. By starting with washed gel under conditions that cause it to relax (even without any relaxing protein), we can demonstrate that the addition of calcium together with troponin-tropomyosin (neither one alone) markedly potentiates contraction. The formation of a calcium-troponin-tropomyosin complex, then, must do something more than simply counterbalance the relaxing influence of troponin-tropomyosin.

From these observations we begin to see a dual control mechanism; in one direction, the control proteins without calcium favor relaxation, and, in the other, these same proteins with calcium favor contraction.

Depending on conditions, one or the other side of this control mechanism is more apparent. If, as in the past work, most measurements are made without added calcium and under conditions in which the gel, free of control protein, is able to contract (the low pH of 6.8 to 7.0 used in most of these studies favors this), then the relaxing action of troponin-tropomyosin will be most apparent (7, 8, 10, 14, 15, 19, 25-27). In the experiments described here, however, the washed gel was markedly inhibited by 50 \( \mu \text{M} \) ATP (the relatively high pH of 7.4 contributes to this), and under these circumstances the activating effect of calcium-troponin-tropomyosin can be demonstrated. The relaxing
Fig. 10. A proposed mechanism for the control of muscular contraction. The operation of this system is discussed in the text. Only the control system is shown here; the active contractile system has been omitted, and the sites that appear to take part in the contractile system have been described in earlier papers (16, 18, 44). Tn-Tm, troponin-tropomyosin.
effect of troponin-tropomyosin without calcium, although less striking, can also be seen under these same conditions if we consider a longer time period, as in Fig. 9. Thus, in accordance with past work, Fig. 9 demonstrates that troponin-tropomyosin facilitates relaxation, and Fig. 6, in sharp contrast, demonstrates that calcium-troponin-tropomyosin potentiates contraction.

α-Actinin—Another muscle protein, α-actinin, has been reported to potentiate superprecipitation and facilitate recovery from ATP-induced clearing (17, 28-35). It comes to mind then, that the effects we attribute to calcium-troponin-tropomyosin may be due to α-actinin in the muscle extract. This possibility was minimized, if not ruled out, by the observation that a partially purified preparation of troponin-tropomyosin, from which most of the α-actinin was removed by NH₄SO₄ fractionation (described under “Methods”), gave the same effects as the whole extract. Moreover, a number of detailed studies on α-actinin itself indicate that any effects of this protein would be minimized by our experimental conditions, i.e. relatively high salt, high pH, and low ATP concentration (33, 34). In addition, from these studies, we would expect the effects of α-actinin to be independent of calcium and to be antagonized by the troponin-tropomyosin in the muscle extracts (34). All of this taken together, we believe, makes it most probable that the troponin-tropomyosin complex is the protein responsible for the effects we describe here. Parenthetically, it occurs to us that α-actinin, apparently located at the z line in muscle (35), may under certain experimental circumstances be replacing the troponin-tropomyosin that is normally on actin, and, in this way, independent of calcium, it might mimic the physiological action of calcium-troponin-tropomyosin.

General Interpretation of Control System—Fig. 10 graphically depicts our interpretation of the various experimental conditions we have studied. Considerable evidence supports the general alignment of components indicated in these diagrams. For example, it seems most probable that troponin is bound to actin (36, 37), that troponin is bound to tropomyosin (38-40), that calcium binds to troponin (41-45), and that ATP inhibits contraction by binding to an inhibitory site, i.e. the relaxing site, that is separate and distinct from the site where ATP is hydrolyzed (5, 18). Whether this inhibitory site is on actin or myosin or both is not known.

Fig. 10 shows the native protein with an inhibitory (relaxing) site, where the binding of ATP inhibits ATP hydrolysis and contraction, and a blocking site comprised of the troponin-tropomyosin complex. When calcium binds to troponin, access of ATP to the inhibitory site is hindered, and contraction proceeds. Fig. 10 shows the washed protein. There is an inhibitory site still fairly accessible to ATP, but the control site has been removed by washing. Under our experimental conditions, the inhibitory site of the washed protein is virtually saturated at 50 μM ATP, and the gel is relaxed with or without calcium. Fig. 10 shows the heated protein. It has a modified inhibitory site where ATP binding is markedly hindered. Binding at this inhibitory site is no longer under normal control, and the gel contracts with or without calcium. In effect, the heating (oxidation) has mimicked the action of the calcium-troponin-tropomyosin complex, and only reduction (treatment with dithiothreitol) will allow the protein to be relaxed.

This mechanism is also consistent with the effect of the calcium-troponin-tropomyosin complex on the clearing response of the gel. As shown in Fig. 7a, the clearing response without troponin-tropomyosin was the same with or without calcium. Under these experimental conditions, which favor clearing of washed gel, the addition of the troponin-tropomyosin without calcium had no significant facilitating effect (the upper curve of Fig. 7b is the same as the curve in Fig. 7a). However, in the presence of calcium, the troponin-tropomyosin complex inhibited clearing (bottom curve of Fig. 7b). Apparently, the calcium-troponin-tropomyosin complex hinders the clearing effect of PPi as it hinders the inhibitory effect of ATP.

Hypothetical Mechanism of Contraction-Relaxation Cycle—The diagrams of Fig. 10 are not intended to indicate an actual physical mechanism for the contraction-relaxation cycle. Physically, as we have outlined in earlier papers (e.g. References 5 and 18), it seems to us that MgATP at the relaxing site prevents hydrolysis at the catalytic site by moving the actin and myosin parts of the catalytic machinery out of proper alignment. When this happens, new links cannot form between myosin and actin, but old links holding the elements of the gel together are still broken by the binding of MgATP to various functional sites of the actomyosin system; under these conditions the gel clears, i.e. it relaxes. This is prevented, we believe, by the hindering action of calcium-troponin-tropomyosin at the relaxing site. By acting to keep MgATP off this inhibitory site, the calcium-troponin-tropomyosin complex allows actin and myosin to come into proper alignment for ATP hydrolysis and the formation of new links. Now contraction occurs through the concerted action of MgATP to form new links, through hydrolysis at the catalytic site by moving the actin and myosin parts of the catalytic machinery out of proper alignment. When this happens, new links cannot form between myosin and actin, but old links holding the elements of the gel together are still broken by the binding of MgATP to various functional sites of the actomyosin system; under these conditions the gel clears, i.e. it relaxes.

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