Ca BINDING OF INTESTINAL SMOOTH MUSCLE MYOSIN B

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Abstract By means of both centrifugation and filtration techniques, the Ca binding activity of intestinal myosin B was studied. The binding capacity of myosin B was Ca dependent and was approximately linear when the concentration of Ca in the medium ranged from $10^{-4}$ to $10^{-7}$ M. The Ca sensitivity of ATPase activity in the same range of Ca concentration exhibited a sigmoid curve. The Scatchard plot of Ca binding showed that intestinal myosin B had at least two types of binding sites. One of these was defined as a high affinity site with an apparent affinity constant of $2.5 \times 10^6$ M$^{-1}$. The other was supposed to be a low affinity site of Ca binding. Mild trypsin treatment reduced the Ca binding capacity of intestinal myosin B by 1.45-2.44 nmol/mg protein. These values are approximately the concentration of the high affinity Ca binding sites in the intestinal myosin B. A major concern regarding the effect of trypsin is that the reduction of Ca binding surely accompanied the elimination of Ca sensitivity of myosin B ATPase activity. From these results, it seems likely that the high affinity sites of Ca binding identified in this study are based on the troponin-like component included in intestinal myosin B.

It is well known that the contraction of smooth muscle is regulated by a change in intracellular Ca ion in the same way as in skeletal muscle. This is illustrated by the fact that superprecipitation and ATPase activity of intestinal myosin B showed a Ca sensitivity like that of skeletal myosin B (MATSUMOTO et al., 1974). What is most certain is that in skeletal muscle an initial occurrence in the contractile reaction of actomyosin-ATP system is the binding of Ca to troponin. EBASHI et al. (1968) and FUCHS and BRIGGS (1968) established the physiological role of troponin as a Ca-binding protein in the contraction-relaxation cycle of...
skeletal muscle and also suggested that among various contractile proteins, troponin was the only protein exhibiting a strong Ca-binding activity.

The existence of a protein component which is similar to the skeletal troponin in the intestinal myosin B has been postulated by MATSUMOTO et al. (1975a). Then MATSUMOTO et al. (1975b) showed that Ca regulatory protein of pig's intestinal smooth muscle was associated with its actin. In the meantime, EBASHI et al. (1975) separated the native tropomyosin from gizzard smooth muscle and ITO and HOTTA (1976) isolated the native tropomyosin from bovine tracheal smooth muscle.

Evidence, on the other hand, has been presented which indicates that in molluscan muscle (KENDRICK-JONES et al., 1972) and in vertebrate smooth muscle (SOBIESZEK and SMALL, 1976) at least one of the myosin light chains is involved in the Ca regulation process.

We have been focussing our attention on the purification and distribution of the protein component responsible for the Ca sensitivity of the contractile system in the intestinal smooth muscle. In this article, with these discussions in mind, we studied the Ca-binding properties and ATPase activity of intestinal myosin B.

Our results show that bound Ca on the high affinity sites of intestinal myosin B, which were supposed to be lost by mild trypsin treatment, are responsible for the Ca sensitivity of ATPase activity.

MATERIAL AND METHODS

The circular and longitudinal muscle layers isolated from pig jejunum and ileum were rinsed in 0.9% NaCl solution and cut with scissors into small pieces. The cut muscle was blended in a Waring blender and then triturated with sea sand in an equal volume of extraction solution. The extraction solution contained 80 mM KCl, 4 mM MgCl₂, 4 mM EGTA (pH 7.0), 10 mM NaHCO₃, 4 mM ATP (pH 7.0) and 0.5 mM dithiothreitol. The triturated muscle was added to two volumes of the extraction solution and left to stand overnight at 0-4°C. Further purification of myosin B was carried out by the same method as that used by SPARROW et al. (1970). In this investigation, however, the solution containing (mM); KCl, 0.5; NaHCO₃, 0.3; dithiothreitol, 0.5 and MgCl₂, 0.1 was used for the purification and suspension of myosin B. The purified myosin B was stored in a refrigerator in the form of a suspension with a protein concentration of about 10 mg/ml.

Protein concentrations were determined by the biuret method using purified bovine plasma albumin as a standard (previously calibrated by the micro-Kjeldahl method for nitrogen determination).

ATPase activity is expressed as μg of inorganic phosphate (Pi) released/mg myosin B protein in 16 min. Released Pi was measured by the FISKE-SUBBAROW method (1925). Superprecipitation of myosin B was measured turbidometrically using a spectrophotometer (Hitachi 101) by following the changes in optical
density at 660 nm after the addition of ATP (EBASHI, 1961). Free Ca concentration in the medium can be calculated sufficiently accurately from the equation (IMAI and TAKEDA, 1967), \[ p[Ca^{+}] = 2pH - 7.28 + \log \left( \frac{[\text{EGTA}]_{\text{added}}}{[\text{CaCl}_2]_{\text{added}}} - 1 \right) \]. In the present experiment, 0.5 mM EGTA were used.

Ca-binding activity of myosin B was measured by means of filtration (glass fiber filter paper, GH-100, Toyo Co.) and of the centrifugation technique (EBASHI, 1968). Myosin B was incubated in a solution consisting of 30 mM KCl, 4 mM MgCl₂, 40 mM Tris maleate buffer (pH 6.8), varied concentrations of Ca which were determined by using Ca-CaEGTA buffer and 0.03 μCi⁴⁵Ca/ml for 20-30 min at 20°C. In the centrifugation technique, the reaction mixture thus incubated was centrifuged for 20 min at 105,000 × g. The radioactivity of the resultant precipitate dissolved by SDS and of the supernatant were measured.

In the filtration technique, each sample was then placed by the Pasteur pipette in a filtration suction apparatus containing glass fiber paper (24 mm diameter). The mixture was completely filtered by vacuum within 5 sec and then the filter paper was washed twice with 5.0 ml of Tris HCl buffer (pH 7.0). The filter papers were removed and placed in scintillation vials containing 10 ml of Triton-toluene scintillation cocktail (PATTERSON and GREENE, 1965). Then the radioactivity of each vial was measured.

ATP-2Na, SDS, trypsin and soybean trypsin inhibitor were obtained from Sigma Chemicals Co. (U.S.A.). EGTA was purchased from Dojin Pharmaceutical Laboratories (Kumamoto, Japan). ⁴⁵Ca was obtained from Radiochemical Centre (England). Other chemical reagents were commercial products (Wako Chemicals Co.) of the best reagent grade available.

RESULTS

ATPase activity and superprecipitation of intestinal myosin B

It has been previously shown that pig intestinal myosin B extracted by the procedure of SPARROW et al. (1970) exhibited Ca sensitivity which was similar to that of skeletal myosin B (MATSUMOTO et al., 1974). The data in the present report confirmed their observation. As shown in Fig. 1, the superprecipitation (Fig. 1(A)) and ATPase activity (Fig. 1(B)) were clearly dependent upon the concentrations of Ca in the medium. The relationships between ATPase activity or superprecipitation and the concentration of Ca were of typical sigmoid curves (Fig. 1(C)).

Ca-binding activity of intestinal myosin B

Ca binding was fairly linear with the concentration of myosin B in the range 1 to 5 mg protein/ml (Fig. 2). From the slopes of both lines, Ca-binding activities were determined as 2.1 nmol/mg protein/30 min by the filtration technique and 1.8 nmol/mg protein/30 min by the centrifugation technique, respectively.
Fig. 1. Superprecipitation and ATPase activity of intestinal myosin B.

(A) Superprecipitation at varied concentration of Ca.
Reaction mixture; 0.4 mg myosin B/ml, 4 mM MgCl₂, 30 mM KCl, 0.5 mM ATP, 40 mM Tris-maleate buffer (pH 6.8) and Ca as indicated on each curve in the figure. The concentrations of Ca were adjusted by Ca-CaEGTA buffer.

(B) Mg activated ATPase activity in the presence of $1.86 \times 10^{-5}$ M Ca and $2.51 \times 10^{-8}$ M Ca. Reaction mixtures for this investigation were the same as those in (A), except that 1.5 mg myosin B/ml and 1 mM ATP were added.

(C) Mg activated ATPase activity and superprecipitation as a function of pCa.

The experimental conditions were identical with those of (B) and ATPase activity was determined at 16 min after an addition of ATP. They were expressed as a percent of the ATPase activity in the presence of $1.86 \times 10^{-5}$ M Ca. ○, ATPase activity; ●, superprecipitation; these points were depicted from Fig. 1 (A). Each value of these points was calculated by the equation, $X/Y \times 100$, and plotted on the ordinate. In this equation, $X$ is the time necessary for the half maximum activation of superprecipitation in the presence of $1.86 \times 10^{-5}$ M Ca. $Y$ is the time necessary for the maximum activation of superprecipitation at varied concentrations of Ca. The value obtained from the equation, $X/Y \times 100$, at $1.86 \times 10^{-5}$ M Ca was designated as 100%.
In this observation the Ca concentration of $1.86 \times 10^{-5}$ M was used. This concentration was necessary for the maximum activation of both ATPase activity and superprecipitation. We also investigated the Ca-binding activity of myosin B at $10^{-2}$ M Ca. In this case, 1 mM CaCl$_2$ was added instead of Ca-CaEGTA buffer. The values thus obtained are shown in Table 1 together with those obtained at $1.86 \times 10^{-5}$ M Ca. There was no significant difference between the two values obtained by different techniques. Unless otherwise stated, thereafter, all Ca-binding experiments were performed using the centrifugation technique.

![Graph](image)

**Fig. 2. Ca-binding activity at varying concentrations of myosin B.**

In this experiment, the concentration of Ca was set at $1.86 \times 10^{-5}$ M by Ca-CaEGTA buffer. The incubation solution containing 4 mM MgCl$_2$, 30 mM KCl, 0.02 $\mu$Ci $^{45}$Ca/ml, 40 mM Tris-maleate buffer (pH 6.8) and myosin B were used. Details of experimental conditions are shown in text. ○, determined by the centrifugation technique; ●, determined by the filtration technique.

**Ca-binding at various concentrations of Ca**

As shown in Fig. 3, the amount of Ca bound increased linearly with the increase of Ca concentration in the range $10^{-7}$ to $10^{-4}$ M. This relation is different from the sigmoid curve for Ca activation of ATPase activity and superprecipitation at the same range of Ca concentration. Table 1 shows that with further increase of Ca from $10^{-4}$ to $10^{-3}$ M the Ca-binding activity of myosin B increased to four times the amount of Ca bound at $10^{-4}$ M Ca.

**Scatchard plot of Ca binding**

Scatchard et al. (1957) devised a method for estimation of the relative affinity and the number of binding sites. Figure 4 shows the Scatchard plot of Ca-binding activity obtained from those in Fig. 3. The plot is biphasic and is apparently composed from two straight lines, (a) and (b), indicating the presence
Fig. 3. Ca binding at various Ca concentration.
Ca-binding activity was determined by the centrifugation technique. Experimental conditions were the same as those described in Fig. 2. The concentration of myosin B in the reaction mixture was set at 3 mg/ml.

of two types of binding sites. The possibility of another set of Ca-binding sites with an intermediate affinity sites exist could not be excluded. The extrapolated intercepts of the linear segments of the plot were used to calculate the number of binding sites and the association constants. The intercepts on the abscissa, $n$ represent the number of binding sites per gram of myosin B protein. The intercept on the ordinate is $nK$ where $K$ is the association constant of the Ca-binding sites. The binding sites (a) of higher affinity, with an association constant of $2.5 \times 10^6$ M$^{-1}$ accomodated about 2 nmol Ca/mg protein. Because (b) segment in our Scatchard plot was constructed by only six points, the interception of lower affinity sites seems less precise than that of (a) segment. We dared to calculate the binding parameters of low affinity sites. Their association constant was thought to be about $2.6 \times 10^4$ M$^{-1}$ and their accomodation was in the vicinity of 8 nmol Ca/mg protein.

As already suggested by Fuchs and Briggs (1968), the association constant which is determined in the presence of MgCl$_2$ should be considered to be the apparent rather than the true constant because magnesium in the medium competes with calcium at the binding sites of protein (Weber and Herz, 1963). We observed that the amount of Ca-binding in the presence of magnesium was about 10% lower than that in the absence of magnesium. We had to use the reaction mixture including magnesium because this ion was known to be necessary for the demonstration of both Ca sensitivity of ATPase activity and superprecipitation.
Furthermore, the concentration of magnesium used in our study was exactly twice that used in the experiment of Fuchs and Briggs (1968). The reason why 4 mM MgCl₂ were introduced was that intestinal myosin B (Takase, 1973) as well as glycerinated vascular muscle (Fitó et al., 1965) required a greater amount of magnesium to get the maximum ATPase activity than did skeletal myosin B.

**Effect of trypsin treatment on the Ca-binding activity and Ca sensitivity**

Mild trypsin treatment was found to eliminate only the Ca-binding protein in myosin B, without affecting actin and myosin, and to yield Ca desensitized actomyosin (Ebashi and Ebashi, 1964). We also observed that the Ca sensitivity of intestinal myosin B ATPase was negated completely by the treatment with 4 µg of trypsin/mg myosin B protein as shown in Fig. 5. The Ca-binding activity decreased in proportion to the increase of trypsin up to 4 µg/mg protein. Trypsin (10 µg/ml) produced a 80% decrease in the amount of Ca bound when Ca ions in the reaction mixture were 1.86 × 10⁻⁵ M and in the medium containing 10⁻³ M Ca the decrease in the amount of Ca bound was about 40% as shown in Table 1. At both concentrations of Ca tested the decrease in Ca binding by trypsin treatment was 1.44–2.55 nmol/mg myosin B.
Fig. 5. Effects of mild trypsin treatment on the Ca binding and the Ca sensitivity. Myosin B was treated with various concentrations of trypsin for 10 min at 20°C in the presence of 0.1 M KCl and trypsic digestion was then stopped by adding trypsin inhibitor twice the trypsin by weight. The Ca binding and the Ca sensitivity of Mg activated ATPase of trypsin treated myosin B are expressed as a percent of those of non-treated myosin B. The Ca sensitivity of Mg activated ATPase (○) is expressed as a ratio of the activity in the presence of $2.51 \times 10^{-8} \text{M Ca}$ to that in the presence of $1.86 \times 10^{-5} \text{M}$ Ca. Such a ratio of ATPase activity on non-trypsin-treated myosin B is designated as 100%. The Ca-binding activities were determined in the presence of $1.86 \times 10^{-5} \text{M Ca}$ which was determined by Ca-CaEGTA buffer (△) and in the presence of $10^{-3} \text{M Ca}$ which was settled by the addition of 1 mM CaCl$_2$ instead of Ca-CaEGTA buffer (○). Other experimental conditions for Ca binding and ATPase activity were the same as those in Fig. 2 and in Fig. 1 (B), respectively.

Table 1. The amount of Ca bound to intestinal myosin B was determined both in the presence of $1.86 \times 10^{-5} \text{M Ca}$ which was determined by Ca-CaEGTA buffer and in the presence of $10^{-3} \text{M Ca}$ which was settled by the addition of 1 mM CaCl$_2$ instead of Ca-CaEGTA buffer. These values obtained by both techniques were calculated from Fig. 2 and those of trypsin-treated myosin B were calculated from the data in Figs. 2 and 5.

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<th>Experiments</th>
<th>Ca binding nmol/mg myosin B</th>
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<td>$1.86 \times 10^{-5} \text{M Ca}$</td>
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<tr>
<td>Control</td>
<td>2.10</td>
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<td>Filtration technique</td>
<td>1.80</td>
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<td>Centrifugation technique</td>
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<td>Treated by trypsin (10 µg/mg myosin B)</td>
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<td>(Centrifugation technique)</td>
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<td>Loss of Ca by trypsin treatment</td>
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Pig intestinal myosin B exhibited a typical Ca sensitivity and superprecipitation (Fig. 1) and was shown to bind Ca strongly (Fig. 2). As shown in Fig. 3, in contrast to the sigmoid curve of Ca dependence in ATPase activity and superprecipitation, the Ca-binding activity showed a linear increase when the Ca concentration was in the range $10^{-4}$ to $10^{-7}$ M. In the presence of $1.86 \times 10^{-5}$ M Ca, which gave maximum ATPase activity and superprecipitation, the amount of Ca bound to myosin B was about 2 nmol/mg myosin B. This approximated to the values reported on skeletal myosin B by Weber and Herz (1963) and by Ebashi et al. (1968) (around 2 nmol/mg protein).

Scatchard plot analysis showed the existence of at least two different types in Ca binding, the higher and lower affinity sites. The apparent affinity constants obtained from the slopes were $2.5 \times 10^6$ M$^{-1}$ for the higher affinity sites and around $2.6 \times 10^4$ M$^{-1}$ for lower affinity sites. These apparent affinity constants resembled the respective values reported on the skeletal myofibrils which was found to have two types of Ca-binding sites (Fuchs and Briggs, 1968). Fuchs and Briggs (1968) assumed that Ca sensitivity of skeletal myosin B originated from the high-affinity Ca-binding sites and that Ca binding at low-affinity sites based mainly on the interaction between Ca and some protein components other than troponin. If their assumption is correct in the case of intestinal myosin B, we should expect to find a difference between the trypsin-treated and non-treated myosin B in regard to the capacity of Ca binding. As expected, treatment with trypsin reduced both Ca binding and Ca sensitivity of ATPase activity, suggesting the existence of a troponin-like protein in intestinal myosin B. With trypsin treatment, though Ca was retained to some degree, Ca sensitivity was completely negated. Forty percent (about 2.55 nmol/mg myosin B) of total Ca binding in the presence of $10^{-5}$ M Ca and 80% (1.44 nmol/mg protein) of that in the presence of $1.86 \times 10^{-5}$ M Ca were lost by the trypsin treatment. On the other hand, the binding capacity of Ca on the high-affinity sites was calculated as about 2 nmol/mg myosin B by a Scatchard plot. The amount of Ca lost by trypsin treatment was nearly equal the concentration of high-affinity Ca-binding sites. It now seems likely that Ca ions lost by trypsin was supposed to be bound on the high-affinity sites of intestinal myosin B.

From the effects of trypsin (Table 1) and some similarities of Ca-binding kinetics between the skeletal myofibrils (Fuchs and Briggs, 1968) and the intestinal myosin B, we arrived at the conclusion that the high-affinity Ca-binding sites of intestinal myosin B originated largely from the troponin-like component included in it.

Then our experimental result, the loss of Ca by trypsin is surely accompanied by the elimination of Ca sensitivity, supports this view.

One object of a series of our experiments is to determine the distribution of troponin-like regulatory protein in the intestinal contractile proteins. In the
present study making use of myosin B, it remained uncertain whether the regulatory protein was linked to myosin or to actin. Though the work of Matsumoto et al. (1975b), in which the pig’s intestinal actin possessing a Ca regulatory function was extracted, led to the suggestion that a troponin-like component was associated with actin in intestinal myosin B.

REFERENCES


