PARTICIPATION OF NATIVE TROPOMYOSIN IN THE ATP-CONTRACTION OF AN INTESTINAL GLYCERINATED MUSCLE BUNDLE

Hideyo YABU, Isamu UCHIDA and Eisaku MIYAZAKI

Department of Physiology, Sapporo Medical College, Sapporo, Japan

Summary Observations were made on the Ca-sensitivity of glycerinated muscle bundles from dog intestine. The results obtained were as follows: 1) The Ca-sensitivity of glycerinated muscle bundles depends on the duration of glycerol extraction. With long-term extraction, the muscle bundles lost a large part of the Ca-sensitivity of the ATP contractility. 2) When the bundles that had a typical Ca-sensitivity were treated with a low concentration of trypsin, their ATP-contractility became insensitive to varying concentrations of Ca ions. 3) On treatment with intestinal native tropomyosin, the Ca-sensitivity of the ATP-contractility of 2-year-glycerinated muscle bundles was restored, to a certain extent. With the same treatment, the Ca-sensitivity of trypsin-treated 2-year-glycerinated muscle bundle was restored to the same level as that of 2-year-glycerinated bundles.

From these results, it is concluded that the Ca-sensitivity of the ATP-contractility of intestinal glycerinated muscle is due to the native tropomyosin it contains.

It is now clear that the contractile system of intestinal muscle is based mainly on actin and myosin, and that the elementary process of physiological contraction is dependent on the interaction of both these proteins and ATP (TOMITA, 1965a, b; MATSUKI, 1967; TANEDA, 1967a, b). However, the role of a new contractile protein in smooth muscle, native tropomyosin, *viz.*, the complex of troponin and tropomyosin (EBASHI and KODAMA, 1966), has not yet been established on the molecular level.

Recently, IWAKURA (1965) extracted from chicken gizzard muscle a protein preparation having characteristics similar to those of native tropomyosin isolated from skeletal muscle.

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藪 英世, 内田 勇, 宮崎英策

Abbreviations: ATP, adenosine triphosphate; EGTA, ethylene glycol-bis (aminoethyl)-N-N-tetra-acetic acid.

In an experimental series related to native tropomyosin, it was shown that trypsin is a useful tool for eliminating the activity of native tropomyosin from myosin B of skeletal (EBASHI and EBASHI, 1964), cardiac (OOI, 1965), and gizzard muscle (IWAKURA, 1965). It was expected, therefore, that a trypsin-treated, glycerinated muscle bundle of mammalian smooth muscle will be induced to contract by Mg-ATP, at the same time losing its Ca-sensitivity.

In the experiments described in this article, to examine the existence of native tropomyosin in the intestinal smooth muscle, the effects of tryptic digestion on the ATP-induced tension development of glycerinated muscle bundles of dogs were observed.

EXPERIMENTAL MATERIALS AND METHODS

Glycerinated muscle bundles of dog intestine were prepared from the small intestinal muscle layer according to the method of TOMITA (1965a). For measurements, the bundles, stripped from the mucous layer and teased to a diameter of $100\ \mu$ and a length of 15–20 mm each, were mounted on a tensionmeter (WEBER, 1949; NAGAI and UCHIDA, 1960) after the washing-out of glycerol. Dog intestinal muscle myosin B was prepared according to the modified Edsall method by using a Weber-Edsall solution (YABU *et al.*, 1969). Intestinal native tropomyosin was prepared according to the method of IWAKURA (1965).

ATP, trypsin (twice crystallized), and soy bean trypsin inhibitor were obtained from Sigma Co. Other reagents, such as EGTA (Dojin Co.), were commercial products of the best reagent grade. The composition of the reaction mixtures and the conditions of trypsin digestion are indicated in each figure.

Superprecipitation of myosin B was estimated by following the change in the absorbance at $660\ m\mu$ after the addition of Mg-ATP. The absorbance measurements were performed using a Hitachi 101 spectrophotometer.

EXPERIMENTAL RESULTS

The pCa dependence of the tension development induced by ATP

Intestinal muscle bundles which were treated with glycerol during a period ranging from 1 day to 2 years developed a maximum tension (about $500\ g/cm^2$) with 2 mM Mg-ATP and $5 \times 10^{-5} M$ Ca^{++} at pH 6.6. This maximum tension is represented as 100% on the ordinate in each figure.

The Ca^{++} dependence of ATP-contractility of glycerinated muscle bundles depends on the duration of glycerol extraction. As shown in Fig. 1, with the increase of pCa ($= -\log [Ca^{++}]$) briefly glycerinated muscle bundles lost their contractility response to Mg-ATP. The bundles that were glycerinated for a long period, however, contracted on addition of Mg-ATP even in the absence of a minute amount of Ca ions. The pCa dependence of the ATP-contractility of

Fig. 1. Calcium requirement for the ATP-induced tension of glycerinated intestinal muscle bundles as a function of extraction period. Reaction mixtures contained: 2 mM Mg-ATP, 70 mM KCl, and 25 mM histidine buffer (pH 6.6). Calcium concentrations were buffered by the EGTA-CaEGTA system (IMAI and TAKEDA, 1967) in which the total EGTA concentration was 3 mM. The ordinate indicates the degree of tension induced by ATP as a relative value. The pCa plots on the abscissa show the free Ca ion concentration in the medium. —▲—, glycerinated 1 day; —△—, glycerinated 2 weeks; —●—, glycerinated 1 month; —□—, glycerinated 3 months; —○—, glycerinated 2 years.

brieflyglycerinated (1 day–1 month) intestinal muscle bundles is virtually identical with that of rabbit psoas skeletal or bovine vascular smooth muscle (FILO *et al.*, 1965) and is similar to that of the ATPase activity of skeletal myofibrils (PORTZEHL *et al.*, 1969).

Effect of the duration of tryptic digestion on the tension development

As shown in Fig. 2, without trypsin digestion the glycerinated muscle bundle extracted 2 years before testing developed a maximal tension in the standard solution at pCa 6.32, and just one-half maximal tension in the absence of Ca ions. Tryptic digestion with a lapse of time (5, 10, 15, and 20 min) had no effect on the tension development at pCa 6.32. With the elongation of tryptic digestion, the degree of tension development increased gradually in the absence of Ca ions.

Effect of trypsin concentration on the tension development of brieflyglycerinated muscle bundles

As shown in Fig. 3, muscle bundles that had been glycerinated for three weeks and digested by 10 and 20 $\mu\text{g/ml}$ of trypsin for 10 min developed maximal tension at pCa 6.32. With further increases of trypsin concentration at a fixed

Fig. 2. The effect of trypsin digestion on ATP-induced tension in glycerinated intestinal muscle bundles. Two-year-glycerinated muscle bundles were digested with $10 \mu\text{g/ml}$ trypsin at 20°C in standard solution for the time indicated on the abscissa. Digestion was stopped with $10 \mu\text{g/ml}$ trypsin inhibitor, after which the tension induced by ATP was measured immediately. For other details see the legend of Fig. 1. —○—, pCa 6.32; —●—, in the absence of Ca ions.

Fig. 3. The effect of trypsin digestion on the tension of glycerinated intestinal muscle bundles. Three-week-glycerinated muscle bundles were digested for 10 min at 20°C at varying concentrations of trypsin. Digestion was stopped with trypsin inhibitor of equivalent concentration, and the tension was measured immediately after the addition of ATP. For other experimental details, see the legend of Fig. 1. —○—, pCa 6.32; —●—, in the absence of Ca ions.

duration of digestion time, the ATP-induced tension gradually decreased. On the other hand, without tryptic digestion, 3-week-glycerinated muscle bundles produced a tension of 10% of the maximum in the absence of free Ca ions.

With the increase of trypsin concentration up to 20 $\mu\text{g/ml}$, the trypsin-digested glycerinated muscle bundles produced a larger tension with the addition of Mg-ATP than did non-trypsin-digested bundles. Thereafter, the tension decreased inversely with further increases in trypsin concentration from 20 μg to 40 $\mu\text{g/ml}$.

It is, therefore, suggested that concentrations of trypsin above 20 $\mu\text{g/ml}$ not only eliminated the activity of native tropomyosin but caused some conformational changes of actomyosin in 3-week-glycerinated muscle bundles.

Functional restoration of the intestinal native tropomyosin in the trypsin-digested glycerinated muscle bundles

In this experimental series, two kinds of experiments were made. The first one was an attempt to restore the extracted intestinal native tropomyosin to the 2-year-glycerinated muscle bundles in which half the Ca-sensitivity was lost, as shown in Fig. 1. The second was an identical trial on muscle bundles exposed to mild tryptic digestion whose Ca-sensitivity was completely lost, as shown in Figs. 2 and 3. In both trials, successful results were obtained, as shown in Figs. 4 and 5. In the first trial, shown in Fig. 4, the treatment of 2-year-glycerinated muscle bundles with KCl-histidine buffer solution containing native tropomyosin reduced the ATP-induced tension in the absence of Ca ions, this decrease in tension

Fig. 4. The effect of intestinal native tropomyosin on ATP-induced tension in glycerinated muscle bundles. Two-year-glycerinated muscle bundles were immersed in a solution containing 100 mM KCl, 25 mM histidine buffer (pH 6.6), and 1 mg/ml of intestinal native tropomyosin for 30 min at 25°C. These bundles were washed with the buffer solution before tension measurement was conducted. —●—, in the absence of Ca ions; —○—, in the presence of $10^{-6.32}\text{M Ca}^{++}$.

Fig. 5. The effect of intestinal native tropomyosin on ATP-induced tension in trypsin-treated glycerinated muscle bundles. Control: ATP-induced tension of 2-year-glycerinated muscle bundle without trypsin digestion. Trypsin treated: With trypsin digestion. Trypsin treated + native tropomyosin: In this case, two-year-glycerinated muscle bundles pretreated with trypsin were immersed in a solution containing 100 mM KCl, 25 mM histidine buffer (pH 6.6), and 1 mg/ml of intestinal native tropomyosin for 30 min at 25°C. The bundles were washed with the buffer solution before tension measurement was conducted. ---●---, in the absence of Ca ions; ---○---, in the presence of $10^{-6.32}M$ Ca^{++} .

being proportional to the increase in the concentration of native tropomyosin added. In the second trial, shown in Fig. 5, similar treatment was applied to the trypsin-digested glycerinated muscle bundles. This decreased the ATP-induced tension to the same level as of that of the glycerinated muscle bundles without tryptic digestion (Fig. 5). Thus, it has been shown that extracted intestinal native tropomyosin can restore Ca -sensitivity in such desensitized glycerinated muscle bundles.

The Ca-desensitizing effect of tryptic digestion on intestinal myosin B

After tryptic digestion, the optical density of the actomyosin gel suspension decreased to some degree, even if a very low concentration of trypsin (0.1–0.5 μ g/ml) was used. Moreover, with the addition of ATP, the digested intestinal myosin B could not produce a significant increase in optical density. Therefore, the participation of native tropomyosin in superprecipitation could not be demonstrated turbidometrically. It is probable that the high solubility of intestinal myosin B by tryptic digestion is partially due to the dissociation of actin and myosin.

DISCUSSION

To clarify the relationship between the contractile mechanism of smooth muscle and Ca ions, the existence of native tropomyosin, which sensitizes the interaction of myosin and actin to Ca ions, was examined in glycerinated muscle bundles of dog intestine, through the observation of ATP-induced tension under various conditions.

Because trypsin is known to be a useful tool for the elimination of the activity of native tropomyosin from skeletal (EBASHI and KODAMA, 1966), cardiac (Ooi, 1965), and gizzard muscle myosin B (IWAKURA, 1965), a weak tryptic-digestion technique was used throughout these experiments. It has been reported that glycerinated psoas muscle fibers treated with trypsin failed to produce a significant tension (EBASHI and EBASHI, 1964). As shown in Figs. 2 and 3, the ATP-contraction of briefly glycerinated intestinal muscle bundles showed a characteristic pCa dependence. That is, these bundles were unable to contract at a low concentration of Ca ions (pCa 8.0), while the trypsin-digested bundles contracted to some degree and showed less pCa dependence. The present results certainly indicate that a componental protein resembling native tropomyosin is participating in the regulation of tension development of the intestinal glycerinated muscle bundles.

Gradual loss of pCa dependence with increased duration of glycerination also suggests the existence of native tropomyosin in intestinal muscle (Fig. 1), because the Ca-sensitizing factor disappeared or was inactivated during glycerol extraction, as suggested by MURPHY *et al.* (1969), SCHAUB (1967), and SCHAUB *et al.* (1967). This may further explain the weak relaxing effect of a $17,500\text{--}67,500\times g$ fraction of intestinal muscle homogenates on the ATP-induced contraction of muscle bundles glycerinated for a long period (MIYAZAKI *et al.*, 1965).

The present results, therefore, suggest the possibility that the glycerinated intestinal muscle bundle is a suitable preparation for examining the role of native tropomyosin in the contraction-relaxation cycle of smooth muscle.

To prove the participation of a Ca-sensitizing factor in the ATP-induced tension development, an experiment was made in which Ca-sensitized glycerinated muscles were reconstituted from Ca-desensitized bundles by the addition of extracted native tropomyosin from intestinal smooth muscle.

Such reconstituting experiments were successful. As shown in Figs. 4 and 5, the ATP-contraction of long-term glycerinated and trypsin-digested glycerinated muscle bundles was sensitized to the Ca ions by treatment with intestinal native tropomyosin. It is clear that, as in the case of superprecipitation of skeletal (EBASHI and KODAMA, 1966), cardiac (Ooi, 1965), and gizzard muscle myosin B (IWAKURA, 1965), the Ca-sensitivity of ATP-contraction of the intestinal muscle is not a property of the actomyosin but depends on the property of an additional protein component, so called native tropomyosin, which is essential for the physiological action of Ca ions in the regulation of the contraction-relaxation cycle of intestinal smooth muscle.

On the other hand, morphological localization of tropomyosin on the Z-line of skeletal muscle and in the dense body of smooth muscle cells has been speculated (HANSON and LOWY, 1963; PANNER and HONIG, 1967). Following procedures of trypsin digestion similar to those described above in "EXPERIMENTAL METHODS AND MATERIALS," electron-microscopic observations showed that the dense body of the glycerinated intestinal muscle remained (MIURA, 1969a, b) though the Ca-sensitivity of ATP-contractility was completely lost, as shown in Figs. 3 and 4.

Two-month-glycerinated muscle bundles demonstrated incomplete Ca-sensitivity, as shown in Fig. 1. On the other hand, the dense body of 2-month or still longer glycerinated muscle has an electron density similar to that of the control muscle (TAKAHASHI *et al.*, 1968). These facts suggest that troponin and tropomyosin are, at least, not located in the dense body of the smooth muscle cell. According to OHTSUKI *et al.* (1967), and NONOMURA and DRABIKOWSKI (1968), the localization of troponin in the skeletal muscle was speculated to be on the F-actin filament and tropomyosin paracrystals. Straub-type actin prepared from skeletal muscle contained native tropomyosin, while that prepared from intestinal muscle did not (YABU *et al.*, 1969). These facts also indicate that the localization of troponin and tropomyosin in smooth muscle cell remains undetermined. Further investigation to clarify the morphological localization of these proteins in the smooth muscle cell will be necessary. This kind of investigation throws light on the contractile mechanism of the smooth muscle and its relation to Ca ions.

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