CALCIUM SENSITIVITY OF ATPase ACTIVITY OF ACTOMYOSIN TOGETHER WITH TROPOMYOSIN-TROPONIN SYSTEM FROM ASCIDIAN SMOOTH MUSCLE

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CALCIUM SENSITIVITY OF ATPase ACTIVITY OF ACTOMYOSIN TOGETHER WITH TROPOMYOSIN-TROPONIN SYSTEM FROM ASCIDIAN SMOOTH MUSCLE

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The effect of the tropomyosin-troponin system from ascidian smooth muscle on the ATPase activity of actomyosin was studied. Ascidian native tropomyosin and a mixture of ascidian tropomysin and troponin repressed the Mg^{2+} – ATPase activity of actomyosin in the absence of calcium ions and the repression was relieved by the addition of calcium ions. When skeletal muscle myosin was mixed with either ascidian native thin filaments or reconstituted thin filaments, the ATPase activity of the mixture was increased to that of the mixture of skeletal muscle myosin and ascidian natural F-actin by adding calcium ions. The latter mixture was insensitive to calcium ions. In the absence of calcium ions, the repression of the ATPase activity of a mixture of ascidian natural F-actin, tropomyosin and rabbit skeletal myosin was progressed by increasing of added troponin.

These results indicate that troponin from ascidian smooth muscle is a repressor for actin-myosin interaction but not a Ca^{2+} – dependent activator for the interaction. This indication contradicts the proposal by Obinaoa's groups that troponin from ascidian smooth muscle is a Ca^{2+} – dependent activator for actin-myosin interaction <Endo and Obinata (1981) J. Biochem. **98**, 1599-1608 and Obinata *et al.* (1983) Comp. Biochem. Physiol., **76B**, 437-442>

The body-wall muscle of an ascidian, *Halocynthia roretzi*, is an unique smooth muscle because this muscle is multinucleated and regulated by tropomyosin-troponin system (Toyota *et al.* 1979, Endo and Obinata 1981, Obinata *et al.* 1983, Shinohara and Konishi 1982, Miyakawa and Konishi 1984). Endo and Obinata (1981) proposed that the function of ascidian troponin differs from that of skeletal troponin : ascidian troponin is a Ca^{2+}-dependent activator for actin-myosin interaction, whereas skeletal muscle troponin is an inhibitor for the interaction and the inhibition is relieved by the binding of calcium ions to troponin. Endo and Obinata (1981) also asserted that Ca^{2+}-dependent activation for actin-myosin

Abbreviations: EGTA, ethylene-glycol-bis (β-aminoethyl-ether)-N, N, N′ N′-tetraacetate; SDS, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; Mops, 4-morpholinepropanesulphonic acid

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interaction is the regulatory mode in vertebrate smooth muscle of which contraction is triggered by the binding of calcium ions to leiotonin C (MIKAWA *et al.* 1977, MIKAWA *et al.* 1978) and/or calmodulin which is a subunit of myosin light chain kinase (SOBIESZEK and BREMEL 1975, CHACKO *et al.* 1977, SHERRY *et al.* 1978, IKEBE *et al.* 1978, ADELSTEIN and KLEE 1981). Thus, the characteristics of ascidian troponin is of interest, compared with those of skeletal muscle troponin. In the previous paper, however, MIYAKAWA and KONISHI (1984) presented that the desensitization of ascidian myosin B was obtained as the result of activation of Mg^{2+}–ATPase activity in the absence of calcium ions. Contrary to the proposal by ENDO and OBINATA (1981), our results in the previous paper suggest that the regulatory mode of ascidian troponin is skeletal muscle troponin type.

In the present work, we presented the effect of ascidian tropomyosin-troponin system on the ATPase activity of actomyosin prepared by combining myosin from rabbit skeletal muscle and actin from either rabbit skeletal muscle or ascidian smooth muscle. Results indicate that ascidian troponin is a repressor for actin-myosin interaction in the absence of calcium ions.

MATERIALS AND METHODS

Preparation of proteins: Myosin and F-actin from rabbit skeletal muscle, natural F-actin and thin filaments from the body-wall muscle of ascidian, *Halocynthia roretzi*, were used for the present study. Myosin was prepared from rabbit skeletal muscle according to the method of PERRY (1955). Prior to use, myosin in 0.5 M KCl containing 10 mM Mops, (pH 7.0), was centrifuged at 40,000 rpm for 1 h to remove insoluble matter. Rabbit skeletal muscle actin was prepared according to the method of SPUDICH and WATT (1971). G-actin in 0.1 M sucrose was freeze-dried and stored at −20℃. After removing sucrose by dialysis, the actin was polymerized into F-form by dialyzing against 0.1 M KCl. Native thin filaments, natural F-actin, native tropomyosin, tropomyosin and troponin were prepared from the body-wall muscle of fresh ascidian, *Halocynthia roretzi*, according to the method of TOYOTA *et al.* (1979) and ENDO and OBINATA (1981). To prevent from proteolysis by intrinsic proteases the muscle was homogenized and washed in the solution containing 0.1 mM DFP.

SDS-gel electrophoresis: SDS-gel electrophoresis was performed by the method of WEBER and OSBORN (1969).

Measurement of ATPase activity: The steady state rate of ATPase was determined from the time course of Pi liberation in the medium of 50 mM KCl, 20 mM Mops, (pH 7.0), 2 mM $MgCl_2$ and 1 mM ATP at 25°C in the presence of either 0.1 mM $CaCl_2$ or 1 mM EGTA. Pi was determined by the method of ALLEN modified by NAKAMURA (1950).

Others: The concentration of protein was determined using a biuret reaction

(GRONALL *et al.* 1949). The concentration of free calcium ions in the medium was adjusted with EGTA based on the apparent dissociation constant of 1.0×10^{-6} M for CaEGTA determined by HARAFUJI and OGAWA (1980). Contaminated calcium ions in the medium was assumed to 20×10^{-6} M.

RESULTS

Effects of ascidian native tropomyosin, troponin together with tropomyosin on the ATPase activity of actomyosin: As shown in Fig. 1, ascidian native tropomyosin as well as troponin together with ascidian tropomyosin repressed markedly the ATPase activity in the absence of calcium ions and showed almost no effect in the presence of calcium ions. These results indicate that the mode of regulation by ascidian tropomyosin-troponin system is similar to that of skeletal muscle; the system acts as a repressor for actin-myosin interaction in the absence of calcium ions and calcium ions relieve the repression.

Results in Fig. 1, were obtained by using actomyosin prepared with myosin from rabbit skeletal muscle and ascidian natural F-actin. ENDO and OBINATA (1981), however, used actomyosin reconstituted by combining myosin and actin from

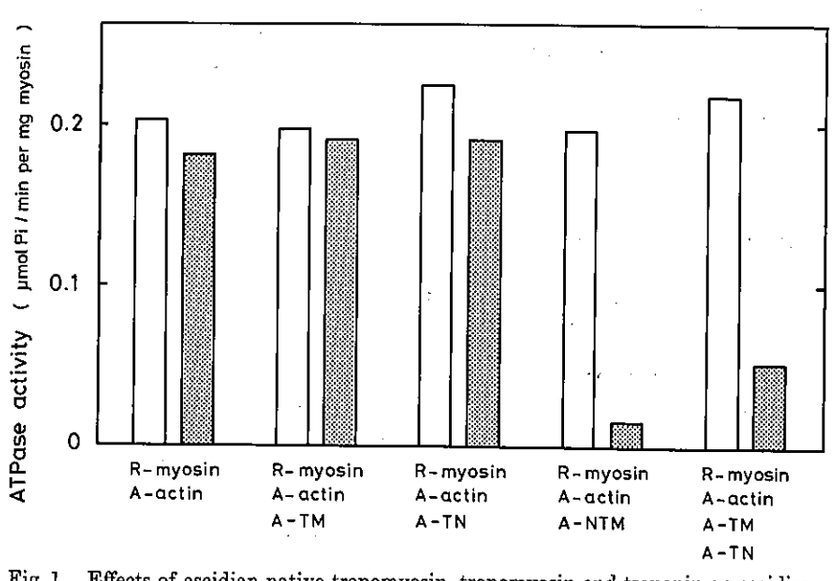

Fig. 1. Effects of ascidian native tropomyosin, tropomyosin and troponin on ascidian acto-rabbit myosin ATPase activity. Reaction mixture contained: 50 mM KCl, 2 mM $MgCl_2$, 20 mM Mops (pH 7.0), 1 mM ATP, 0.1 mM $CaCl_2$ (open bars) or 1 mM EGTA (dotted bars), rabbit skeletal myosin (0.1 mg/ml) and ascidian actin (0.05 mg/ml) and ascidian native tropomyosin (A-NTM, 0.05 mg/ml) or tropomyosin with troponin. Regulatory proteins were incubated with actin in an ice box overnight. ATPase activity was measured at 25°C.

rabbit skeletal muscle. Thus, actin from rabbit skeletal muscle was used in the following experiments.

The ATPase activities of reconstituted rabbit skeletal muscle actomyosin together with ascidian tropomyosin were 0.36 and 0.37 (μmol Pi/min per mg myosin) in the absence and presence of calcium ions, respectively. Ascidian troponin reduced the ATPase activity in the absence of calcium ions to 0.068 (μmol Pi/min per mg myosin) while the activity in the presence of calcium ions was not affected by this regulatory protein. Contrary to ENDO and OBINATA's report (1981), these results also indicate that ascidian troponin is a repressor for actin-myosin

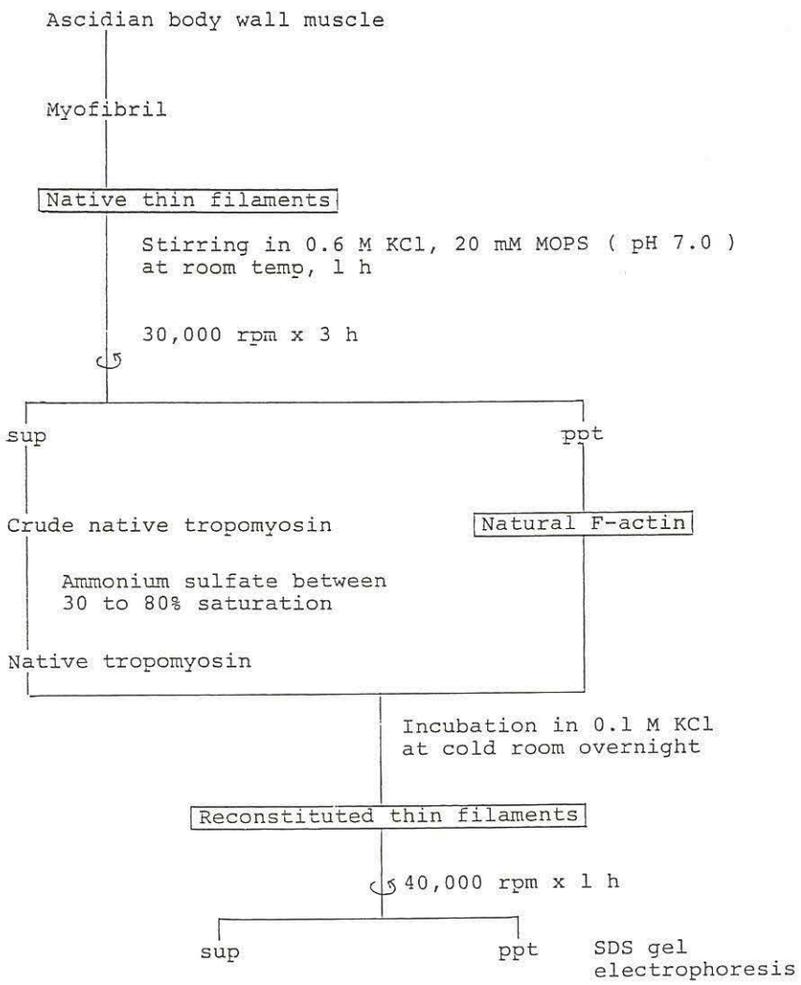

Fig. 2. Preparation procedures for reconstituted thin filaments.

interaction in the absence of calcium ions.

Ca^{2+}-sensitivity of Mg^{2+}-ATPase activity of skeletal muscle myosin with either ascidian native or reconstituted thin filaments: One of the causes of discrepancy between the results by ENDO and OBINATA (1981) and those presented here was assumed to the different extent of reconstitution of the tropomyosin-troponin system. Thus, the mode of Ca^{2+}-sensitization induced by ascidian native thin filaments is of great interest. We studied the Ca^{2+}-sensitivity of ATPase activity of rabbit skeletal muscle myosin with either ascidian native or reconstituted thin filaments. The preparation procedure of reconstituted thin filaments was presented in Fig. 2. Ascidian native tropomyosin and natural F-actin which were separated from native thin filaments were mixed together in a weight ratio of 1 : 1 in 0.1 M KCl. After settled in a cold room overnight, the mixture was used as reconstituted thin filaments. For SDS-gel electrophoresis, reconstituted thin filaments collected by centrifugation at 40,000 rpm for 1 h were applied.

Fig. 3 shows SDS-gel electrophoretic patterns of ascidian natural F-actin, native tropomyosin, reconstituted and native thin filaments. Natural F-actin consists of actin and X-protein mainly. Main components in native tropomyosin were tropomyosin and three components of troponin. Native and reconstituted thin filaments did not differ each other in their components which were actin, tropomyosin, three components of troponin (T, I and C) and X-protein. However, the amounts of troponin components were less in native thin filaments than in reconstituted thin filaments.

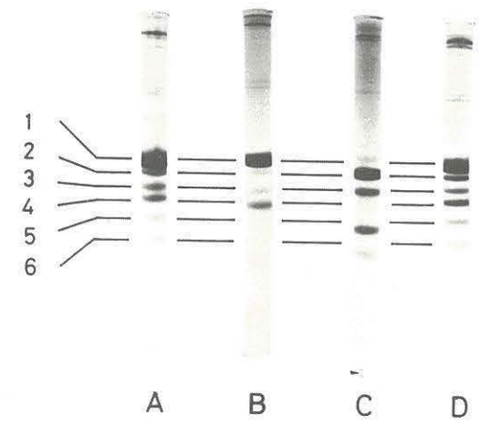

Fig. 3. SDS-gel electrophoretic patterns of ascidian proteins. A) native thin filaments (50 μg); B) ascidian actin (10 μg); C) native tropomyosin (30 μg); D) reconstituted thin filaments (50 μg). 7.5% gel rods were used. (1)-(6) represent actin, tropomyosin, troponin-T, X-protein (unidentified protein), troponin-I and troponin-C respectively.

Table 1.
Ca^{2+}-sensitivity of Mg^{2+}-ATPase activity of rabbit myosin with components from ascidian thin filaments

	components from thin filaments		
	actin	native thin filaments	actin+native tropomyosin
$-Ca^{2+}$	0.40	0.16	0.073
$+Ca^{2+}$	0.40	0.40	0.41

Conditions: 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA ($-Ca^{2+}$) or 0.1 mM $CaCl_2$ ($+Ca^{2+}$), 20 mM Mops (pH 6.8), 0.1 mg/ml rabbit myosin, 0.1 mg/ml ascidian actin, 0.1 mg/ml ascidian native thin filaments, 0.1 mg/ml ascidian native tropomyosin and 1 mM ATP. The activity is expressed in (μ molPi/min per mg myosin).

Table 1 shows the Mg^{2+}-ATPase activity of skeletal muscle myosin which was mixed with either ascidian natural F-actin or native thin filaments or reconstituted thin filaments. When skeletal muscle myosin was incubated with ascidian native thin filaments, Mg^{2+}-ATPase activities were 0.16 and 0.40 (μmol Pi/min per mg myosin) in the absence and presence of calcium ions respectively. When ascidian natural F-actin was used, the activity was 0.40 (μmol Pi/min per mg myosin) regardless the concentration of calcium ions. When reconstituted thin filaments

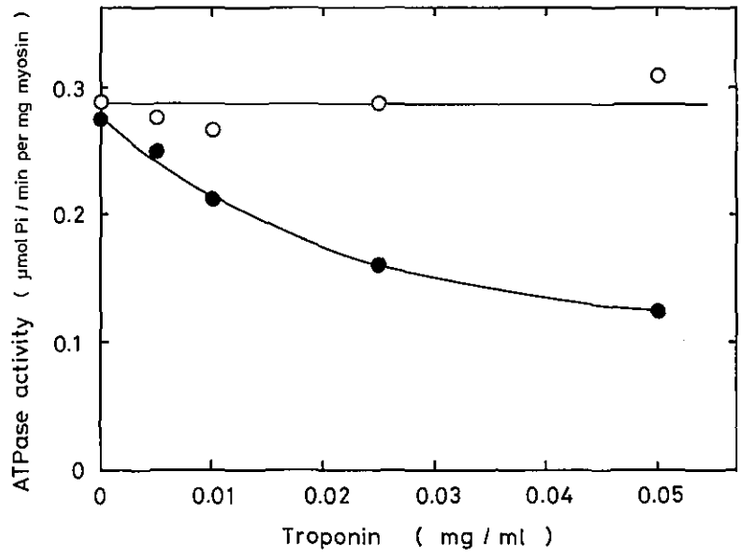

Fig. 4. Inhibition profile of the ATPase activity of ascidian acto-rabbit myosin with ascidian tropomyosin by ascidian troponin. The reaction conditions were the same as those for Fig. 1. (○) with 0.1 mM $CaCl_2$; (●) with 1 mM EGTA.

were used, the activities were 0.073 and 0.41 (μmol Pi/min per mg myosin) in the absence and presence of calcium ions perspectively. These results clearly indicate that ascidian troponin acts as a repressor for actin-myosin interaction in the absence of calcium ions when this protein is situated in position in vivo. In the absence of calcium ions, the ATPase activity of myosin with the reconstituted thin filaments was lower than that obtained with native thin filaments. This result suggests that the inhibitory component of regulatory system of this muscle released from thin filaments during the preparation. This suggestion is supported by the results in Fig. 3 which show that the concentration of regulatory protein in native thin filaments is not as much as in reconstituted thin filaments.

Effect of troponin concentration in ATPase activity: Fig. 4 shows the effect of various amounts of ascidian troponin on the ATPase activity of a reconstituted actomyosin, a mixure of ascidian natural F-actin, tropomyosin and rabbit skeletal myosin. In the presence of calcium ions, the actin activated Mg^{2+}-ATPase activity

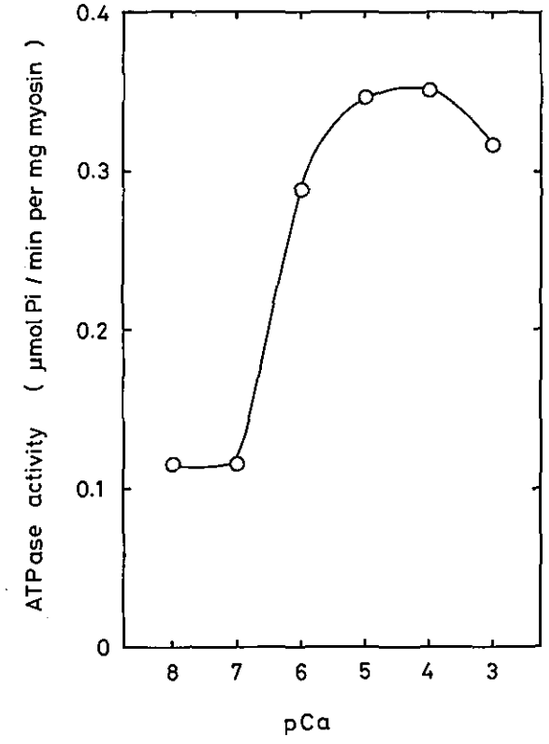

Fig. 5. Dependence on Ca^{2+} concentration of the ATPase activity of ascidian acto-rabbit skeletal myosin with ascidian tropomyosin and troponin. The reaction conditions were the same as those for Fig. 1 except for the use of 20 mM Mops (pH 6.8) instead of 20 mM Mops (pH 7.0).

of myosin remained constant irrespective of the amount of added troponin. In the absence of calcium ions, however, the repression of the ATPase activity was progressed by increasing added troponin.

Effect of calcium ion concentration on ATPase activity: Fig. 5 shows the actin activated Mg^{2+}-ATPase activity of a reconstituted actomyosin, a mixture of rabbit skeletal myosin, ascidian natural F-actin, tropomyosin and troponin at various concentrations of calcium ions. At pCa above 7, repression of ATPase activity by tropomyosin-troponin system was completed. At around pCa6, the release of the repression was initiated and the activity reached to the maximum at pCa in the range from 5 to 4. The activity decreased again at pCa3.

Results in Fig. 4 and 5 also indicate that ascidian troponin is a repressor for actin-myosin interaction in the absence of calcium ions.

Discussion

All results presented in this paper indicate that troponin from ascidian smooth muscle is a repressor for actin-myosin interaction. This evidence is in marked contrast to the conclusion of Endo and Obinata (1981). Although a cause of the contrast still remains to be solved, the difficulty of the complete reconstitution of ascidian tropomyosin-troponin system from their components may be a candidate for the cause of this contrast. In the previous paper, it was presented that ascidian myosin B was desensitized by EDTA treatment accompanying the release of troponin C and resensitized by the addition of this protein (Miyakawa and Konishi 1984). In that case, the desensitization was accomplished by activation of ATPase in the absence of calcium ions whereas the resensitization was established by activating of ATPase in the presence of calcium ions but not by the repression of the activity measured in the absence of calcium ions. This discrepancy may be due to the topological incompletion of reconstitution of the regulatory system. However, results obtained with native thin filaments suggest that ascidian regulatory system is a repressor for actin-myosin interaction, but not a Ca^{2+}-dependent activator for the interaction in vivo (Table 1).

There are two distinct different systems for the regulation of the contraction of various muscles. In vertebrate striated muscle, tropomyosin-troponin system is a only system of the regulation and the regulation is actin-linked (Ebashi and Endo 1968). In vertebrate smooth muscle, Ca^{2+}-dependent phosphorylation of myosin light chain initiates the contraction (Sobieszek and Bremel 1975, Chacko *et al.* 1977, Sherry *et al.* 1978, Ikebe *et al.* 1978). In invertebrate, the binding of calcium ions to myosin light chains is essntial for the initiation of the contraction of both striated and smooth muscles (Lehman *et al.* 1972, Lehman and Szent-Gyorgyi 1975). The regulation in these muscles is myosin-linked. In smooth muscles of both vertebrate and invertebrate, tropomyosin-troponin system has not

been regarded as a regulatory system. Ascidian body-wall muscle is only one smooth muscle of which regulatory system is identified as tropomyosin-troponin system (Toyota *et al.* 1979, Endo and Obinata 1981, Obinata *et al.* 1983, Shinohara and Konishi 1982, Miyakawa and Konishi 1984). Ascidian is situated at the very important position on phylogenic tree. Thus, the study on the regulatory mode and the relation between the regulatory mode and the physiological properties of this muscle is important for understanding of the evolutionary aspect for the regulatory system and the physiological significance of the existence of two distinct different regulatory systems. For further study on the function of ascidian tropomyosin-troponin system, a system reconstituted from ascidian myosin, actin and regulatory proteins is essential. Recently, Obinata *et al.* (1983) reported that ascidian tropomyosin-troponin system activated the ATPase activity of ascidian reconstituted actomyosin in the presence of calcium ions. Their results are consistent with their results obtained with rabbit reconstituted actomyosin (Endo and Obinata 1981). Results in this paper, however, clearly shows that ascidian tropomyosin-troponin system is a repressor for actin-myosin interaction in the absence of calcium ions. Thus, the effect of ascidian tropomyosin-troponin system on ascidian reconstituted actomyosin should be reinvestigated.

Under our experimetal conditions, ascidian tropomyosin showed no effect on ATPase activity of actomyosin. Effect of tropomyosin, especially from smooth muscle, on actin-myosin interaction is complicated (Sobieszek and Small 1981, Sobieszek 1982). Thus, the effect of ascidian tropomyosin on the interaction under various conditions should be studied.

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