THE Ca^{2+} -ACTIVATION PROFILE OF RABBIT FAST SKELETAL MYOFIBRILS IS NOT AFFECTED BY TROPONIN T ISOFORMS

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ABSTRACT

In order to clarify the role of troponin T isoforms in the Ca^{2+} -regulation of fast skeletal muscle contraction, two myofibrillar preparations were prepared from rabbit fast skeletal myofibrils, by the use of a novel procedure for exchanging troponin T isoforms in myofibrils; more than 80% of troponin T isoforms consisted of troponin T_{1f} in one myofibrillar preparation, while almost all of the troponin T consisted of troponin T_{2f} in the other preparation. These two myofibrillar preparations showed exactly the same activation profiles of Ca^{2+} -sensitive Mg-ATPase activity. This indicates that troponin T isoforms do not affect the Ca^{2+} -activation profiles of rabbit fast skeletal muscle contraction under physiological conditions.

Troponin is a Ca^{2+} -receptive protein of the myofibrillar contraction in vertebrate striated muscles. This protein is a complex of three different components, called troponin C, I and T. Troponin C (TN·C) is a Ca^{2+} -binding component, troponin I (TN·I) is an inhibitory component of myosin-actin interaction in the presence of tropomyosin and troponin T (TN·T) is a tropomyosin binding component. All three components are required for the Ca^{2+} -regulation of contraction (9).

Accumulating evidence has revealed the presence of several types of TN·T isoforms, owing to the variety of the amino-terminal amino acid residues (1, 5, 13). Regarding the role of such TN·T isoforms in the Ca^{2+} -regulated contraction of fast skeletal muscles, certain correlations have been suggested to exist between the relative content of the specific isoforms of both TN·T (TN· T_{2f}) and tropomyosin ($\alpha\alpha$ -tropomyosin) and the Ca^{2+} -sensitivity and/or the cooperativity of the

skinned fiber contraction (14). Fibers expressing a high content of TN· T_{2f} isoform and $\alpha\alpha$ -tropomyosin exhibit a higher Hill coefficient and Ca^{2+} -sensitivity. (14)

However, biochemical evidence has also been presented, based on the studies of troponin T fragments devoid of amino-terminal residues, which indicated that the variable amino-terminal region of troponin T is not directly related to the Ca^{2+} -activation profiles of either actomyosin or actomyosin subfragment-1 (3, 10, 12). A 26 kDa fragment of TN·T, lacking NH_2 -terminal 45 residues, showed the same properties as intact TN·T in calcium-activated superprecipitation and actomyosin ATPase activities of rabbit fast skeletal muscle (10). The velocity of ATP hydrolysis was lower for actoS1 ATPase regulated by 26 kDa fragment of TN·T than that by intact TN· T_{2f} (12). However, there was very little difference between the effect of the 26 kDa fragment and TN·T after the data were normalized (12). Furthermore, a 25 kDa fragment of β -TN·T, *i.e.*, neonate fast skeletal TN·T fragment lacking NH_2 -terminal 58 residues, showed the same actoS1 Mg-ATPase activity as that of intact β -TN·T (3).

One of the possible reasons for the discrepancy between physiological and biochemical findings

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Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; TN, troponin; TN·T, troponin T; TN·I, troponin I; TN·C, troponin C; TN·CI, troponin CI; actoS1, actomyosin subfragment-1

above mentioned, is that there are many kinds of isoforms in the contractile and regulatory proteins. Thus it is difficult to identify the responsible proteins for the contractile characteristics in the experiments using muscle fibers. While it is possible to control the species of the specific protein in the experiments using the reconstituted actomyosin system, the ATPase measurements are carried out under conditions which are rather different from the actual physiological situation. Thus, the role of TN·T isoforms in the Ca^{2+} -regulated contraction in the physiological myofibrillar lattice of skeletal muscle remains uncertain.

Recently, we found that three troponin components could be hybridized by the exogenously added troponin components in either myofibrils or single skinned fibers, without changing the composition of other myofibrillar proteins (6, 7, 16). In the present study, this procedure was applied to our investigation of the role of two physiologically predominant TN·T isoforms (TN·T_{1f} and T_{2f}) regarding the Ca^{2+} -regulation of myofibrils from the rabbit psoas muscle. For this purpose, we prepared two myofibrillar preparations, of which TN·T was hybridized almost exclusively with either TN·T_{1f} or T_{2f}, and then examined the Ca^{2+} -regulated Mg-ATPase of these myofibrillar preparations. The results provided evidence that the profiles of Ca^{2+} -activation were not affected by the species of TN·T isoforms.

EXPERIMENTAL PROCEDURE

Preparation of Troponin and Troponin Components

TN was prepared from rabbit white skeletal muscle by the procedure of Ebashi *et al.* (2). Three troponin components were separated by using the FPLC system (Pharmacia, Sweden) as follows. A crude troponin preparation dissolved in a solution (solution B) containing 8 M urea, 20 mM Tris-HCl, pH 7.50, 1 mM EDTA and 13 mM β -mercaptoethanol was applied to a MONO S column (Pharmacia, Sweden), a strong cation exchanger, and then was eluted with the solution B using a linear gradient of 0–500 mM NaCl. TN·I, T and C were eluted in order. The TN·T fraction was dialyzed against solution B and applied to a MONO Q column (Pharmacia, Sweden), a strong anion exchanger, and then was eluted by solution B using a linear gradient of 0–500 mM NaCl. TN·T was then separated into

five peaks. The SDS-polyacrylamide gel electrophoretic analysis indicated that most of first and second peaks (peaks I and II) demonstrated lower molecular weight TN·T, while peaks IV and V mainly contained higher molecular weight TN·T, and peaks III consisted of a mixture of them. Based on previous reports (2–4), the lower molecular weight TN·T was identified as TN·T_{2f} and the higher molecular weight TN·T was TN·T_{1f}. When necessary, further purification was carried out by the same procedure. Peaks II (TN·T_{2f} > 90%) (Fig. 1a) and IV (TN·T_{1f} > 90%) (Fig. 1b) were used for the experiments.

TN·C was further purified by applying it to a MONO Q column and then eluting it in solution B with a linear gradient of 0–500 mM NaCl. TN·I was dialyzed against solution B and applied to a MONO S column, and then was eluted in solution B with a linear gradient of 0–500 mM NaCl.

Troponin CI complex (TN·CI) was prepared by mixing equimolar TN·C and TN·I.

Preparation of Myofibrils

Male albino rabbits were anesthetized with pentobarbitone sodium (40 mg/kg, i.v.) and then exsanguinated. Myofibrils were prepared from psoas muscle by the method of Harigaya *et al.* (4) and stored in a 50% glycerol solution at -20°C .

Troponin T-Treatment of Myofibrils

A myofibrillar preparation stored in 50% glycerol was washed with 50 vol of a solution (solution A) containing 100 mM KCl, 1 mM NaHCO_3 , 0.5 mM dithiothreitol and 0.1 mg/ml pepstatin and was centrifuged at 2,500 rpm (Hitachi 05PR-2, D-assembly) for 7 min at 4°C . TN·T treatment was carried out at slightly acidic conditions essentially the same as reported previously (6, 7, 16). The myofibrillar suspension containing 0.32 mg/ml myofibrils, 250 mM KCl, 6 mM MgCl_2 , 20 mM 4-morpholinepropanesulfonic acid (MOPS)-KOH, pH 6.51, 0.5 mM dithiothreitol, 0.1 $\mu\text{g/ml}$ pepstatin, and 0.04 mg/ml TN·T was incubated for 60 min at 25°C , centrifuged at 2,500 rpm for 7 min at 4°C and then suspended in solution A.

Control myofibrils were prepared as follows: The myofibrillar suspension containing 0.32 mg/ml myofibrils, 250 mM KCl, 6 mM MgCl_2 , 20 mM MOPS-KOH, pH 6.51, 0.5 mM dithiothreitol, 0.1 $\mu\text{g/ml}$ pepstatin A was incubated for 60 min at

Fig.1 SDS polyacrylamide gel electrophoretic patterns of rabbit psoas myofibrils after successive treatments with TN·T and TN·CI. Lanes a-h indicate TN·T_{2f}, TN·T_{1f}, intact myofibrils, control myofibrils, myofibrils treated with TN·T_{1f}, myofibrils reconstituted with TN·T_{1f}, myofibrils treated with TN·T_{2f}, myofibrils reconstituted with TN·T_{2f}. Myofibrillar preparations reconstituted with TN·CI for SDS gel electrophoresis were prepared as follows. One-tenth the amount of TN·CI to myofibrils was added in the suspension of myofibrils treated with TN·T_{1f} or T_{2f}, centrifuged at 2,500 rpm after 30-min incubation at 4°C and then washed by the solution A. HC, myosin heavy chain; A, actin; TM, tropomyosin; LC1, 2 and 3, myosin light chains 1, 2 and 3; TN·T_{1f} and T_{2f}, troponin T_{1f} and T_{2f}; TN·I and C, troponins I and C

25°C, centrifuged at 2,500 rpm for 7 min at 4°C and then suspended in solution A.

Myofibrillar Mg-ATPase Activity Measurement

The reaction mixture (1 ml) contained 6 mM MgCl_2 , 4 mM Na_2ATP , 20 mM MOPS-KOH, pH 7.00, 2 mM Ca^{2+} -EGTA buffer, 0.2 mg myofibrils, and various amounts of KCl to adjust the ionic strength to 0.16 M. When the Mg-ATPase activity of myofibrils reconstituted with TN·CI was measured, 0.02 mg TN·CI was added to the reaction mixture. The free calcium ion concentrations and ionic strength were calculated as previously described (7).

The reaction was started by the addition of Na_2ATP and then was terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. The liberated inorganic phosphate was determined by the procedure of Pagani and Solaro (11). All pro-

cedures were carried out at 25°C.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out by the method of Laemmli (8) with an acrylamide concentration of 12%. The gels were stained with Coomassie brilliant blue R-250 and scanned with a densitometer (CS-900, Shimadzu, Japan).

Protein Concentration

The protein concentration was determined by using a protein assay kit (BIO-RAD, U.S.A.) with the bovine serum albumin as standard.

Curve Fitting

The data were fitted to the equation described

Table 1 Relative Contents of Troponin Components in the Myofibrils (%)

Myofibrils	Troponin components			
	TN · T _{1f}	TN · T _{2f}	TN · I	TN · C
1) Intact	26.5	73.5	100	100
2) Treated with TN · T _{1f}	74.7	25.3	< 20	< 20
3) Reconstituted with TN · CIT _{1f}	82.1	17.9	> 100	> 100
4) Treated with TN · T _{2f}	~ 0	100	< 20	< 20
5) Reconstituted with TN · CIT _{2f}	~ 0	100	> 100	> 100

Protein density was normalized based on the density of myosin light chain

1. Data were taken from Fig. 1.

below by using the least-squares method.

$$\text{Relative Ca}^{2+}\text{-sensitive Mg-ATPase activity} = [\text{Ca}^{2+}]^n / (K^n + [\text{Ca}^{2+}]^n)$$

where n is the Hill coefficient, and K is a constant. pK ($-\log K$) is the pCa at half maximal Ca^{2+} -sensitive Mg-ATPase activity.

Statistics

The values measured were expressed as mean $\pm$ SEM. All differences were evaluated by an analysis of variance. Values of $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

In the present study, we attempted to investigate the role of TN · T isoforms in the Ca^{2+} -regulation of fast skeletal muscle contraction. For this purpose, we chose rabbit psoas muscle consisting exclusively of type IIB fiber and in which TN · T_{1f} and T_{2f} isoforms are predominant (1, 5, 13, 15). A SDS-gel electrophoretic analysis showed that TN · T_{2f} and T_{1f} were 73.5% and 26.5% of the total TN · T, respectively (Fig. 1c) (Table 1). Since other TN · T isoforms were only faintly observed on the gel, the physiological Ca^{2+} -regulation is indicated to be most closely correlated with these two TN · T isoforms. Both TN · T_{1f} and T_{2f} were isolated from rabbit fast skeletal muscle (Fig. 1, a and b). The myofibrils were then treated with an excess amount of isolated TN · T_{1f} or T_{2f}. After treatment with TN · T_{2f}, most of the densities of TN · C, I, and T_{1f} disappeared while that of TN · T_{2f} increased (Fig. 1g). After treatment with TN · T_{1f}, most of the density in both TN · C and TN · I was removed from the myofibrils while the density of TN · T_{1f} increased to almost 80% of all TN · T densities and TN · T_{2f} density decreased to about 20% (Fig. 1e,

Fig. 2 The effect of TN · CI on the Ca^{2+} -sensitive Mg-ATPase activity of TN · T-treated myofibrils. ▼, ▽, intact myofibrils; ▲, △, control myofibrils; □, ■, myofibrils reconstituted with TN · CIT_{1f}; ○, ●, myofibrils reconstituted with TN · CIT_{2f}. The open symbols (▽, △, □, ○) indicate the Mg-ATPase activity at pCa 8.71, while the closed symbols (▼, ▲, ■, ●) indicate the Mg-ATPase activity at pCa 4.43.

Table 1). These findings indicated that most of the TN · CIT complexes in the myofibrils were replaced by the added TN T_{1f} or T_{2f}, in agreement with our previous findings (6, 7, 16). Both TN · C and I were then reconstituted by the subsequent treatment with TN · CI (Fig. 1, f and h; Table 1). We thus prepared two different kinds of myofibrils; more than 80% of TN · T was TN · T_{1f} in the one myofibrillar preparation, while almost all the TN · T was TN · T_{2f} in the other preparation. The compositions of the other myofibrillar proteins were all the same.

The myofibrillar Mg-ATPase activity was desensitized to calcium ion after TN · T-treatment and then was resensitized to calcium ion after reconsti-

Fig. 3 The effect of TN·T isoforms on the Ca^{2+} -sensitive Mg-ATPase activity of rabbit psoas myofibrils. ●, intact myofibrils; ○, control myofibrils; □, myofibrils reconstituted with TN·CIT_{1f}; Δ, myofibrils reconstituted with TN·CIT_{2f}. The values were expressed as the means $\pm$ SEM from six experiments. Upper; absolute myofibrillar ATPase activity. Lower; relative myofibrillar ATPase activity

tution with TN·CI (13) (Fig. 2). The results correlated with our previous findings (6, 7, 16). Fig. 2 also showed that more than 20 μg TN·CI (to 0.2 mg myofibrils) was required for maximum inhibition at low Ca^{2+} -concentrations. Thus, the Ca^{2+} -activated Mg-ATPase activity of the TN·T reconstituted myofibrils was examined in the presence of 20 μg TN·CI (one tenth the weight of the myofibrils) (Fig. 3). Both TN·CIT-reconstituted and the control myofibrils showed a slightly higher Mg-ATPase activity at lower calcium concentrations and a slightly higher calcium sensitivity (larger pK values), compared with the intact myofibrils (Table 2). These slight changes would be due to a certain deterioration of the myofibrils during the TN·T treatment procedure.

The Mg-ATPase activity of the myofibrils reconstituted with either TN·CIT_{1f} or TN·CIT_{2f} showed almost the same Ca^{2+} -activation profiles. No significant difference was observed in either the Hill coefficient (*n*) or the calcium sensitivity (pK) of myofibrillar Mg-ATPase activity (Fig. 3, Table 2). These findings clearly indicate that the physiologically predominant TN·T isoforms in the psoas myofibrils do not affect the Ca^{2+} -activation profiles of contraction.

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Table 2 Properties of Myofibrillar Mg-ATPase Activity

Myofibrils	Hill coefficient	pK	Mg-ATPase activity (nmol Pi/mg min)	
			at pCa 8.71	at pCa 4.43
Intact	3.52 ± 0.07	$5.62 \pm 0.01^*$	28.0 ± 3.3	427.0 ± 6.9
Control	3.31 ± 0.25	$5.66 \pm 0.01^*$	$33.9 \pm 1.2^*$	426.7 ± 5.3
Reconstituted with TN·CIT _{1f}	3.24 ± 0.15	$5.67 \pm 0.02^*$	$85.7 \pm 3.9^{*\#}$	419.6 ± 6.8
Reconstituted with TN·CIT _{2f}	3.24 ± 0.16	$5.67 \pm 0.01^*$	$91.1 \pm 3.0^{*\#}$	424.3 ± 2.2

* $P < 0.05$ compared with intact myofibrils. # $P < 0.05$ compared with control myofibrils. Each value was expressed as the mean $\pm$ SEM from six experiments.

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