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Effects of Removal and Reconstitution of Myosin Regulatory Light Chain and Troponin C on the Ca^{2+}-sensitive ATPase Activity of Myofibrils from Scallop Striated Muscle[1]

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In order to examine the involvement of troponin-linked Ca^{2+}-regulation, in addition to well-known myosin-linked Ca^{2+}-regulation, in the contraction of molluscan striated muscle, myofibrils from Ezo-giant scallop striated muscle were desensitized to Ca^{2+} by removing both myosin regulatory light chain and troponin C by treatment with a strong divalent cation chelator, CDTA. The ATPase level in the desensitized myofibrils was about half the maximum level in intact myofibrils regardless of the Ca^{2+}-concentration at 25 and 15°C. In the absence of Ca^{2+}, the ATPase of the desensitized myofibrils was suppressed by myosin regulatory light chain but not affected by troponin C at either temperature. The ATPase was activated at higher Ca^{2+}-concentrations by both myosin regulatory light chain and troponin C, but the activating effects of these two proteins were affected differently by temperature. The activation of ATPase by myosin regulatory light chain was much greater than that by troponin C at 25°C, whereas the activation by troponin C was much greater than that by myosin regulatory light chain at 15°C. The maximum activation was only obtained in the presence of both myosin regulatory light chain and troponin C at these temperatures. These findings strongly suggest that the contraction of scallop striated muscle is regulated through both myosin-linked and troponin-linked Ca^{2+}-regulation, and that the troponin-linked Ca^{2+}-regulation is more significant at lower temperature.

Key words: Ca^{2+}-regulation, myofibrillar ATPase, myosin regulatory light chain, scallop striated muscle, troponin C.

There are two types of Ca^{2+}-regulation for the contraction of striated muscle in the animal kingdom. One is Ca^{2+}-regulation through specific regulatory proteins called troponin and tropomyosin distributed along the actin double strands in the thin filament (*1, 2*). Troponin is the Ca^{2+}-receptive protein complex which triggers contraction and consists of three different components; a Ca^{2+}-binding component (troponin C; TN·C), an inhibitory component (troponin I), and a tropomyosin binding component (troponin T) (*2*). The essential processes of this type of Ca^{2+}-regulation are depression of the contractile interaction of myosin and actin-tropomyosin by troponin I, and reversal of the depression by troponin I through the activating action of troponin C. The activating action of troponin C itself is almost completely insensitive to Ca^{2+} and full Ca^{2+}-sensitivity is only acquired in the presence of troponin T. All three components of troponin are therefore required for the Ca^{2+}-regulation of contraction. The other type is Ca^{2+}-regulation, that is linked to myosin (*3*). In this case, the Ca^{2+}-receptive site is the essential light chain of the myosin molecule (*4*), and the ATPase of myofibrils has been found to be desensitized to Ca^{2+} on removal of the regulatory light chain (RLC) from myosin (*5*).

Myosin-linked Ca^{2+}-regulation was first found in the striated muscle of a molluscan and has long been considered to be the sole mechanism that regulates the contraction of this muscle (*3*). However, the presence of troponin and tropomyosin was demonstrated later in molluscan striated muscle, although the functional significance of these proteins remains obscure (*6, 7*). The present study was thus undertaken to examine the role of troponin-tropomyosin in the Ca^{2+}-regulation of contraction of molluscan striated muscle. For this purpose, myofibrillar preparations of Ezo-giant scallop adductor striated muscle were desensitized to Ca^{2+} by removing RLC and TN·C by treatment with CDTA, and then the Ca^{2+}-activation profiles of their ATPase activity were examined before and after reconstitution with RLC and TN·C. The results strongly suggested that both myosin-linked and troponin-linked Ca^{2+}-regulation are involved in the contraction of the scallop striated muscle and that the troponin-linked Ca^{2+}-regulation is more prominent at lower temperatures.

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Abbreviations: RLC, regulatory light chain of myosin; TN·C, troponin C; CDTA, *trans*-1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; pCa, $-\log [Ca^{2+}]$; pCa_{50}, pCa at 50% Ca^{2+}-sensitive ATPase activity.

EXPERIMENTAL PROCEDURES

Preparation of Myofibrils—Myofibrils were prepared from striated adductor muscle of Ezo-giant scallops (*Patinopecten yessoensis*) as follows. The muscle mince was homogenized with 4 volumes of a solution comprising 60 mM KCl, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 15 mM β-mercaptoethanol, and 1 μg/ml pepstatin A, for 15 s three times with 45 s intervals using a Waring blender, and then was centrifuged at 2,500 rpm for 10 min at 2°C. This homogenization and centrifugation procedure was repeated three times. The final precipitate was suspended in a solution comprising 60 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $NaHCO_3$, and 15 mM β-mercaptoethanol. The myofibrillar suspension was mixed with an equal volume of glycerol and stored at −20°C.

Preparation of Desensitized Myofibrils—Desensitized myofibrils were prepared from a myofibrillar preparation of Ezo-giant scallop striated muscle by the method described previously with several modifications (*8*). The myofibrillar suspension comprising 5 mM CDTA (pH adjusted to 8.4 with Tris), 15 mM β-mercaptoethanol, 1 μg/ml pepstatin A, and 0.5 mg/ml myofibrils was gently stirred for 5 min, and then centrifuged at 2,700 rpm for 6 min at 2°C. This washing procedure was repeated twice. The final sediment was washed twice with a solution comprising 60 mM KCl, 1 mM $NaHCO_3$, and 15 mM β-mercaptoethanol, and then used for the experiments.

Preparation of Proteins—The myosin regulatory light chain of Ezo-giant scallop striated muscle was prepared essentially according to the procedure of Kendrick-Jones *et al.* (*5*). The myofibrils were incubated in a solution comprising 40 mM NaCl, 5 mM K-phosphate (pH 7.0), and 10 mM EDTA for 20 min at 0°C, and then centrifuged. The supernatant was saturated with ammonium sulfate and then centrifuged. The resulting pellet was dialyzed against a solution containing 1 mM $NaHCO_3$. Troponin C was prepared from the Ezo-giant scallop striated muscle according to the procedures described previously (*1, 6, 7*).

ATPase Assay—The reaction mixture (0.5 ml) for the ATPase assay comprised 90 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM Ca^{2+}-EGTA, 20 mM MOPS (pH 6.8), and 100 μg myofibrils (intact or CDTA-treated desensitized myofibrils). Other proteins such as myosin regulatory light chain and troponin C were added to the reaction mixture in 10 μg amounts unless otherwise indicated, followed by incubation overnight at 2°C. The reaction was started by the addition of ATP at 25 or 15°C, and was terminated by the addition of 4 ml of a mixture of 50% acetone, 2.5 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, and 1.25 N H_2SO_4 after 2.5 min at 25°C or 5 min at 15°C. The contents of each tube were mixed carefully, and then 0.4 ml of 1 M citric acid was added and the yellow color was measured at OD 355 nm (*9*). The Ca^{2+}-concentration in the reaction mixture was determined as described previously (*8*).

Binding Experiments—The binding of myosin regulatory light chain and troponin C to the desensitized myofibrils was examined by means of a cosedimentation assay. The reaction mixture (0.5 ml) comprised 90 mM KCl, 5 mM $MgCl_2$, 1 mM Ca^{2+}-EGTA, 20 mM MOPS (pH 6.8), 100 μg/ml desensitized myofibrils, and 10 μg/ml of myosin regulatory light chain and/or troponin C. The mixture was incubated for 120 min and then centrifuged at 2,700 rpm at 2°C. Thereafter the precipitate was examined by SDS-PAGE and alkaline urea gel-electrophoreses.

Gel-Electrophoreses—SDS-gel electrophoresis was carried out according to the procedure of Laemmli (*10*) with an acrylamide concentration of 12%. Alkaline urea-gel electrophoresis was performed according to the procedure of Pan and Solaro, with slight modifications, with an acrylamide concentration of 13.5% at pH 8.8 (*11*).

RESULTS

Figure 1A shows the SDS-gel electrophoretic patterns of scallop myofibrils demonstrating that both RLC and TN·C were removed from the myofibrils after the CDTA-treatment. Alkaline urea-gel electrophoresis also confirmed the complete removal of both RLC and TN·C from the myofibrils on CDTA-treatment (Fig. 1B). In the present

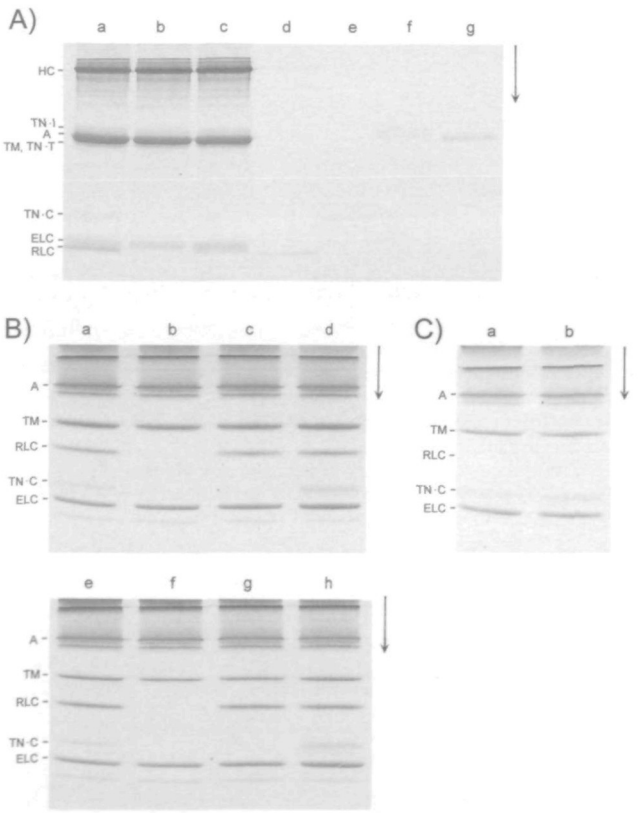

Fig. 1. **SDS- and alkaline urea-gel electrophoretic patterns of scallop striated myofibrils before and after removal and reconstitution of myosin regulatory light chain (RLC) and troponin C (TN·C).** Desensitized myofibrils were prepared by CDTA-treatment, and the binding of RLC and TN·C to the desensitized myofibrils was examined as described under "EXPERIMENTAL PROCEDURES." (A) SDS-gel. a, intact myofibrils; b, desensitized myofibrils; c, desensitized myofibrils+RLC; d, RLC; e, TN·C; f, troponin (troponin C·I·T complex); g, tropomyosin. (B) Alkaline urea-gels. a and e, intact myofibrils; b and f, desensitized myofibrils; c and g, desensitized myofibrils+RLC; d and h, desensitized myofibrils+RLC+TN·C. The binding of RLC and TN·C was examined at pCa 8.2 (a–d) and pCa 4.4 (e–h). (C) Alkaline urea-gel of desensitized scallop striated myofibrils after reconstitution with troponin C at pCa 8.2 (a) and pCa 4.4 (b). Abbreviations. HC, myosin heavy chain; A, actin; TM, tropomyosin; TN·I, T and C, troponins I, T, and C; ELC, myosin essential light chain; RLC, myosin regulatory light chain.

study, the effect of reconstitution of CDTA-treated myofibrils with RLC and TN·C on the Ca^{2+}-sensitive myofibrillar ATPase activity was investigated at 25 and 15°C. The maximum ATPase activity of both intact and CDTA-treated myofibrils at 25°C was approximately three times higher than that at 15°C (Table I).

The ATPase of the CDTA-treated myofibrils was desensitized to Ca^{2+} to a level of about half that of the maximum activity of the intact myofibrils at 25°C (Figs. 2 and 3, and Table I). After the reconstitution of these desensitized myofibrils with RLC, the ATPase was mostly inhibited at low Ca^{2+} (pCa 8.2) and was activated at high Ca^{2+} (pCa 4.4) to a level of about 80% of the maximum activity of the intact myofibrils, whereas the ATPase of the desensitized myofibrils was not affected at low Ca^{2+} but slightly activated at high Ca^{2+}, on reconstitution with TN·C (Fig. 2 and Table I). When the desensitized myofibrils were reconstituted with both RLC and TN·C, the ATPase was inhibited at low Ca^{2+} to a level which was the same as that after reconstitution with RLC alone, while at higher Ca^{2+}-concentrations, the ATPase level exceeded that obtained after reconstitution with RLC alone and almost reached the level of intact myofibrils (Fig. 3 and Table I).

At the low temperature of 15°C, the ATPase of the desensitized myofibrils was also about half the maximum activation level of the intact myofibrils regardless of the

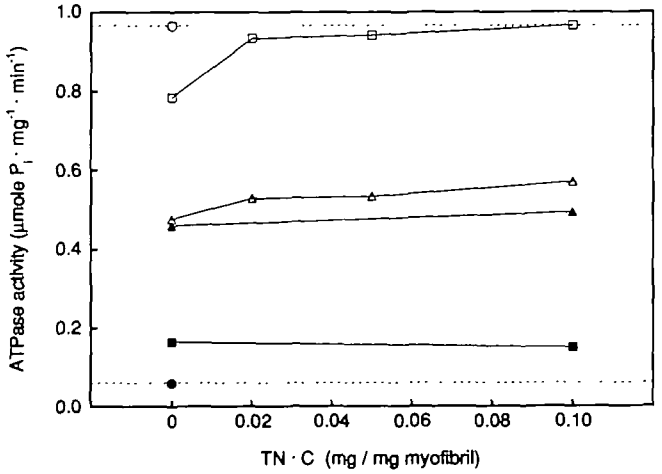

Fig. 2. **Effect of reconstitution with myosin regulatory light chain and troponin C on the Ca^{2+}-activated ATPase of desensitized scallop striated myofibrils at 25°C.** ○, ●, intact myofibrils; △, ▲, desensitized myofibrils; □, ■, desensitized myofibrils reconstituted with RLC (0.1 mg/mg desensitized myofibrils). ○, △, □, pCa 4.4; ●, ▲, ■, pCa 8.2.

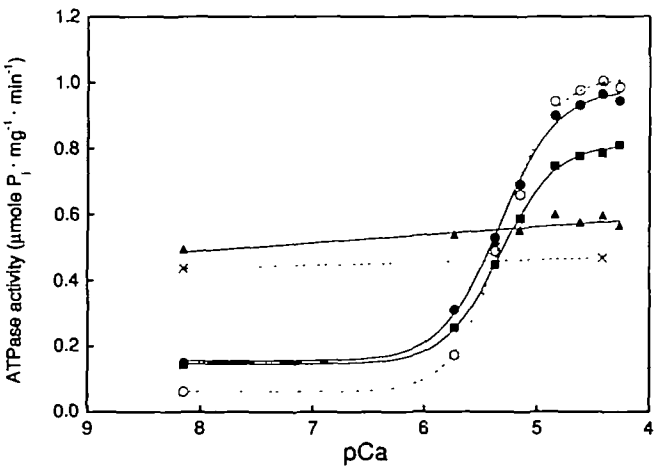

Fig. 3. **Effects of reconstitution with myosin regulatory light chain and troponin C on the Ca^{2+}-activation profiles of the ATPase of desensitized scallop striated myofibrils at 25°C.** □, intact myofibrils (pCa_{50}, 5.30); ×, desensitized myofibrils; ■, desensitized myofibrils+RLC (pCa_{50}, 5.32); ▲, desensitized myofibrils + TN·C; ●, desensitized myofibrils+RLC+TN·C (pCa_{50}, 5.32). The amount of RLC or TN·C in the reaction mixtures was 0.1 mg/mg desensitized myofibrils.

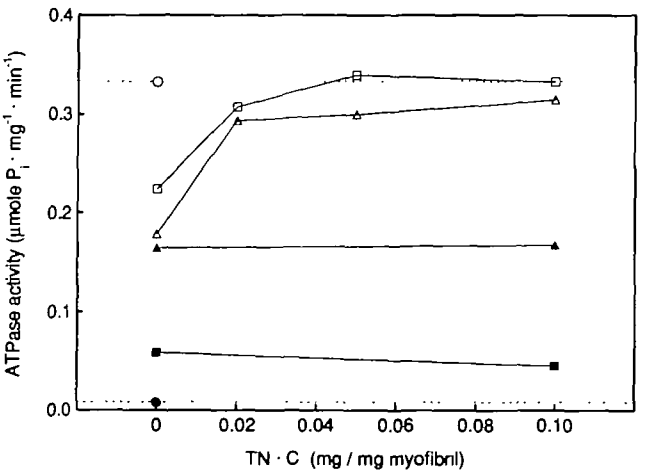

Fig. 4. **Effect of reconstitution with myosin regulatory light chain and troponin C on the Ca^{2+}-activated ATPase of desensitized scallop striated myofibrils at 15°C.** ○, ●, intact myofibrils; △, ▲, desensitized myofibrils; □, ■, desensitized myofibrils reconstituted with RLC (0.1 mg/mg desensitized myofibrils). ○, △, □, pCa 4.4; ●, ▲, ■, pCa 8.2.

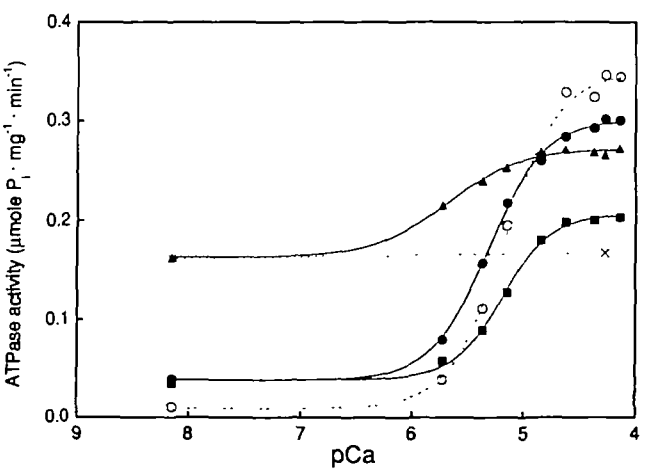

Fig. 5. **Effects of reconstitution with myosin regulatory light chain and troponin C on the Ca^{2+}-activation profiles of the ATPase activity of desensitized scallop striated myofibrils at 15°C.** □, intact myofibrils (pCa_{50}, 5.17); ×, desensitized myofibrils; ■, desensitized myofibrils+RLC (pCa_{50}, 5.17); ▲, desensitized myofibrils+TN·C (pCa_{50}, 5.69); ●, desensitized myofibrils+RLC+TN·C (pCa_{50}, 5.32). The amount of RLC or TN·C in the reaction mixture was 0.1 mg/mg desensitized myofibrils.

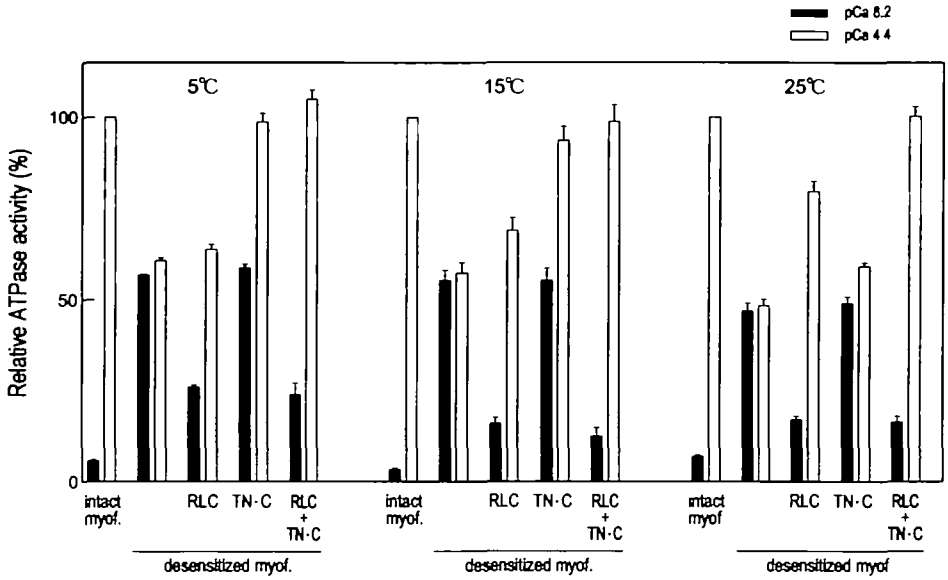

Fig. 6. **Comparison of the activating effect of RLC and TN·C on the ATPase activity of desensitized myofibrils at different temperatures.** The data at 15 and 25 C are cited from Table I. The data at 5 C are the means±SEM for three measurements. The ATPase activities of intact myofibrils at 5°C were 5.26±0.21 nmol P_i/mg/min at pCa 8.2 and 93.67±2.14 nmol P_i/mg/min at pCa 4.4, respectively. The ordinate shows the relative ATPase activity normalized by the ATPase activity of intact myofibrils at each temperature.

TABLE I. **Effect of reconstitution of myosin regulatory light chain and troponin C on the Ca^{2+}-ATPase activity of CDTA-treated desensitized myofibrils from scallop striated muscle at different temperatures.**[a]

Myofibrillar preparation	25°C			15°C		
	ATPase activity (μmol P_i·mg^{-1}·min^{-1})		pCa_{50}[b]	ATPase activity (μmol P_i·mg^{-1}·min^{-1})		pCa_{50}[b]
	pCa 8.2	pCa 4.4		pCa 8.2	pCa 4.4	
1) Intact myof.	0.07±0.01 (8)	1.04±0.03 (8)	5.39±0.03 (5)	0.01±0.00 (8)	0.32±0.02 (8)	5.20±0.02 (6)
2) CDTA-treated myof.	0.47±0.01 (5)	0.48±0.01 (5)	n.d.[c]	0.17±0.00 (7)	0.17±0.00 (7)	n.d.[c]
3) CDTA-treated myof.+RLC	0.18±0.01 (4)	0.79±0.01 (5)	5.33±0.02 (4)	0.05±0.01 (5)	0.21±0.01 (5)	5.17±0.03 (3)
4) CDTA-treated myof.+TN·C	0.52±0.01 (4)	0.57±0.01 (4)	n.d.[c]	0.17±0.00 (7)	0.29±0.01 (7)	5.63±0.13 (4)
5) CDTA-treated myof.+RLC+TN·C	0.17±0.01 (4)	0.97±0.01 (4)	5.33[d] (2)	0.04±0.01 (6)	0.31±0.01 (6)	5.28±0.03 (4)

[a]Each value is the mean±SEM. Numbers in parentheses indicate the numbers of measurements. [b]pCa at 50% Ca^{2+}-sensitive ATPase activation. [c]Not determined. [d]Average of two measurements (5.32, 5.34).

Ca^{2+}-concentration, and was also inhibited at low Ca^{2+} on the reconstitution with RLC (Figs. 4 and 5, and Table I). However, the activation caused by the reconstitution with RLC was lower than that at 25°C, while the activation on the reconstitution with TN·C was much greater than that at 25°C (Figs. 2-5, and Table I). The ATPase level on reconstitution with both RLC and TN·C was the same as that after reconstitution with RLC alone at low Ca^{2+}, but the maximum activity at high Ca^{2+} was higher than that on reconstitution with RLC or TN·C. The Ca^{2+}-affinity (pCa_{50}) of the ATPase of the desensitized myofibrils reconstituted with both RLC and TN·C was lower than that on reconstitution with TN·C, but was higher than that on reconstitution with RLC (Fig. 5 and Table I). The activating effect of TN·C was also much higher than that of RLC at 5°C (Fig. 6).

The binding of RLC and TN·C to the desensitized myofibrils was examined by means of the cosedimentation assay. The alkaline urea-gel electrophoretic patterns of the desensitized myofibrils after incubation with RLC and TN·C for two hours at 2°C clearly demonstrated that both RLC and TN·C were reconstituted with the desensitized myofibrils regardless of the Ca^{2+}-concentration (Fig. 1, B and C). Essentially the same results were obtained with the desensitized myofibrils after reconstitution with RLC and TN·C under the conditions for the ATPase assay, *i.e.*, incubation at 25 or 15°C for 5 min after preincubation overnight at 2°C (data not shown).

DISCUSSION

In the present study, Ca^{2+}-desensitized myofibrils were prepared by removing RLC and TN·C from the myofibrils of molluscan striated muscle by treatment with a strong divalent cation chelator, CDTA (*3*). The effects of the removal and reconstitution of RLC and TN·C on the ATPase activity of the desensitized myofibrils were then examined at two different temperatures in order to examine the involvement of troponin-linked Ca^{2+}-regulation, in addition to myosin-linked Ca^{2+}-regulation, in the contraction of scallop striated muscle.

The ATPase level in the desensitized myofibrils was about half the maximum ATPase activity in the intact myofibrils, and the ATPase was almost fully inhibited on the reconstitution with RLC but was not affected by the reconstitution with TN·C in the absence of Ca^{2+} at 25 and 15°C (Figs. 2-5). On the other hand, the ATPase of the desensitized myofibrils was activated on the reconstitution with RLC and TN·C at higher Ca^{2+}-concentrations, but the extents of activation caused by RLC and TN·C were dependent on the temperature. At 25°C, great activation of the ATPase was observed on the reconstitution with RLC, while the ATPase was only slightly activated by TN·C (Figs. 2 and 3, and Table I). At the lower temperature of

15°C, however, the ATPase was only slightly activated by RLC but was greatly activated by TN•C (Figs. 4 and 5, and Table I). The myosin-linked Ca^{2+}-regulation was therefore indicated to be mainly involved in the phase of activation of the ATPase at 25°C, while the troponin-linked Ca^{2+}-regulation is mainly involved at 15°C. Essentially the same activating effects of RLC and TN•C as that at 15°C were observed at 5°C (Fig. 6).

It was also noted that, at 15°C, the Ca^{2+}-affinity of the ATPase on reconstitution with both RLC and TN•C was lower than that after reconstitution with TN•C, but was a little higher than that on reconstitution with RLC (Fig. 5 and Table I), suggesting that the entire Ca^{2+}-activation profile of the ATPase is determined by the myosin-linked and troponin-linked Ca^{2+}-regulation with different Ca^{2+}-affinities.

Early studies demonstrated that the contraction of the molluscan adductor striated muscle was regulated by the action of Ca^{2+} on myosin filaments but not on thin filaments, *i.e.*, troponin-tropomyosin-actin filaments (*3, 5, 12*). However, the ATPase measurements in these studies were performed at temperatures of 23-25°C, at which the participation of troponin-linked Ca^{2+}-regulation was found to be relatively low in the present study (Figs. 2 and 3, and Table I). This might explain why the troponin-linked Ca^{2+}-regulation could not previously be detected in the contraction of scallop striated muscle (*12*).

It is well known that the contraction of striated muscle of scallops causes rapid expulsion of sea water through the adduction of the shells and consequently the swimming movement of the scallop with jet propulsion. This characteristic swimming movement of Ezo-giant scallops has been reported to be observed only in the range of relatively low temperatures of 5 to 20°C (*13*). It was also found, in the present study on the ATPase activity of myofibrils from the adductor striated muscle of Ezo-giant scallops, that the troponin-linked Ca^{2+}-regulation occurs at low temperatures from 5 to 15°C (Figs. 4, 5, and 6 and Table I). These findings strongly suggest that the troponin-linked Ca^{2+}-regulation is involved in the physiological contractile activity of the striated muscle in the scallop.

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