INTERACTION OF TROPONIN C AND CALMODULIN WITH TROPONIN T: A COMPARATIVE STUDY OF SKELETAL AND CARDIAC MUSCLE TROPONINS

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ABSTRACT

1) We investigated the interactions between troponin C and troponin T, and between calmodulin and troponin T, in the presence of Ca^{2+} and Sr^{2+} . Polyacrylamide-gel electrophoresis under non-denaturing conditions and ultracentrifugation methods was used to examine the interactions. Calmodulin and skeletal troponin C bound to skeletal troponin T only in the presence of bivalent cations, whereas calmodulin and cardiac troponin C bound to cardiac troponin T in both the presence and absence of bivalent cations. 2) Skeletal troponin C bound to the skeletal troponin T-tropomyosin-actin complex only in the presence of bivalent cations, although cardiac troponin C hardly bound to the cardiac troponin T-tropomyosin-actin complex even in the presence of bivalent cations. 3) Calmodulin bound to the skeletal and cardiac troponin T-tropomyosin-actin complexes only in the presence of bivalent cations. 4) Trifluoperazine, a calmodulin antagonist, inhibited the bivalent-cation-dependent interaction between calmodulin and skeletal troponin T. 5) The results suggest that the conformation of cardiac troponin T coupled with the tropomyosin-actin complex differs considerably from that of troponin T alone, and that the interaction between troponin C and troponin T does not play a major role in the troponin regulation of muscle contraction. The bivalent-cation-induced exposure of the hydrophobic region may enable calmodulin to bind to skeletal troponin T.

The contraction-relaxation cycle of striated muscles is regulated by Ca^{2+} through the troponin-tropomyosin system (6). Troponin which consists of three subunits, troponin C, troponin I and troponin T, locates on the thin filaments (27). The interactions between troponin C and troponin I, and between troponin I and the tropomyosin-actin complex are well-known and their significance in the troponin

regulation of muscle contraction is well established (18, 20). A number of workers have investigated the interaction between troponin C and troponin T (7, 9, 10, 13, 15, 19, 22, 26). However, it is not clear what role the interaction between troponin C and troponin T plays in the troponin regulation (5). In the present study, we investigated the interaction between troponin C and troponin T and performed a comparative study of skeletal and cardiac-muscle troponin in an attempt to provide information about the role of troponin C-troponin T interaction.

Abbreviations: TNT, troponin T; TNI, troponin I; TNC, troponin C; CaM, calmodulin

Fig. 1 Electrophoretic pattern of the mixture of troponin C and troponin T in the presence of bivalent cations under non-denaturing conditions. A mixture of troponin C and troponin T in a 1.5 : 1 molar ratio (approximately 10 μ g) was applied to a 7% polyacrylamide-gel in 25 mM Tris/240 mM glycine buffer, pH 8.5, containing: lanes a, d and g, 2 mM EGTA; lanes b, e and h, 2 mM CaCl_2 ; lanes c, f and i, 2 mM EGTA and 10 mM SrCl_2 . Lanes a, b and c; skeletal troponin C and skeletal troponin T; lanes d, e and f, cardiac troponin C and cardiac troponin T; lanes g, h and i, skeletal troponin C and cardiac troponin T

Calmodulin, one of Ca^{2+} -binding proteins, has been reported to be able to replace troponin C in the regulation of muscle contraction *in vitro* (1, 29). We showed that the mode of the calmodulin regulation was considerably different from that of the troponin regulation (29, 30). In the present study, we investigated the interaction between calmodulin and troponin T and compared it with the results of troponin C and troponin T to obtain further knowledge about troponin T.

MATERIALS AND METHODS

Materials

Trifluoperazine was purchased from Yoshitomi Chemical (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of Proteins

Skeletal troponin was prepared from rabbit skeletal muscle by the method of Ebashi *et al.* (8) and cardiac troponin was from porcine cardiac muscle by the method of Tsukui and

Ebashi (25). Troponin was fractionated into components by the method of Perry and Cole (21). Calmodulin was prepared from bovine brain by the method of Yazawa *et al.* (33). Tropomyosin was prepared from rabbit skeletal muscle as described previously (31). Actin was prepared from acetone-dried powder of rabbit skeletal muscle by the method of Spudich and Watt (23).

Ultracentrifugation Study

Troponin components, calmodulin, tropomyosin and actin were mixed in a final volume of 3 ml of solution containing 50 mM Tris/HCl, pH 7.4 at 4°C, 2 mM MgCl_2 , 100 mM KCl and 1 mM EGTA or 1 mM CaCl_2 or 1 mM EGTA and 2 mM SrCl_2 . Ultracentrifugation was carried out at 100,000 g for 2 h at 4°C. After centrifugation, 0.2 ml of a solution containing 10 mM sodium phosphate buffer, pH 7.0, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) 2-mercaptoethanol, 50% (v/v) glycerol and 0.05% (w/v) Bromophenol Blue was added to the pellet, which was redissolved vi-

Fig. 2 Interaction of skeletal troponin C with the skeletal troponin T-tropomyosin-actin complex. Proteins were mixed in a solution containing 50 mM Tris/HCl buffer, pH 7.4, 2 mM $MgCl_2$, 100 mM KCl and 1 mM EGTA (lanes a and b), 1 mM $CaCl_2$ (lanes c and d), or 1 mM EGTA and 2 mM $SrCl_2$ (lanes e and f). Protein concentrations in (a): actin, 0.51 mg/ml; tropomyosin, 0.14 mg/ml; troponin T, 0.08 mg/ml; and troponin C, 0.06 mg/ml. Protein concentrations in (b): actin, 0.45 mg/ml; tropomyosin, 0.12 mg/ml; troponin T, 0.08 mg/ml; and troponin C, 0.37 mg/ml. Ultracentrifugation was carried out at 100,000 g for 2 h at 4°C. The pellet and supernatant were treated for 9% SDS-polyacrylamide-gel electrophoresis in 0.1 M sodium phosphate buffer, pH 7.0. Lanes a, c and e, pellets; lanes b, d and f, supernatants; and lane g, skeletal troponin T, I and C.

Fig. 3 Interaction of cardiac troponin C with the cardiac troponin T-tropomyosin-actin complex. Actin and tropomyosin were from rabbit skeletal muscle. Proteins were mixed in a solution containing 50 mM Tris/HCl buffer, pH 7.4, 2 mM $MgCl_2$, 100 mM KCl and 1 mM EGTA (lanes a and b), 1 mM $CaCl_2$ (lanes c and d) or 1 mM EGTA and 2 mM $SrCl_2$ (lanes e and f). Ultracentrifugation and electrophoresis were carried out as in Fig. 2. Protein concentrations in (a): actin, 0.63 mg/ml; tropomyosin, 0.17 mg/ml; troponin T, 0.09 mg/ml; and troponin C, 0.05 mg/ml. Protein concentrations in (b): actin, 0.39 mg/ml; tropomyosin, 0.12 mg/ml; troponin T, 0.05 mg/ml; and troponin C, 0.23 mg/ml. Lanes a, c and e, pellets; lanes b, d and f, supernatants; and lane g, cardiac troponin T, I and C

Fig. 4 Electrophoretic pattern of the mixture of calmodulin and troponin T in the presence of bivalent cations under non-denaturing conditions. A mixture of calmodulin and troponin T in a 1.5:1 molar ratio (approximately 10 μ g) was applied to a 7% polyacrylamide-gel in 25 mM Tris/240 mM glycine buffer, pH 8.5, containing: lanes a and f, 2 mM EGTA; lanes b and g, 2 mM CaCl_2 ; lanes c and h, 2 mM EGTA and 10 mM SrCl_2 ; lane d, 2 mM CaCl_2 and 100 μ M trifluoperazine; and lane e, 2 mM EGTA, 10 mM SrCl_2 and 100 μ M trifluoperazine. Lanes a, b, c, d and e, calmodulin and skeletal troponin T; and lanes f, g and h, calmodulin and cardiac troponin T

gorously. A 5 ml volume of 10% (w/v) trichloroacetic acid was added to the supernatant, which was centrifuged at 2,000 g for 5 min. To the precipitate was added 0.2 ml of the 1% SDS solution described above.

Polyacrylamide-Gel Electrophoresis

SDS-polyacrylamide-gel electrophoresis was performed in 100 mM sodium phosphate buffer, pH 7.0, as described by Weber and Osborn (28). The sample solution (15–20 μ l) was applied to a 9% polyacrylamide slab-gel. Tris-glycine gel electrophoresis was carried out on a 7% polyacrylamide-gel as described previously (30). The protein concentration was determined as described previously (29).

RESULTS

Electrophoretic Study on the Interaction of Troponin C with Troponin T under Non-denaturing Conditions

Skeletal troponin C formed a complex with

skeletal troponin T in the presence of Ca^{2+} (Fig. 1, lane b) and Sr^{2+} (Fig. 1, lane c), but not in the absence of bivalent cations (Fig. 1, lane a). Trifluoperazine did not inhibit the bivalent-cation-dependent interaction between skeletal troponin C and troponin T (data not shown). Cardiac troponin C formed a complex with cardiac troponin T in both the presence and absence of bivalent cations (Fig. 1, lanes d, e and f). Skeletal troponin C formed a complex with cardiac troponin T regardless of the concentrations of bivalent cations (Fig. 1, lanes g, h and i).

Interaction of Skeletal Troponin C with the Skeletal Troponin T-Tropomyosin-Actin Complex

Skeletal troponin C bound to the troponin T-tropomyosin-actin complex in the presence of Ca^{2+} (Fig. 2a, lanes c and d) and Sr^{2+} (Fig. 2a, lanes e and f), but not in the absence of bivalent cations (Fig. 2a, lanes a and b). When a large amount of troponin C was added, troponin

Fig. 5 Interaction of calmodulin with the skeletal troponin T-tropomyosin-actin complex in the presence of bivalent cations. Proteins were mixed in a solution containing 50 mM Tris/HCl buffer, pH 7.4, 2 mM $MgCl_2$, 100 mM KCl and 1 mM EGTA (lanes a and b), 1 mM $CaCl_2$ (lanes c and d), or 1 mM EGTA and 2 mM $SrCl_2$ (lanes e and f). Protein concentrations in (a): actin, 0.51 mg/ml; tropomyosin, 0.17 mg/ml; troponin T, 0.10 mg/ml; and calmodulin, 0.06 mg/ml. Protein concentrations in (b): actin, 0.45 mg/ml; tropomyosin, 0.12 mg/ml; troponin T, 0.08 mg/ml; and calmodulin, 0.37 mg/ml. Ultracentrifugation and electrophoresis were carried out as in Fig. 2. Lanes a, c and e, pellets; lanes, b, d and f, supernatants; and lane g, skeletal troponin T, I and calmodulin

Fig. 6 Interaction of calmodulin with the cardiac troponin T-tropomyosin-actin complex in the presence of bivalent cations. Actin and tropomyosin were from rabbit skeletal muscle. Proteins were mixed in a solution containing 50 mM Tris/HCl, 2 mM $MgCl_2$, 100 mM KCl and 1 mM EGTA (lanes a and b), 1 mM $CaCl_2$ (lanes c and d), or 1 mM EGTA and 2 mM $SrCl_2$ (lanes e and f). Protein concentrations in (a): actin, 0.56 mg/ml; tropomyosin, 0.15 mg/ml; troponin T, 0.08 mg/ml; and calmodulin, 0.04 mg/ml. Protein concentrations in (b): actin, 0.49 mg/ml; tropomyosin, 0.16 mg/ml; troponin T, 0.07 mg/ml; and calmodulin, 0.36 mg/ml. Ultracentrifugation and electrophoresis were carried out as in Fig. 2. Lanes a, c and e, pellets; lanes b, d and f, supernatants; and lane g, cardiac troponin T, I and calmodulin

nin C bound to troponin T in the presence of Ca^{2+} and Sr^{2+} (Fig. 2b, lanes c and e), but not in the absence of bivalent cations (Fig. 2b, lanes a and b), indicating that the amount of troponin C added has little effect on the interaction.

Interaction of Cardiac Troponin C with the Cardiac Troponin T-Tropomyosin-Actin Complex

Cardiac troponin C did not bind to the cardiac troponin T-tropomyosin-actin complex regardless of the concentrations of bivalent cations (Fig. 3a, lanes a, c and e). When a large amount of troponin C was added, troponin C hardly bound to the troponin T-tropomyosin-actin complex in both the presence and absence of bivalent cations (Fig. 3b, lanes a, c and e). The result shows that the amount of cardiac troponin C added has little effect on the interaction.

Electrophoretic Study on the Interaction of Calmodulin with Troponin T under Non-denaturing Conditions

Calmodulin formed a complex with skeletal troponin T in the presence of Ca^{2+} (Fig. 4, lane b), but not in its absence (Fig. 4, lane a). Calmodulin bound to skeletal troponin T but did not produce a clear band in the presence of Sr^{2+} (Fig. 4, lane c). Trifluoperazine inhibited the bivalent-cation-dependent interaction between calmodulin and skeletal troponin T (Fig. 4, lanes d and e). Calmodulin formed a complex with cardiac troponin T in both the presence and absence of bivalent cations (Fig. 4, lanes f, g and h). Trifluoperazine did not inhibit the bivalent-cation-independent interaction between calmodulin and cardiac troponin T (data not shown).

Interaction of Calmodulin with the Skeletal and Cardiac Troponin T-Tropomyosin-Actin Complexes

Calmodulin bound to the skeletal troponin T-tropomyosin-actin complex in the presence of Ca^{2+} (Fig. 5a, lane c) and Sr^{2+} (Fig. 5a, lane e), but not in the absence of bivalent cations (Fig. 5a, lane a). When a large amount of calmodulin was added, calmodulin bound to

skeletal troponin T in the presence of bivalent cations (Fig. 5b, lanes c and e), but not in their absence (Fig. 5b, lane a). Calmodulin showed the same bivalent-cation-dependent interaction with cardiac troponin T (Fig. 6a, lanes a, c and e) as with skeletal troponin T. When a large amount of calmodulin was added, calmodulin bound to cardiac troponin T in the presence of bivalent cations (Fig. 6b, lanes c and e), but not in the absence of bivalent cations (Fig. 6b, lane a). The results show that the amount of calmodulin added has little effect on the interaction.

DISCUSSION

It has been shown that skeletal troponin C binds to skeletal troponin T and that the interaction between troponin C and troponin T is intensified by Ca^{2+} (7, 9, 13, 15-17, 26). Jackson *et al.* (11) showed that the complex of troponin C and troponin T was not formed in the presence of tropomyosin. Furthermore, it has been shown that troponin C hardly bound to troponin T regardless of whether Ca^{2+} was present or not when both troponin C and troponin T were applied to a tropomyosin-Sepharose column (19, 24). However, Margossian and Cohen (14) obtained the results that troponin C bound to the troponin T-tropomyosin paracrystals. In the present study, we demonstrated that skeletal troponin C bound to skeletal troponin T and to the skeletal troponin T-tropomyosin-actin complex in a bivalent-cation-dependent manner.

Cardiac troponin C has been shown to bind to cardiac troponin T (2, 4, 12) and the interaction was strengthened by Ca^{2+} (4). However, Byers and Kay (3) observed no interaction between cardiac troponin C and troponin T in both the presence and absence of Ca^{2+} using bovine cardiac troponin. We have shown that porcine cardiac troponin C bound to cardiac troponin T regardless of the concentrations of bivalent cations (32). The present results show that both skeletal and cardiac troponin Cs bind to cardiac troponin T in a bivalent-cation-independent manner. Unexpectedly, cardiac troponin C did not bind to cardiac troponin T coupled with the tropomyosin-actin complex. These findings suggest that the conformation of cardiac troponin T combined with the tropomyosin-actin complex differs

considerably from that of troponin T alone. Our present results also suggest that the interaction of troponin C with troponin T differs between skeletal and cardiac troponins. We have shown that all hybrid troponins prepared from skeletal and cardiac muscles confer Ca^{2+} or Sr^{2+} sensitivity on actomyosin ATPase (29). Ca^{2+} or Sr^{2+} sensitivity of actomyosin ATPase activity which contained skeletal troponin T was almost the same as that of actomyosin ATPase activity which contained cardiac troponin T (29). Thus Ca^{2+} or Sr^{2+} sensitivity of ATPase was hardly determined by the properties of troponin T. These findings suggest that the interaction between troponin C and troponin T does not play a major role in the troponin regulation of muscle contraction.

Amphlett *et al.* (1) showed a Ca^{2+} -dependent interaction between calmodulin and skeletal troponin T. Our results are in agreement with theirs in case of skeletal troponin T and show that the bivalent-cation-induced exposure of the hydrophobic region is important for calmodulin to bind to skeletal troponin T. We showed a bivalent-cation-independent interaction between calmodulin and cardiac troponin T, although calmodulin bound to cardiac troponin T coupled with the tropomyosin-actin complex in a bivalent-cation-dependent manner. These findings indicate that the interaction between calmodulin and cardiac troponin T differs from that between calmodulin and cardiac troponin T on the thin filaments.

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