Interaction of calmodulin with troponin I and the troponin–tropomyosin–actin complex

Effect of Ca^{2+} and Sr^{2+} ions

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1. In an effort to elucidate the mechanism of calmodulin regulation of muscle contraction, we investigated the interaction between calmodulin and troponin components in the presence of Ca^{2+} or Sr^{2+} by the use of ultracentrifugation methods and polyacrylamide-gel electrophoresis. Skeletal-muscle troponin C bound to troponin I and dissociated it from the tropomyosin–actin complex in the presence of Ca^{2+} or Sr^{2+} . 2. When troponin T was absent, calmodulin bound to troponin I and dissociated it from the tropomyosin–actin complex in the presence of Ca^{2+} or Sr^{2+} . When troponin T was present, calmodulin hardly bound to troponin I even in the presence of bivalent cations. 3. Trifluoperazine, a calmodulin antagonist, inhibited the bivalent-cation-dependent interaction between calmodulin and troponin I. 4. Calmodulin migrated more slowly in the presence of Sr^{2+} than it did in the presence of EGTA but faster than it did in the presence of Ca^{2+} on polyacrylamide-gel electrophoresis under non-denaturing conditions. 5. It is concluded that troponin T is not required in the calmodulin regulation of muscle contraction because troponin T inhibits the bivalent-cation-dependent interaction between calmodulin and troponin I and because calmodulin binds to troponin I and dissociates it from the tropomyosin–actin complex in a bivalent-cation-dependent manner. Sr^{2+} -induced exposure of the hydrophobic region enables calmodulin to bind to troponin I, as is the case with Ca^{2+} .

INTRODUCTION

It has been shown that the Ca^{2+} -binding protein calmodulin has structural homologies to troponin C (Watterson *et al.*, 1976, 1980; Grand & Perry, 1978), being capable of replacing troponin C in the regulation of muscle contraction *in vitro* (Fine *et al.*, 1975; Amphlett *et al.*, 1976; Dedman *et al.*, 1977; Castellani *et al.*, 1980; Yamamoto, 1983). Troponin consists of three components, troponin C, troponin I and troponin T, all of which are required for the regulation of muscle contraction (Ebashi *et al.*, 1972), although troponin T is not required when troponin C is replaced by calmodulin (Amphlett *et al.*, 1976; Castellani *et al.*, 1980; Yamamoto, 1983). We have shown that calmodulin hardly neutralizes the inhibitory action of skeletal-muscle troponin I even in the presence of bivalent cations when skeletal-muscle troponin T is present (Yamamoto, 1983). Thus calmodulin regulation of muscle contraction is considerably different from the regulation by troponin. To shed some light on the regulation by calmodulin, we investigated the interaction of calmodulin with troponin I and the troponin–tropomyosin–actin complex in the presence of bivalent cations.

In previous studies we suggested that the Ca^{2+} -sensitizing action of troponin was not solely determined by the properties of troponin C but was also affected by the properties of troponin I (Yamamoto, 1983; Ebashi & Ohtsuki, 1983). However, the Sr^{2+} -sensitizing action of troponin was determined by troponin C alone (Yamamoto, 1983). When troponin C was replaced by calmodulin, there existed little difference between

Ca^{2+} - and Sr^{2+} -stimulated contractions (Yamamoto, 1983). In the present study, we used not only Ca^{2+} but Sr^{2+} to investigate the effect of bivalent cations on the calmodulin regulation of muscle contraction.

MATERIALS AND METHODS

Materials

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

Preparation of proteins

Troponin was prepared from rabbit skeletal muscle by the method of Ebashi *et al.* (1971) and fractionated into components by the method of Perry & Cole (1974). Calmodulin was prepared from bovine brain by the procedure of Yazawa *et al.* (1980). Tropomyosin was purified from rabbit skeletal muscle as described previously (Yamamoto & Ohtsuki, 1982). Actin was prepared from acetone-dried powder of rabbit skeletal muscle by the method of Spudich & Watt (1971).

Ultracentrifugation study

Calmodulin, troponin components, tropomyosin and actin were mixed in a final volume of 3 ml of solution containing 50 mM-Tris/HCl buffer, pH 7.4 at 4 °C, 2 mM- MgCl_2 , 50–100 mM-KCl and either 1 mM-EGTA or 1 mM- CaCl_2 or 1 mM-EGTA and 2 mM- SrCl_2 . Concentrations of each protein are indicated in the Figure legends. Ultracentrifugation was carried out at 100 000 g

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Fig. 1. Effect of Ca^{2+} and Sr^{2+} on the interaction of troponin C with the troponin I-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 either 1 mM-EGTA (lanes a and b) or 1 mM- CaCl_2 (lanes c and d) or 1 mM-EGTA and 2 mM- SrCl_2 (lanes e and f). Protein concentrations: actin, 0.25 mg/ml; tropomyosin, 0.07 mg/ml; troponin I, 0.02 mg/ml; troponin C, 0.02 mg/ml. After ultracentrifugation at 100000 g for 2 h at 4 °C, the pellets and supernatants were prepared for SDS/polyacrylamide-gel electrophoresis. Electrophoresis was carried out on SDS/9% polyacrylamide gel in 0.1 M-sodium phosphate buffer, pH 7.0. Lanes a, c and e, pellets; lanes b, d and f, supernatants; lane g, actin; lane h, troponins T, I and C. Abbreviations: TNT, troponin T; TNI, troponin I; TNC, troponin C.

for 2 h at 4 °C. After centrifugation, 0.2–0.3 ml of a solution containing 10 mM-sodium phosphate buffer, pH 7.0, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 50% (v/v) glycerol and 0.05% Bromophenol Blue was added to the pellet, which was redissolved vigorously. A 5 ml volume of 10% (w/v) trichloroacetic acid was added to the supernatant, which was centrifuged at 2000 g for 5 min. To the precipitate 0.1–0.2 ml of the 1% SDS solution described above was added.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out in 0.1 M-sodium phosphate buffer, pH 7.0, under the conditions described by Weber & Osborn (1969). A 20–30 μl volume of the sample solution was applied to a 9% polyacrylamide slab gel. Tris/glycine gel electrophoresis was performed on a 7–9% polyacrylamide slab gel both in the presence and in the absence of detergent by using a 25 mM-Tris/240 mM-glycine buffer solution, pH 8.5. Whenever trifluoperazine was used, the light was kept dim to prevent breakdown of the phenothiazine. The protein concentration was determined as described previously (Yamamoto, 1983).

RESULTS AND DISCUSSION

Interaction of calmodulin with the troponin I-tropomyosin-actin complex

Fig. 1 (lanes c, d, e and f) shows that troponin C bound to troponin I in the presence of Ca^{2+} or Sr^{2+} and dissociated it from the tropomyosin-actin complex. In the absence of bivalent cations some proportion of the troponin C-troponin I complex was associated with the tropomyosin-actin complex (Fig. 1, lane a). It has been

Fig. 2. Interaction of calmodulin with the troponin I-tropomyosin-actin complex

Proteins were mixed in a solution containing 50 mM-Tris/HCl buffer, pH 7.4, 2 mM- MgCl_2 , 50 mM-KCl and either 1 mM-EGTA (lanes a and b) or 1 mM- CaCl_2 (lanes c and d) or 1 mM-EGTA and 2 mM- SrCl_2 (lanes e and f). Protein concentrations: actin, 0.34 mg/ml; tropomyosin, 0.16 mg/ml; troponin I, 0.04 mg/ml; calmodulin, 0.03 mg/ml. Ultracentrifugation and electrophoresis were carried out as indicated in Fig. 1 legend. Lanes a, c and e, pellets; lanes b, d and f, supernatants; lane g, actin; lane h, troponin T-troponin I-calmodulin complex. Abbreviations: TNT, troponin T; TNI, troponin I; CalM, calmodulin.

shown that troponin C binds to troponin I in the presence of Ca^{2+} and dissociates it from the tropomyosin-actin complex (Potter & Gergely, 1974; Hitchcock, 1975), suggesting that troponin C abolishes the inhibitory action of troponin I on the actin-myosin interaction. Our results indicate that the tight interaction of troponin C with troponin I upon the binding of Sr^{2+} prevents troponin I from exerting its inhibitory action on the tropomyosin-actin complex, allowing the actin-myosin interaction to occur. These findings suggest that Sr^{2+} plays a role similar to that of Ca^{2+} in the fundamental mechanism of the troponin regulation of muscle contraction.

Interaction of calmodulin with the troponin I-tropomyosin-actin complex

Fig. 2 (lanes d and f) shows that calmodulin bound to troponin I in the presence of Ca^{2+} or Sr^{2+} . The calmodulin-troponin I complex was dissociated from the tropomyosin-actin complex (Fig. 2, lanes c and e). However, calmodulin did not bind to troponin I in the absence of bivalent cations (Fig. 2, lanes a and b). Our results indicate that calmodulin binds to troponin I in a bivalent-cation-dependent flip-flop manner, probably releasing the inhibitory action of troponin I when the concentration of bivalent cations is increased. These findings may explain results obtained in a study of actomyosin ATPase (Amphlett *et al.*, 1976; Castellani *et al.*, 1980; Yamamoto, 1983), showing that troponin T is not required in the calmodulin regulation of muscle contraction.

Interaction of calmodulin with the troponin I-troponin T-tropomyosin-actin complex

Fig. 3(a) (lanes c, d, e and f) shows that calmodulin hardly bound to the troponin I-troponin T-tropomyosin-actin complex even in the presence of Ca^{2+} or Sr^{2+} .

Fig. 3. Effect of troponin T on the interaction of calmodulin with the troponin-tropomyosin-actin complex

Proteins were mixed in a solution containing 50 mM-Tris/HCl buffer, pH 7.4, 2 mM-MgCl₂, 50 mM-KCl and either 1 mM-EGTA (lanes a and b) or 1 mM-CaCl₂ (lanes c and d) or 1 mM-EGTA and 2 mM-SrCl₂ (lanes e and f). Protein concentrations in (a): actin, 0.20 mg/ml; tropomyosin, 0.12 mg/ml; troponin T, 0.03 mg/ml; troponin I, 0.02 mg/ml; calmodulin, 0.02 mg/ml. Protein concentrations in (b): actin, 0.45 mg/ml; tropomyosin, 0.09 mg/ml; troponin T, 0.04 mg/ml; troponin I, 0.03 mg/ml; calmodulin, 0.40 mg/ml. Ultracentrifugation and electrophoresis were carried out as described in Fig. 1 legend. Lanes a, c and e, pellets; lanes b, d and f, supernatants; lane g, troponin T-troponin I-calmodulin complex. Abbreviations: TNT, troponin T; TNI, troponin I; CalM, calmodulin.

Fig. 4. Effect of trifluoperazine on the interaction of calmodulin with the troponin I-tropomyosin-actin complex

Proteins were mixed in a solution containing 50 mM-Tris/HCl buffer, pH 7.4, 2 mM-MgCl₂, 50 mM-KCl, 50 μ M-trifluoperazine and either 1 mM-EGTA (lanes a and b) or 1 mM-CaCl₂ (lanes c and d) or 1 mM-EGTA and 2 mM-SrCl₂ (lanes e and f). Protein concentrations: actin, 0.43 mg/ml; tropomyosin, 0.14 mg/ml; troponin I, 0.05 mg/ml; calmodulin, 0.05 mg/ml. Ultracentrifugation and electrophoresis were carried out as described in Fig. 1 legend. Lanes a, c and e, pellets; lanes b, d and f, supernatants; lane g, troponin T-troponin I-calmodulin complex. Abbreviations: TNT, troponin T; TNI, troponin I; CalM, calmodulin.

Calmodulin did not bind to the troponin I-troponin T-tropomyosin-actin complex in the absence of bivalent cations [Fig. 3(a), lanes a and b]. These results indicate that troponin T inhibits the bivalent-cation-dependent interaction between calmodulin and troponin I, implying that troponin I would exert inhibitory action on the actin-myosin interaction regardless of the concentration

Fig. 5. Effect of trifluoperazine on the interaction of calmodulin with troponin I in the presence of bivalent cations

Calmodulin was mixed with an equimolar amount of troponin I, and 5–10 μ g of the mixture was applied to a 7% polyacrylamide gel in 25 mM-Tris/240 mM-glycine buffer, pH 8.5, containing: lane a, 2 mM-EGTA; lane b, 2 mM-CaCl₂; lane c, 2 mM-EGTA and 10 mM-SrCl₂; lane d, 2 mM-CaCl₂ and 100 μ M-trifluoperazine; lane e, 2 mM-EGTA, 10 mM-SrCl₂ and 100 μ M-trifluoperazine.

of the bivalent cations. These findings are consistent with our previous results showing that the calmodulin-skeletal-muscle troponin I-troponin T complex hardly sensitized actomyosin ATPase to bivalent cations (Yamamoto, 1983). Calmodulin bound to the troponin I-troponin T-tropomyosin-actin complex in the presence of bivalent cations when the amount of calmodulin included in the reaction mixture was more than equimolar to the troponin I [Fig. 3(b), lanes c, d, e and f], but did not do so in the absence of bivalent cations [Fig. 3(b), lanes a and b]. In the presence of troponin T,

Fig. 6. Effect of detergents on the electrophoretic patterns of calmodulin in the presence of bivalent cations

Approx. 10 μ g of calmodulin was applied to a 7% polyacrylamide gel containing no detergent (a), a 9% polyacrylamide gel containing 0.1% SDS (b) and a 9% polyacrylamide gel containing 3 M-urea (c) in 25 mM-Tris/240 mM-glycine buffer, pH 8.5, containing: lane a, 2 mM-EGTA; lane b, 2 mM- CaCl_2 ; lane c, 2 mM-EGTA and 10 mM- SrCl_2 .

the calmodulin-troponin I complex was not removed from the tropomyosin-actin complex [Fig. 3(b), lanes c and e]. Under these conditions calmodulin would bind to troponin I by the mass effect of calmodulin in spite of the inhibitory effect of troponin I on the bivalent-cation-dependent interaction between calmodulin and troponin I.

Effect of trifluoperazine on the interaction of calmodulin with troponin I

It has been shown that calmodulin antagonists bind to calmodulin in a Ca^{2+} -dependent manner (Levine & Weiss, 1977; Tanaka *et al.*, 1982) and inhibit the Ca^{2+} -dependent interaction of calmodulin with skeletal-muscle troponin I (LaPorte *et al.*, 1980; Tanaka *et al.*, 1983). Fig. 4 (lanes c, d, e and f) shows that calmodulin hardly bound to troponin I, even in the presence of Ca^{2+} or Sr^{2+} , when trifluoperazine was added. Troponin I was not removed from the tropomyosin-actin complex in the presence of bivalent cations (Fig. 4, lanes c and e). Polyacrylamide-gel electrophoresis of calmodulin-troponin I complex under non-denaturing conditions also showed that trifluoperazine inhibited the bivalent-cation-dependent interaction between calmodulin and troponin I (Fig. 5, lanes d and e). In the present study a large amount of trifluoperazine was required to inhibit the interaction because there would be a marked decrease in the Ca^{2+} -dependent binding of trifluoperazine to calmodulin in the high-pH conditions used (Levine & Weiss, 1977). Results obtained in the present work indicate that Sr^{2+} -induced exposure of the hydrophobic region enables calmodulin to bind to troponin I, as is the case with Ca^{2+} (LaPorte *et al.*, 1980; Tanaka *et al.*, 1983).

Electrophoretic pattern of calmodulin under non-denaturing and denaturing conditions

It has been shown that calmodulin migrated more slowly in the presence of Ca^{2+} than it did in the presence

of EGTA on the polyacrylamide-gel electrophoresis under non-denaturing conditions (Amphlett *et al.*, 1976; Grand *et al.*, 1979; Kakiuchi *et al.*, 1981; Yamamoto & Ohtsuki, 1985). It has also been shown that the migration of calmodulin was faster in the presence of Ca^{2+} than that in the presence of EGTA on polyacrylamide-gel electrophoresis under denaturing conditions (Burgess *et al.*, 1980; Grand *et al.*, 1980; Wallace *et al.*, 1982). Our results show that under non-denaturing conditions calmodulin migrated faster in the presence of Sr^{2+} than it did in the presence of Ca^{2+} and migrated more slowly than it did in the presence of EGTA [Fig. 6(a), lanes a, b and c]. These results imply that the conformational change of calmodulin upon binding of Ca^{2+} may not be identical with that upon binding of Sr^{2+} . At present, however, it is not certain whether calmodulin is saturated by bivalent cations under these conditions or not. Further study is required to clarify the conformational changes of calmodulin upon binding of Sr^{2+} .

We found that calmodulin migrated more slowly in the presence of Sr^{2+} than it did in the presence of Ca^{2+} when 0.1% SDS was included [Fig. 6(b), lanes b and c]. Wallace *et al.* (1982) also observed the change in mobility of calmodulin on SDS/polyacrylamide-gel electrophoresis upon the binding of Ca^{2+} or Tb^{3+} . When 3 M-urea was included, calmodulin migrated faster in the presence of Ca^{2+} than it did in the presence of Sr^{2+} or EGTA [Fig. 6(c), lanes a, b and c]. Walsh *et al.* (1979) have suggested that calmodulin, partially unfolded in the presence of 3 M-urea, is stabilized against the action of urea by Ca^{2+} and so migrates faster in the presence of Ca^{2+} . Our results suggest that in the presence of 3 M-urea calmodulin will be less stabilized by Sr^{2+} than by Ca^{2+} .

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