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Troponin, Tropomyosin, and Actin Interactions in the Ca^{2+} Regulation of Muscle Contraction†

James D. Potter* and John Gergely

ABSTRACT: The effect of Ca^{2+} on the interaction of troponin (TN) and its components with F-actin, tropomyosin, and the F-actin–tropomyosin complex has been studied. The Ca-binding component of TN (TN-C) did not bind to F-actin, F-actin–tropomyosin, or tropomyosin regardless of whether Ca^{2+} was present. A complex of TN-C and of the inhibitory component (TN-I) bound to F-actin only in the presence of tropomyosin and in the absence of Ca^{2+}. TN-I was weakly bound to F-actin and more strongly to F-actin–tropomyosin. Unfractionated troponin bound tightly to tropomyosin and to F-actin–tropomyosin but only weakly to F-actin, and in both cases the binding was not affected by Ca^{2+}. The tropomyosin-binding component of TN (TN-T) and a complex of TN-C and TN-T bound to tropomyosin and to F-actin–tropomyosin but not to F-actin alone. On the basis of these binding studies and the known positions of tropomyosin on the thin actin-containing filaments during contraction and relaxation, a model is proposed which may explain how troponin and Ca^{2+} regulate muscle contraction.

Troponin, a complex of three subunits (Greaser and Gergely, 1971; Greaser *et al.*, 1972), and tropomyosin are required to confer Ca^{2+} sensitivity on the actin–myosin interaction. Ebashi *et al.* (1969) showed that TN[1] and TM were located in the thin actin-containing filaments with TN spaced at intervals of 40 nm along the thin filament. From this spacing they estimated that TN, TM, and actin are present in a molar ratio of 1:1:7. In good agreement with this stoichiometry, Bremel and Weber (1972) have shown that one TN and one TM can block the combination of myosin with approximately seven actins. More recently Potter (1974) has shown by direct measurement that TN, TM, and actin are present in a 1:1:7 molar ratio in myofibrils and that in each TN the molar ratio of TN-T:TN-I:TN-C is 1:1:1. Hanson and Lowy (1963) originally suggested that TM may be located in the two long pitch grooves of the F-actin double helix. Recently several groups (Hanson *et al.*, 1972; Spudich *et al.*, 1972; Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973) have confirmed this suggestion on the basis of X-ray diffraction data from living muscle and three-dimensional reconstructions from electron micrographs of TN- and TM-containing actin paracrystals. It has been further deduced that TM is localized asymmetrically in the grooves between the two long pitch actin helices of the thin filaments, so that one strand of TM is in close association with one helical set of actin monomers and another TM strand with the other actin helix. In the relaxed state tropomyosin molecules are thought to sterically block the combination of myosin heads with actin. In the activated state, *viz.*, upon combination of Ca^{2+} with TN-C, TM moves into a position closer to the groove and no longer blocks the interaction of myosin.

Hitchcock and Szent-Györgyi (1973) reported that Ca^{2+} inhibited the combination of a TN-T-deficient TN (*i.e.*, a com-

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[1] Abbreviations used are: TM, tropomyosin; TN, unfractionated troponin; TN-T, tropomyosin-binding subunit of troponin; TN-I, ATPase inhibitory subunit; TN-C, Ca^{2+}-binding subunit; CT, the complex of TN-C and TN-T; CI, the complex of TN-C and TN-I; EGTA, ethylene glycol bis(β-aminoethyl ester); EDTA, ethylenediaminetetraacetic acid.

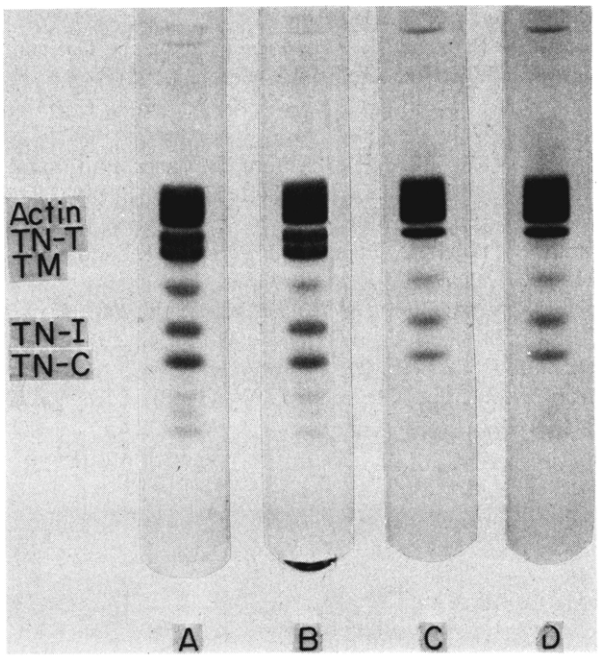

FIGURE 1: Interaction of TN with F-actin and F-actin–TM. Proteins were mixed in a solution containing 0.15 M KCl, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and either 0.01 mM $CaCl_2$ ($+Ca^{2+}$) or 0.1 mM EGTA ($-Ca^{2+}$). (A) F-actin, TM, TN, $+Ca^{2+}$; (B) F-actin, TM, TN, $-Ca^{2+}$; (C) F-actin, TN, $+Ca^{2+}$; (D) F-actin, TN, $-Ca^{2+}$. Protein concentrations: F-actin, 1.04 mg/ml; TM, 0.25 mg/ml; TN, 0.28 mg/ml. After centrifugation at 100,000*g* for 2 hr at 4°, the pellets were treated for electrophoresis as described under Methods. Equal sample volumes containing 10–25 μg of protein were applied to each gel. The material running between TM and TN-I on the gels is probably the breakdown product of TN-T and only represents a small fraction of the total amount.

plex of TN-C and TN-I) with F-actin–TM. In the light of these results we began an investigation of the binding, and the effect of Ca^{2+} on the binding, of purified TN components and their various combinations to F-actin, TM, and the F-actin–TM complex. On the basis of these results, and using the positions of TM during contraction and relaxation mentioned above, a model of the Ca^{2+} regulation of muscle contraction is proposed.

Experimental Section

Materials

Isolation and Purification of TN, TN-T, TN-I, and TN-C. The procedure of Greaser and Gergely (1973) for TN purification was followed with the exception that the pH of the supernatant obtained after the pH 4.6 precipitation step was brought to 2.5 with 1 N HCl for 30 min at 4° to inactivate the catheptic activity present in the extract (Hartshorne and Dreizen, 1972).

For further fractionation TN was dialyzed against two changes of 10 volumes of a solution containing 6 M urea, 50 mM Tris (pH 8.0), 0.1 mM dithiothreitol, and, as described by Eisenberg and Kielley (1972), 1 mM EDTA. The dialyzed TN was chromatographed, to separate the TN components, on DEAE-Sephadex as described by Greaser and Gergely (1973) with the exception that 1 mM EDTA was included in the column and elution buffers.

Purification of Tropomyosin. Tropomyosin was purified from the pH 4.6 precipitate obtained in the course of the preparation of troponin (see above). The precipitate was suspended in a solution containing 1 M KCl–50 mM Tris base, and the pH was adjusted to 8.0 with 2 N KOH. Solid ammonium sulfate was added to produce 40% saturation (230 g/l.), and after centrifugation at 10,000*g* for 10 min the supernatant was brought to 60% saturation with solid ammonium sulfate (125 g/l.). The resulting precipitate was collected by centrifugation at 10,000*g* for 10 min and dissolved in a solution containing 2 mM Tris (pH 8.0). After dialysis against 15 l. of the same solution for 16 hr, KCl was added to a final concentration of 1 M and the pH was lowered to 4.6 with 1 N HCl. The precipitate collected by centrifugation at 10,000*g* for 10 min was dissolved in a solution containing 1 M KCl–50 mM Tris base, and the pH was adjusted to 8.0 with 2 N KOH. Ammonium sulfate was added to 45% saturation, and after centrifugation at 10,000*g* for 10 min the supernatant was brought to 60% saturation. The resulting precipitate was collected by centrifugation at 10,000*g* for 10 min, dissolved in a minimal volume of 2 mM Tris (pH 8.0), and dialyzed for 16 hr against 16 liters of the same solution. This procedure of isoelectric precipitation, ammonium sulfate fractionation, and dialysis was repeated. After the last dialysis the purified TM was lyophilized and stored at −20°.

Actin. Actin was prepared by the method of Spudich and Watt (1972).

Methods

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Densitometry. Electrophoresis was carried out on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Greaser and Gergely (1971). The gels were stained for 3 hr with a solution containing 0.1% Fast Green, 50% methanol, and 9.2% acetic acid. The gels were destained in 50% methanol and 9.2% acetic acid for 16 hr, then for 2 days in 5.0% methanol and 7.5% acetic acid, and finally for 2 days in 7.5% acetic acid. Densitometry of the Fast Green stained gels was performed on a Joyce-Loebl microdensitometer (Model no. S/N 1040) with a 639-nm filter over the light source.

Protein Concentration. Protein nitrogen was determined by micro-Kjeldahl analysis. The nitrogen contents used to convert protein nitrogen values into protein concentrations were 18.0% for TN-I, 18.3% for TN-T, 16.5% for TN-C (Greaser and Gergely, 1973), and 16.5% for actin and TM. These proteins were then used to calibrate the biuret reaction (Gornall *et al.*, 1949).

TN Interactions with F-actin, TM, and F-actin–TM. To study TN interactions with F-actin or F-actin–TM the following procedure was used. Troponin, TN components, or TN component complexes were mixed with either F-actin or with F-actin–TM and centrifuged at 100,000*g* for 2 hr at 4°. For electrophoresis the supernatants were made 1% with sodium dodecyl sulfate and then dialyzed against a large volume of a solution containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. The pellets were suspended in 1% sodium dodecyl sulfate and dialyzed against the above solution. After electrophoresis the stained gels were scanned with a densitometer as described above. In most of the experiments the extent of binding of the TN components to F-actin or F-actin–TM was measured by comparing densitometric scan areas of the TN components in supernatants of mixtures without actin with those of supernatants of actin-containing mixtures. The amount of CI bound to F-actin–TM was also determined by measuring the densitometric scan areas of TN-I, TN-C, actin, and TM in the pellets. The relative staining intensities of these components are known (Potter, 1974) and were used to

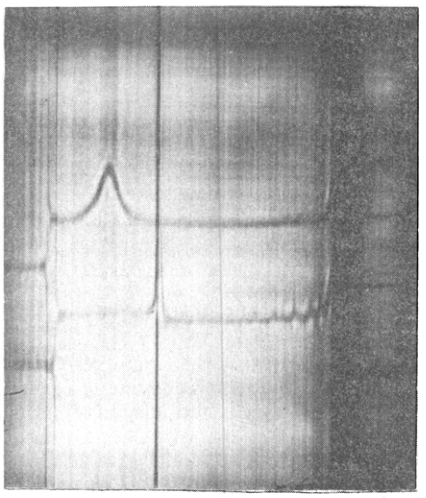

FIGURE 2: Ultracentrifugal analysis of the interaction of TN with TM. The run was made at 20°, rotor speed of 52,640 rpm and bar angle of 65°, sedimentation is from left to right. Photograph taken after 64 min at full speed. The solvent contained 0.15 M KCl, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and 0.1 mM EGTA. Top, 2.9 mg of TM/ml; bottom, a mixture containing 2.9 mg of TM and 3.4 mg of TN/ml.

correct for differences in dye binding. The interactions of the TN components with TM were studied in a Beckman Model E analytical ultracentrifuge.

Results

Interaction of TN with F-actin, TM and F-actin–TM. The interaction of TN with TM and F-actin–TM is well established, but the effect of Ca^{2+} on these interactions has not been studied directly. TN binds to F-actin–TM in the presence or absence of Ca^{2+} (Figure 1A,B) and 1 mol of TN was bound per TM in a complex containing actin and TM in a 7:1 molar ratio in the presence or absence of Ca^{2+} (Table I). Very little TN binds to F-actin alone and the binding was quite variable (Figure 1C,D). This conclusion is based on quantitative densitometry (Potter, 1974) of the gels (Table I), since visual estimates of the amounts of the various components present can be quite misleading. The binding of TN to TM has been studied in the presence of Ca^{2+} (Hartshorne and Mueller, 1967) but not in its absence. Figure 2 shows that TN also forms a complex with TM in the presence of EGTA.

Interaction of TN Components with F-actin, TM, and F-actin–TM. The study of the interactions of TN-T with F-actin, F-actin–TM, or TM at physiological salt concentrations (0.1 M KCl) poses some problems because of the insolubility of TN-T at KCl concentrations less than 0.3 M (Greaser and Gergely, 1973). In 0.4 M KCl TN-T formed a complex with TM in the ultracentrifuge as has been previously reported (Greaser *et al.*, 1972; Greaser and Gergely, 1973). The interaction of TN-T with F-actin and with F-actin–TM was studied at 0.4 M KCl (Figure 3A,B). In the case of F-actin–TM, 0.6 mol of TN-T was bound per TM in a complex containing actin and TM in a 7:1 molar ratio, whereas very little binding to F-actin alone was observed (Table I). Unfortunately, under more physiological conditions, *viz.*, at 0.1 M KCl, TN-T is insoluble; and analysis of pellets obtained by centrifugation shows the presence of TN-T whether or not actin, TM, or both are present. TN-C did not bind to F-actin, TM, or F-actin–TM regardless of whether Ca^{2+} was present (Table I).

TN-I binds to both F-actin and F-actin–TM (Figure 4A,B). It should be noted that the salt concentration used in this

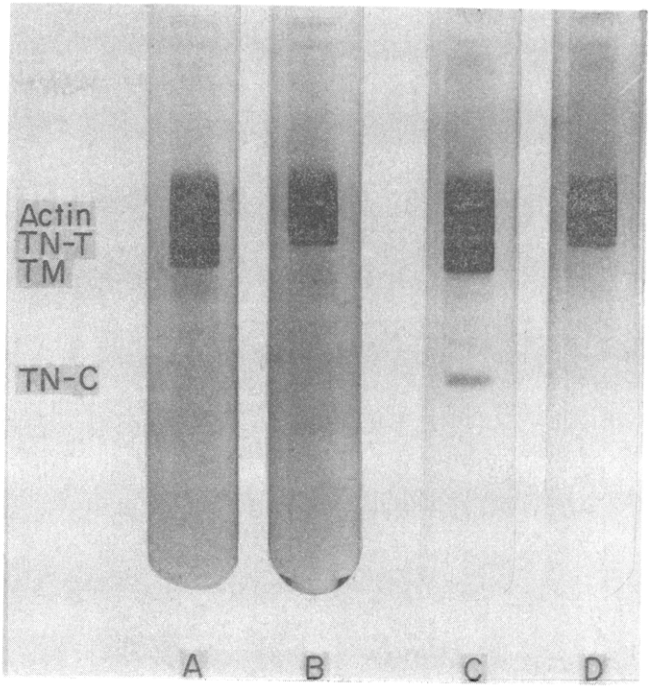

FIGURE 3: Interaction of TN-T and CT with F-actin and F-actin–TM. Proteins were mixed in a solution containing 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 0.01 mM $CaCl_2$, and either 0.4 M KCl (A,B) or 0.1 M KCl (C,D). (A) F-actin, TM, and TN-T; (B) F-actin and TN-T; (C) F-actin, TM, and CT; (D) F-actin and CT. Protein concentrations: F-actin, 0.66 mg/ml; TM, 0.16 mg/ml; TN-T, 0.09 mg/ml; and CT 0.13 mg/ml. The CT complex was prepared as described in the legend to Table I. The samples were centrifuged and the pellets were subjected to gel electrophoresis as described in the legend to Figure 1.

experiment was 0.1 M KCl where TN-I was reported to be insoluble (Greaser and Gergely, 1973). We found, however, that at lower protein concentrations (0.1 mg/ml) than those used previously, TN-I was soluble and did not precipitate

TABLE I: TN Interactions with F-Actin and F-Actin–TM.[a]

Components Added	Binding to F-actin[b]	Binding to F-actin–TM[b]
TN-C ($\pm Ca^{2+}$)	0.00	0.00
TN-I	0.58	0.95
TN-T[d]	0.15	0.60
CT ($+Ca^{2+}$)[c]	0.00	0.90
CI ($+Ca^{2+}$)[c]	0.00	0.00
CI ($-Ca^{2+}$)	0.00	0.95
TN ($\pm Ca^{2+}$)[e]	0.30 (0.1–0.4)	1.00

[a] Proteins were mixed in a solution containing 0.1 M KCl, unless otherwise noted, 2 mM $MgCl_2$, 10 mM imidazole (pH 7.0), and 0.1 mM EGTA ($-Ca^{2+}$) or 0.01 mM $CaCl_2$ ($+Ca^{2+}$) as indicated. Protein concentrations: F-actin, 0.99 mg/ml; TM, 0.24 mg/ml; TN-C, 0.06 mg/ml; TN-I, 0.08 mg/ml; CT, 0.19 mg/ml; CI, 0.14 mg/ml; and TN, 0.27 mg/ml. These mixtures, as well as controls that did not contain actin or TM, were centrifuged at 100,000*g* for 2 hr at 4°. For treatment of the supernatants for gel electrophoresis and the quantitative determination of the various components see Methods. [b] Binding expressed as mol/7 mol of actin. [c] The CT and CI complexes were made by mixing the purified components (1:1 molar ratio) in 6 M urea (see Materials), followed by dialysis against the solvent used in the experiment for the removal of urea. [d] 0.40 M KCl. [e] 0.15 M KCl.

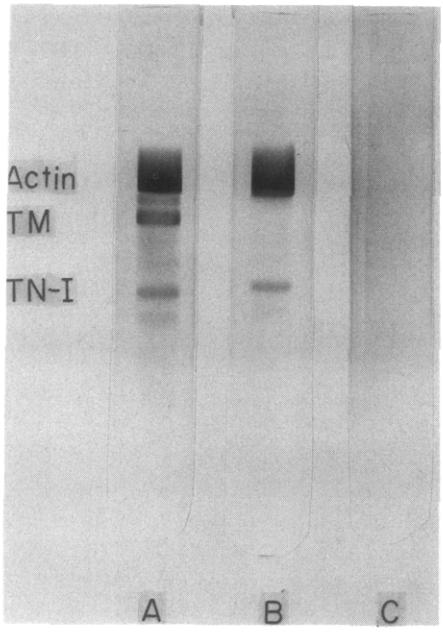

FIGURE 4: Interaction of TN-I with F-actin and F-actin–TM. Proteins were mixed in a solution containing 0.1 M KCl, 10 mM imidazole (pH 7.0), and 2 mM $MgCl_2$. (A) F-actin, TM, and TN-I; (B) F-actin and TN-I; (C) TN-I. Protein concentrations: F-actin, 0.99 mg/ml; TM, 0.24 mg/ml; and TN-I, 0.08 mg/ml. The samples were centrifuged and the pellets were subjected to gel electrophoresis as described in the legend to Figure 1.

(Figure 4C). One mole of TN-I was bound per TM in a complex containing actin and TM in a 7:1 molar ratio whereas only 0.6 mol were bound per 7 actins when no TM was present (Table I). At 0.2 M KCl no interaction was observed between TN-I and TM in the ultracentrifuge.

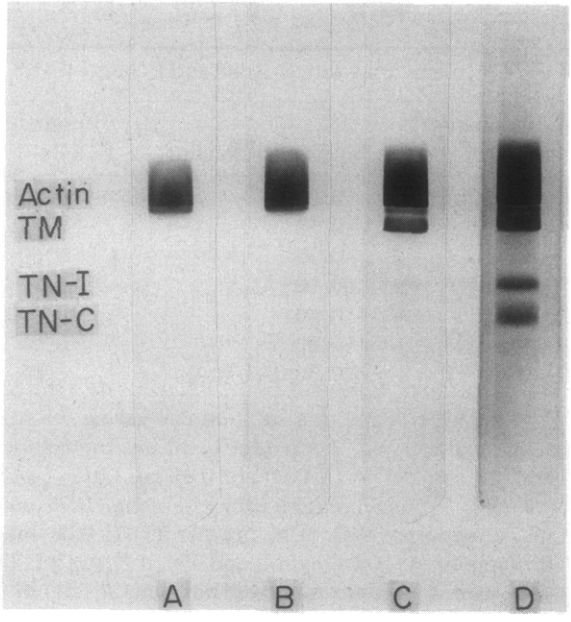

FIGURE 5: Effect of Ca^{2+} on the interaction of CI with F-actin and F-actin–TM. Proteins were mixed in a solution containing 0.1 M KCl, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and either 0.01 mM $CaCl_2$ ($+Ca^{2+}$) or 0.1 mM EGTA ($-Ca^{2+}$) as indicated. (A) F-actin, CI, $+Ca^{2+}$; (B) F-actin, CI, $-Ca^{2+}$; (C) F-actin, TM, CI, $+Ca^{2+}$; (D) F-actin, TM, CI, $-Ca^{2+}$. Protein concentrations: F-actin, 0.99 mg/ml; TM, 0.24 mg/ml; CI, 0.14 mg/ml. The samples were centrifuged and the pellets subjected to gel electrophoresis as described in the legend to Figure 1. The CI complex was prepared as described in the legend to Table I.

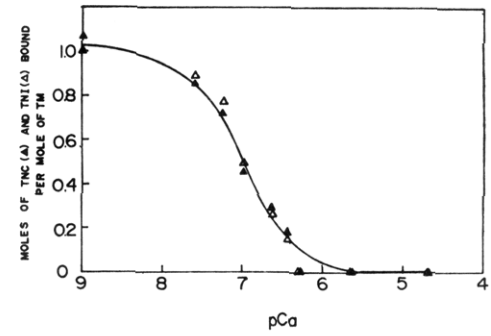

FIGURE 6: The effect of pCa on the binding of CI to F-actin–TM. Proteins were mixed in a solution containing 75 mM KCl, 50 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and 2 mM EGTA. $CaCl_2$ was added to achieve the pCa indicated on the abscissa; pCa was calculated with the use of a computer program (Perrin and Sayce, 1967) and with the following logarithmic association constants for metals and H^+ to EGTA appropriate for the ionic strength used: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68; H^+ to H_3EGTA^-, 2.0. Ca^{2+} to $EGTA^{4-}$, 11.0; Ca^{2+} to $HEGTA^{3-}$, 5.33; Mg^{2+} to $EGTA^{4-}$, 5.21; Mg^{2+} to $HEGTA^{3-}$, 3.37 (Sillén and Martell, 1964). The ordinate shows the moles of TN-C or TN-I bound per mole of TM. Protein concentrations: F-actin, 0.81 mg/ml; TM, 0.19 mg/ml; and CI, 0.12 mg/ml. The molar ratio of actin to TM was 7:1. The samples were centrifuged and the pellets were subjected to electrophoresis as described in the legend to Figure 1. For quantitative determination of proteins in the gels, see Methods. The CI complex was prepared as described in the legend to Table I.

Interaction of CI and CT Complex with F-actin, TM, and F-actin–TM. Of all the interactions studied the only one influenced by Ca^{2+} is the binding of the CI complex to F-actin–TM (Figure 5C,D). In the presence of 0.01 mM Ca^{2+} (Figure 5C) very little binding of CI takes place, whereas in the absence of Ca^{2+} (Figure 5D), CI is bound to F-actin–TM. Approximately 1 mol of CI bound per TM in a complex containing actin and TM in a 7:1 molar ratio in the absence of Ca^{2+}; no detectable binding was observed in the presence of Ca^{2+} (Table I). The binding of CI to F-actin–TM was studied over a wide range of pCa values (Figure 6) and binding occurred between pCa 6–8 with $K_{diss} = 10^{-7}$ M.

CI does not bind to F-actin alone regardless of whether or not Ca^{2+} is present (Figure 5A,B; Table I). Similarly, CI does not bind to TM, as shown by studies in the analytical ultracentrifuge (Figure 7). Equimolar mixtures of TM and CI were sedimented in the presence and absence of Ca^{2+}, and in both cases a leading shoulder was observed. The sedimentation rate of this shoulder corresponded to that of the CI complex, and the unchanged position of the TM peak in the presence of CI also suggested that no interaction took place between TM and CI. Thus CI only interacts with actin when TM is present and in the absence of Ca^{2+}.

The interaction of the CT complex with F-actin, TM, and F-actin–TM could only be studied in the presence of Ca^{2+} since in its absence TN-T is not solubilized by TN-C (Ebashi *et al.*, 1972). The soluble CT complex which exists at 0.1 M KCl in the presence of Ca^{2+} behaves similarly to TN-T and binds only to F-actin–TM (Figure 3C,D) and TM (Figure 8). About 1 mol of CT was bound per TM in a complex containing actin and TM in a 7:1 molar ratio (Table I) and no binding to F-actin alone was observed.

Discussion

The results of this study give some insights into the interaction among the proteins of the thin filaments and may con-

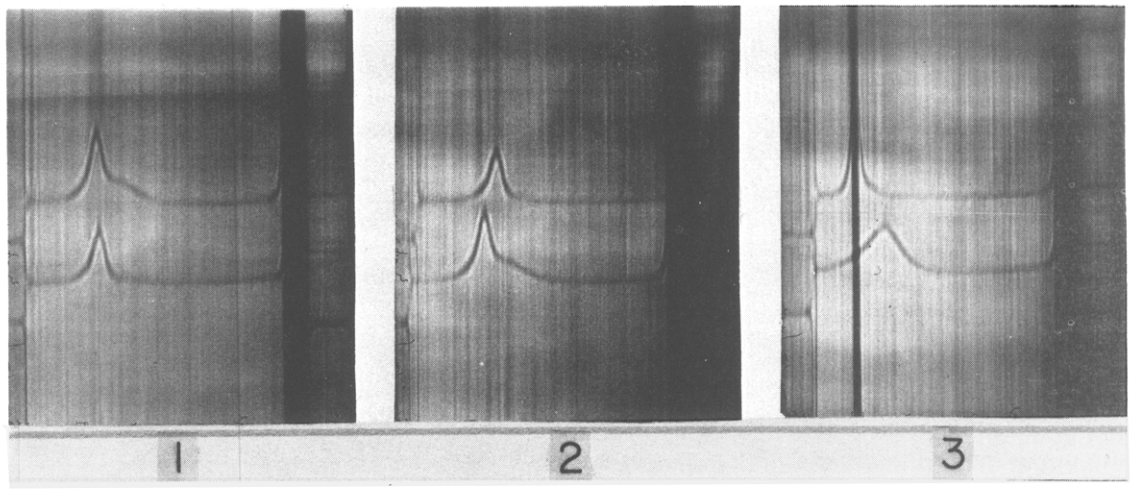

FIGURE 7: Ultracentrifugal analysis of the interaction of CI with TM. The run was made at 20°, rotor speed of 52,640 rpm and bar angle of 65°, sedimentation is from left to right. The solvent contained 0.1 *m* KCl, 10 mM imidazole (pH 7.0), and 2 mM $MgCl_2$. Other additions as indicated below. Frame 1: 96 min at full speed. Top, 4.15 mg of TM and 2.5 mg of CI per ml, and 0.01 mM $CaCl_2$; bottom, 4.15 mg of TM per ml and 0.01 mM $CaCl_2$. Frame 2: 96 min at full speed. Top, 4.15 mg of TM per ml and 0.1 mM EGTA; bottom, 4.15 mg of TM and 2.5 mg of CI per ml and 0.1 mM EGTA. Frame 3: 64 min at full speed. Top, 7.8 mg of TM/ml; bottom, 5.3 mg of CI/ml. The CI complex was prepared as described in the legend to Table I.

tribute to an understanding of the mechanism by which Ca^{2+} regulates contraction and relaxation. We shall try to fit our data into the model that assumes two positions for tropomyosin on the actin filaments corresponding to contraction and relaxation. One key question in any scheme for the control of actin–myosin interaction by Ca^{2+} binding to the Ca–receptor of troponin (TN-C) is the mode of attachment of TN to tropomyosin and actin.

TN probably binds to TM through TN-T since previous studies (Greaser *et al.*, 1972) have shown that TN-T, but not TN-I or TN-C, interacts with TM. The results presented in this paper show that TN-C and CI, with or without Ca^{2+}, and TN-I do not bind to TM whereas TN-T and CT do. They strongly suggest that the binding of TN to TM takes place *via* its TN-T moiety. This conclusion is further supported by the fact that TN-T and CT, like TN itself, do not bind to F-actin but, again like TN, do bind to F-actin–TM.

The existence of a second site involving the TN-C and TN-I subunits at which TN is attached to the thin filament, but only in the absence of Ca^{2+}, was suggested on the basis of experiments with TN-T-deficient, mersalyl-treated TN (Hitchcock and Szent-Györgyi, 1973). They showed that this preparation bound to F-actin–TM in the absence of Ca^{2+} but not in its presence and that it did not bind to TM. Essentially the same results have recently been reported with reconstituted CI and have been incorporated into a "two-site" model for troponin binding (Hitchcock *et al.*, 1973). Our present results show that CI does not bind to either F-actin or TM alone regardless of whether Ca^{2+} is present and that the range of pCa in which CI dissociates from F-actin–TM is the same as that of Ca^{2+} binding to the CI complex (Potter *et al.*, 1974). According to our data the complex of actin–TM–TN-I–TN-C contains these four proteins in a 7:1:1:1 ratio; this ratio is derived from densitometry of Fast Green stained gels and with the use of calibration factors obtained with purified components (Potter, 1974). The recently published data of Hitchcock *et al.* (1973) indicate a 4.2:1:1:0.5 ratio, but the authors assumed the same protein staining intensity values for each component.

In a recent paper Van Eerd and Kawasaki (1973) also reported on their studies relating to the binding of TN components to TM. Changes in the fluorescence of a dye bound to TM indicated that all the TN subunits and their various combinations bind to TM. However, in view of the fact that there were some discrepancies between fluorescence and gel electrophoresis experiments, the authors regarded the interaction of the TN-C and CI with TM as "nonspecific."

The CI complex probably binds *via* its TN-I moiety since TN-C does not bind to F-actin, to TM, or to F-actin–TM in either the presence or absence of Ca^{2+}, but TN-I binds to both

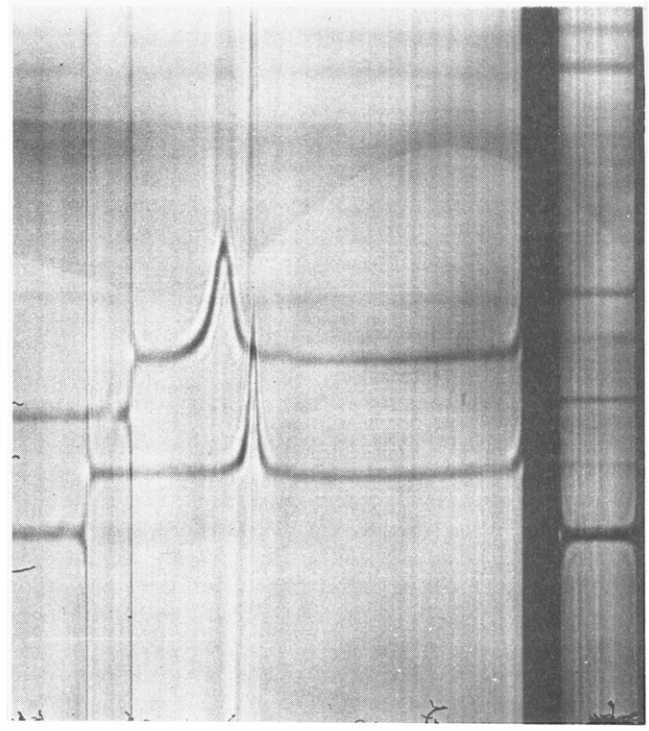

FIGURE 8: Ultracentrifugal analysis of the interaction of CT with TM. The run was made at 20°, rotor speed of 52,640 rpm and bar angle of 65°, sedimentation is from left to right. The solvent contained 0.1 M KCl, 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, and 0.01 mM $CaCl_2$. Photograph taken after 64 min at full speed. Top, 4.9 mg of TM/ml; bottom, 1.94 mg of TM and 1.76 mg of CT/ml. The CT complex was prepared as described in the legend to Table I.

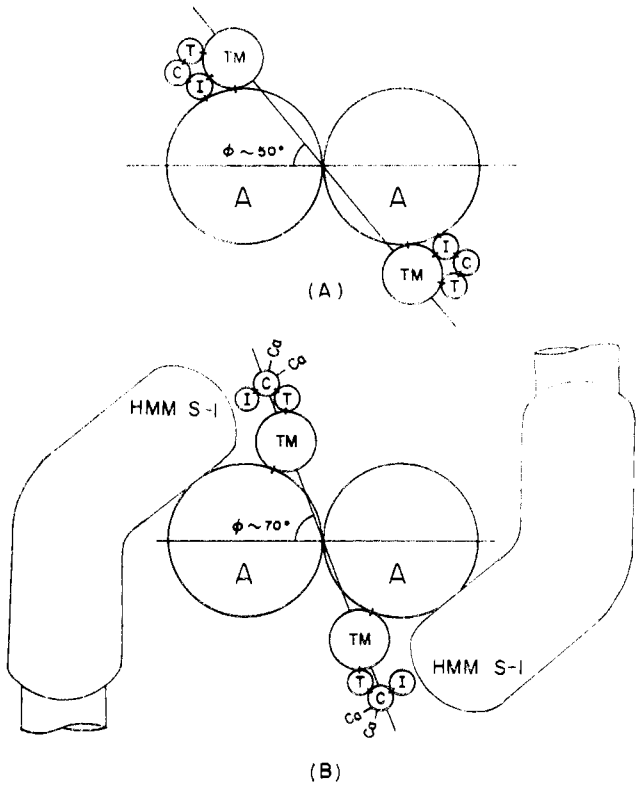

FIGURE 9: Scheme of regulation of muscle contraction by TN and Ca^{2+}. The relative positions of actin, tropomyosin and the head of the myosin molecule (HMM S-1) in the model are essentially as proposed by Spudich *et al.* (1972), Haselgrove (1972), Huxley (1972), and Parry and Squire (1973). Key: A, actin,; TM, tropomyosin; T, TN-T; I, TN-I; C, TN-C; A, relaxation in the absence of Ca^{2+}; B, activation, $[Ca^{2+}] \sim 1$ μM. Suggested interactions between proteins are indicated by a short connecting line.

F-actin and F-actin–TM. The results concerning the binding of TN-I (Table I) are consistent with the inhibition of actin–TM–myosin ATPase by TN-I (Perry *et al.*, 1972) at a TN-I:TM ratio of one. The binding of TN-I to F-actin alone, which occurs to a lesser extent than to F-actin–TM, may be related to the inhibition of actomyosin ATPase in the absence of TM. The latter requires a ratio of TN-I to actin that approaches 1, and it is not clear how this process is related to the inhibition of ATPase and presumably to the actin–myosin interaction occurring in the presence of TM. Since the binding of TN-I to F-actin–TM seems stronger than that to F-actin alone and since CI only binds to F-actin–TM, we propose that TN-I binds to either a site made up jointly of actin and TM or to actin whose conformation has been altered by its interaction with TM.

Thus it seems most likely that *in vivo* the troponin complex binds to the actin–tropomyosin filament in a two pronged fashion, TN-T being attached to tropomyosin and TN-I attached to a site made up jointly of actin and tropomyosin. If the view concerning the two-pronged attachment of troponin to the actin–tropomyosin filament is correct, any scheme that seeks to explain the regulatory role of troponin entirely in terms of troponin–TM interactions must be incomplete (*cf.* Van Eerd and Kawasaki, 1973). Since TN-T does not bind to TN-I (Van Eerd and Kawasaki, 1973), TN-T is probably linked to TN-I *via* TN-C. There is ample evidence for the interaction between TN-C and TN-T and between TN-C and TN-I. TN-C, which is soluble at low ionic strength (<0.1 M), is known to solubilize TN-T and TN-I, which are both rather insoluble at the same ionic strength (Greaser and Gergely, 1973). The interaction of TN-C with TN-T attached to TM has also been shown in work on TM paracrystals (Margossian and Cohen, 1973). The recent results of Potter *et al.* (1974) show that the CI complex is completely soluble in the presence and absence of Ca^{2+} and that the affinity of Ca^{2+} for isolated TN-C can be increased to that of native TN on formation of the CI complex. TN-I also alters the mobility of a spin label attached to TN-C and the effect is maximal when the molar ratio of TN-I:TN-C is one. These results strongly suggest an interaction between TN-I and TN-C in the native complex. Recently Ebashi *et al.* (1972) have shown that solubilization of TN-T by TN-C requires the presence of Ca^{2+}, as was also found in the present study. The requirement for Ca^{2+} in the interaction of TN-C and TN-T also emerges from studies on TN-T-containing TM paracrystals (Margossian and Cohen, 1973). The fact that TN-T does not dissociate from the native TN complex in the absence of Ca^{2+} may mean that the conformation of the isolated subunits of TN is slightly different from that in the native complex, and in the latter TN-I stabilizes the interaction of TN-C and TN-T in the absence of Ca^{2+}.

Mechanism of Regulation. The data discussed above can be incorporated into the current model of myosin–actin tropomyosin interaction that involves the blocking of the combination of the myosin head (HMM S-1) with actin when tropomyosin is in one position but not in the other (Figure 9). It should be noted that the myosin–actin interaction is blocked only in the presence of ATP, when S-1 presumably forms a product complex (Taylor, 1973), but not with myosin or HMM–S-1 alone (rigor) (see, *e.g.*, Huxley, 1972).

In the presence or absence of Ca^{2+}, the TN complex would always be anchored to TM *via* TN-T (Figure 9A,B). In the absence of Ca^{2+}, corresponding to the relaxed state, TN-I would be bound to a site on actin and possibly also to a site on TM; this would lock TM in the "blocking" position ($\phi = 50°$, Figure 9A) preventing the interaction of myosin with the seven actins within each TM domain. Upon activation, *i.e.*, release of Ca^{2+} by the sarcoplasmic reticulum and combination of Ca^{2+} with TN-C, the links between TN-I and actin–TM would be broken and TM would move to the nonblocking position ($\phi = 70°$, Figure 9B) allowing myosin and actin to interact. It should be noted that the position of TN facing away from the groove is not essential; it could equally face toward the groove.

There are two obvious ways in which the movement of TM may take place depending on whether the thermodynamically favored position of TM, when there is no TN present, is (i) in the blocking position ($\phi = 50°$, Figure 9A) or (ii) the nonblocking position ($\phi = 70°$, Figure 9B). Case i is more plausible since X-ray evidence shows that in molluscan muscles which do not contain TN, TM is located at $\phi = 50°$ during relaxation (Parry and Squire, 1973). In case i the shift of TM toward the groove produced by Ca^{2+} would not only have to involve release of TN-I from a site made up jointly of actin and TM but also the induction of a conformational change in TM to account for the new equilibrium position. In case ii the effect of Ca^{2+} need only involve the release of TN-I as above, since *ex hypothesi*, the movement of TM would be spontaneous and would not require a change in conformation of TM. We assume, as discussed by Vibert *et al.* (1972), that Ca^{2+} causes the change in the position of tropomyosin without the involvement of myosin heads since stimulation of muscle causes a change in the thin filament X-ray diffraction pattern even at sarcomere lengths where there is no overlap. Removal of Ca^{2+} would restore TM to the

"blocking" position in case i by reversing the postulated conformational change in TM, or in case ii merely by stabilization of the interaction of TN-I with A-TM in the $\phi = 50°$ position.

This model would also explain the Ca^{2+}-insensitive inhibition of actomyosin ATPase by TN-I. In the absence of TN-T and TN-C, TN-I would inhibit actomyosin ATPase by forming a link between TM and actin (see Figure 9A) locking TM in the "blocking" position. Since TN-I does not bind Ca^{2+}, the inhibition would be Ca^{2+} insensitive. The fact that TN-I inhibits actomyosin ATPase in the presence of tropomyosin at a TM:TN-I ratio of 1 implies, in terms of the model depicted in Figure 9, that the stabilization of tropomyosin in the blocking position is not offset by the interaction of S-1 and actin. In the presence of TN-C the binding of TN-I to A–TM must be altered since the combination of myosin with actin, presumably with the accompanying displacement of TM, can then take place even in the absence of Ca^{2+}, as shown by the reversal by TN-C of the TN-I inhibition of actomyosin–ATPase (Greaser and Gergely, 1971).

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