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Troponin

I. Preparation and Physiological Function*

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1. A method for isolation of troponin from native tropomyosin was described.

2. Troponin in combination with tropomyosin restored the whole activity of native tropomyosin in sensitizing the interaction of myosin and actin to Ca ion.

3. Troponin was found to bind nearly 4 moles of Ca per 10^5 g, of which most were exchangeable. The result of the experiment to determine the binding constant of these Ca binding sites was explained by assuming that half of the binding sites possessed a binding constant of 1.3×10^6 M^{-1} and the remaining half 5×10^4 M^{-1}.

4. The amount of exchangeable Ca in the contractile system was mainly accounted for by the Ca-binding capacity of troponin, which was not influenced by other contractile proteins or ATP.

5. Cardiac troponin showed a much higher affinity for Sr ion than skeletal troponin. The ratio of the former affinity to the latter was in good agreement with the ratio of the sensitivity to Sr ion of a reconstituted contractile system containing cardiac troponin to that containing skeletal troponin. Based on these findings and the results described above, it was concluded that the sensitivity of a contractile system to Ca ion is solely dependent upon the affinity for Ca ion of the troponin molecule present.

6. The mechanism of troponin regulation of the interaction of actin and myosin was discussed.

Native tropomyosin, which sensitizes the interaction of myosin and actin to Ca ion (*1*, *2*), has been shown to consist of two separate proteins (*3*, *4*), one tropomyosin of the Bailey type (*5*) and the other a new structural protein, named troponin. The latter protein alone cannot sensitize the interaction but requires the collaboration of tropomyosin. Further evidence had indicated that troponin is the Ca-receptive protein of the contractile system (*6*, *7*), *i.e.*, the contraction-triggering role of Ca ion is effected *via* troponin.

The purpose of this article is to describe procedures for the preparation of troponin and to make clear some of its properties with special reference to its physiological function. Preliminary accounts of this report have been

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The following abbreviations were used;
GEDTA: Glycoletherdiaminetetraacetic acid (EGTA),
PCMB: *p*-Chloromercuribenzoate,
NEM: N-Ethylmaleimide.

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presented previously (*6*, *7*).

METHODS

Preparation of Troponin—Any preparations of native tropomyosin may be used as the starting material for the preparation of troponin. To obtain a preparation of excellent quality in high yield, however, it is preferable to start from a fresh preparation at high concentrations, *viz.*, 20—30 mg/ml.

Most preparations used in the present experiments were made as follows: The early part of the method for preparing native tropomyosin —up to the point of ammonium sulfate fractionation— was essentially the same as that described in the previous paper on "preparation of active component without acetone treatment" (*2*). To the supernatant of the fraction, containing 1.6 M ammonium sulfate, was further added ammonium sulfate up to 2.6 M (approximately 12 g ammonium sulfate per 100 ml of the supernatant). The resulting precipitate was collected by centrifugation and suspended in a minimum volume of water. The suspension was then dialyzed first against 0.3 mM $NaHCO_3$ for several hours and then against 0.4 M LiCl overnight. The dialyzed solution, whose protein concentration should be more than 20 mg/ml, was brought to pH 4.5 with 1 N HCl at 0°—4°C and centrifuged. The resulting precipitate was thoroughly suspended in 0.6 M LiCl to half of the original solution volume and centrifuged. Both supernatants were combined and their pH was adjusted back to 7.0. To the solution was added solid ammonium sulfate to a final concentration of 3.0 M and the precipitate formed was centrifuged. The resulting sediment was dissolved in a small volume of water and dialyzed against 10 mM LiCl containing 0.3 mM $NaHCO_3$. After about 10 hr' dialysis, the solution was centrifuged to remove some flocculated material and the clear supernatant, usually containing 20—50 mg protein/ml, was used as the crude troponin preparation.

At this stage the troponin preparations were occasionally already fairly homogeneous according to examination by discelectrophoresis and did not require further purification. However, the usual preparations were more or less contaminated by tropomyosin. The latter protein could be removed by repeating the isoelectric precipitation, *i.e.*, after adding LiCl up to 0.4 M to the crude troponin solution, the pH was adjusted to 4.5 and the resulting precipitate, a complex of tropomyosin and somewhat denatured troponin, was eliminated by centrifugation. Alternatively the following procedures could even more effectively remove tropomyosin from the solution: Urea was added to the crude troponin solution up to 2 M and the pH was adjusted to 5.35, the resulting sediment being removed by centrifugation. The supernatant, usually practically free of tropomyosin, was neutralized and then dialyzed against 10 mM LiCl (or NaCl) containing 0.3 mM $NaHCO_3$.

In the early stage of the studies on troponin, 1 M KCl was used in place of 0.4 M LiCl (*3*). Generally speaking, the KCl preparations resulted in a much less yield, sometimes less than half, but showed the same physiological activity as that of LiCl preparations. The use of 0.6 M NaCl gave an intermediate yield. Troponin could be maintained in the frozen state without appreciable loss of activity.

Gel filtration technique was also useful for the purification of troponin. One of the examples will be described in a later paper (*8*).

Cardiac troponin was prepared from native tropomyosin of bovine heart essentially in the same manner as was the case with skeletal troponin.

Preparation of Tropomyosin—Tropomyosin was prepared from the residue of acetone powder from which Straub-type actin had been extracted at 0°C, or from the residue of native tropomyosin from which troponin had been separated. Further procedures of tropomyosin preparation essentially followed the original method of BAILEY (*5*), except that the acid precipitation at pH 4.5 was always performed in 1 M KCl solution.

One of the crucial points of the method for the preparation of a pure sample was the concentration of ammonium sulfate. The crude tropomyosin preparation dialyzed against water was adjusted to about 5 mg/ml. To 100 ml of this solution was added 32 g ammonium sulfate *plus* 25 mg Na_2CO_3 and the resulting precipitate was removed by centrifugation. To the supernatant was added 2 g of ammonium sulfate and left for 15 min. After centrifugation, further 2 g of ammonium sulfate was added to the resulting supernatant, and the solution was left for 1 hr. The resulting precipitate was collected by centrifugation at 11,000×*g* for 20 min and 1 ml of water was added to 5 g of precipitate with thorough mixing. The resulting suspension was centrifuged at 11,000×*g* for 30 min and the supernatant was dialyzed against water. If necessary, the above procedures were repeated.

Preparation of Myosin and Myosin B—They were prepared by the procedures described in the previous paper (*2*).

Preparation of Actin—The water-extract of acetone powder of exhaustively washed muscle residue was subjected twice to MOMMAERTS' purification procedure (*9*) with a slight modification and the resulting actin preparations were used.

Preparation of Native Tropomyosin-free Myosin B—PERRY *et al.* (*10*) have developed an excellent method for eliminating native tropomyosin from myosin B.

The original method was followed with a slight modification: Myosin B was repeatedly washed using a large volume of cold 2 mM $NaHCO_3$. Usually five washes were sufficient to eliminate most of native tropomyosin (see Fig. 6).

Determination of Ca-Binding—Unless otherwise stated, all the experiments to be described below were performed in solutions containing 0.05—0.1 M KCl, 4 mM $MgCl_2$ and 0.02 M Tris-maleate buffer (pH 6.8). In the case of those proteins which could be centrifuged down, *e.g.*, F-actin and myosin in low ionic strength media, the protein solution with 0.05 mM GEDTA and a specified concentration of Ca ion containing Ca^{45} was centrifuged down* and the radioactivities of both the sediment and supernatant were determined. From these data the free Ca ion concentration in the supernatant and the amount of Ca bound to the sediment were calculated under the assumption that the binding constant of GEDTA at pH 6.8 in the Tris-maleate buffer would be $5\times10^5\,M^{-1}$ (*11*), and that the amount of contaminating Ca ions derived from the ingredients except for that from the protein would be 5 mμmoles per ml. The reciprocal value of the concentration of Ca ion which gave the half of the maximum Ca binding capacity was considered as the binding constant of the protein.

In the case of troponin, tropomyosin and their complex, which could not be centrifuged down, the chelating resin method (*12*) was used. Chelex-100 (200—400 mesh) was washed first with 1 mM GEDTA to remove Ca ion and then with water to remove GEDTA. Then the resin was thoroughly equilibrated with the above experimental medium and was dried on a filter paper for several days. A specified amount of the resin thus treated, usually 10 mg, was suspended in 1 ml of the protein solution, and the suspension was mixed for one minute. Then the resin was centrifuged down under low gravity and the upper part of the supernatant was taken for the determination of radioactivity. A part of protein was found to combine with the resin, so that the concentration of the protein in the supernatant was again determined.**

Essentially the same procedures were applied to the determination of Sr bound to troponin using Sr^{90}, except that 0.05 mM GEDTA was always added to the reaction mixture to eliminate contaminating Ca ion from the resin. The binding constant of GEDTA for Sr ion was considered to be 1/800 of that for Ca ion.

As it is expected from a simple calculation,*** the ratio of the amount of Ca in the solution to that bound to the resin is fairly constant within a certain range of Ca concentration, if there is no Ca-binding substance in the supernatant. Therefore, if we know the total amount of Ca, we can easily calculate the free Ca ion concentration in the solution. In the presence of some Ca-binding substance, the radioactivity in the supernatant increases, whearas the ratio of the radioactivity due to free Ca ion in the supernatant to that bound to the resin should remain constant. Then the amount of radioactivity bound to the protein and, therefore, the amount of Ca bound to the protein could be estimated. If we know the maximum binding capacity of the protein for Ca ion, the binding constant of the protein for this ion can also be calculated assuming that each binding site has the same binding constant. For this method it is important to know the amount of contamination by Ca of the whole system including the ingredients of the solution and the chelex resin. This was estimated from experiments using appropriate

* Using a small tube of a high performance index, F-actin could be centrifuged down at $100{,}000\times g$ for 2 hr. In the case of myosin in a low ionic strength, *e.g.*, 0.1, one hour at $100{,}000\times g$ was enough even in the presence of ATP.

** In the case of troponin and tropomyosin, a minor part, less than 20%, was bound to the resin, but a large part of F-actin was shown to co-precipitate with the resin.

*** It is assumed that the resin suspension may behave as a solution of a water-soluble chelating agent. Then we have

$$[\mathrm{Ca\ X}]=K_{Ca}\,[\mathrm{Ca}]'\,[\mathrm{X}]'$$
$$[\mathrm{Mg\ X}]=K_{Mg}\,[\mathrm{Mg}]'\,[\mathrm{X}]'$$

where K_{Ca} and K_{Mg} are the apparent binding constants for Ca and Mg respectively; [Ca]′ and [Mg]′ are the total concentrations of respective substances unbound to the resin in the absence of complex-forming materials in the solution except for the chelating resin, and [X]′ is the concentration of resin free of these divalent cations. [Ca]′ and [Mg]′ are practically equal to $[Ca^{++}]$ and $[Mg^{++}]$, so that

$$\frac{[Ca^{++}]}{[\mathrm{Ca\ X}]}=\frac{[K_{Mg}]\,[Mg^{++}]}{[K_{Ca}]\,[\mathrm{Mg\ X}]}$$

Since K_{Mg} is about $1\times10^3\,M^{-1}$ and the resin is equilibrated with 4×10^{-3} M Mg, [Mg X] is several times higher than [X]′. The total Ca concentration is less than 3×10^{-5} M, so that [X]′ is several times higher than [Ca X]. Therefore,

$$\frac{[\mathrm{Mg\ X}]}{[\mathrm{X}]_{tot}}=\frac{[\mathrm{Mg\ X}]}{[\mathrm{Mg\ X}]+[\mathrm{X}]'+[\mathrm{Ca\ X}]}\fallingdotseq\frac{[\mathrm{Mg\ X}]}{[\mathrm{Mg\ X}]+[\mathrm{X}]'}$$
$$=\frac{K_{Mg}\,[Mg^{++}]}{1+K_{Mg}\,[Mg^{++}]}=\text{constant}$$

Thus the ratio, $[Ca^{++}]$/Ca X], should be practically constant so far as the concentration of total Ca ion is lower than a certain level.

amounts of GEDTA and was shown to be 6 to 10 mμmoles per 10 mg resin.

Determination of total Ca bound to proteins was made by the colorimetric method of Yanagisawa following the procedures described in the previous paper (*13*). To determine the non-exchangeable fraction bound to protein, the protein solution was passed through a chelex-100 column and the Ca remaining in the effluent was determined. In the case of troponin, a considerable part of the exchangeable Ca remained in the effluent after passage through the chelating resin column, due to the strong affinity of the protein for Ca ion unless the resin had been carefully washed with GEDTA beforehand. To avoid erroneous conclusions, therefore, troponin was first equilibrated with Ca^{45} and then passed through the column. The determination of bond Ca was considered reasonable if the remaining fraction of radioactivity was less than 10% of the total.

Determination of Sulfhydryl Content of Protein—Measurements were made according to the method of BOYER (*14*).

For Other Methods and Materials—All other procedures and materials employed were those described in the previous paper (*2*).

RESULTS

Reconstitution of "Native Tropomyosin" from Troponin and Tropomyosin—Troponin prepared by the procedures described in the "METHODS" section was usually found to be homogeneous according to electrophoretic and ultracentrifugal* examinations. Its amino acid composition was different from any of those of the known structural proteins of muscle (Table I). The difference in amino acid composition between native tropomyosin and the Bailey-type tropomyosin, particularly in their proline content, can be explained by the presence of troponin in native tropomyosin. In view of these data, the ratio of troponin to tropomyosin in native tropomyosin by weight appears to be around 0.6.**

TABLE I

Amino acid composition of troponin in comparison with those of tropomyosin and native tropomyosin.

	Troponin (a)	Tropomyosin (b)	Native tropomyosin	$\frac{0.6\times(a)+(b)}{1.6}$
Asp	83	89	83	85
Thr	22	26	20	24
Ser	31	40	34	37
Glu	159	212	197	192
Pro	26	2	11	11
Gly	43	11	23	23
Ala	74	108	92	95
Val	37	27	31	32
Met	27	16	23	20
Ileu	33	29	36	31
Leu	65	95	82	84
Tyr	12	15	15	14
Phe	23	4	12	11
Lys	100	113	106	108
His	17	5	10	10
Arg	66	41	50	50

It has previously been shown (*cf.* (*7*) and (*15*)) that troponin with tropomyosin can exert a Ca-sensitizing action on native tropomyosin-free actomyosin preparations as illustrated in Fig. 1. This has been confirmed by several workers (*16—18*). More quantitative data are presented in Fig. 2. The sensitizing activity of the complex of troponin with tropomyosin was not less than 90 per cent of that of native tropomyosin. Thus, combination of troponin with tropomyosin restores the original activity of native tropomyosin almost completely.

* It was found that the physico-chemical properties of troponin including its sedimentation diagram were greatly influenced by the concentration of free Ca ion in the medium (T. Wakabayashi, personal communication). This may explain considerable differences among the values of sedimentation coefficients of troponin so far reported ($s_{20,w}$ of troponin at 4—5 mg/ml is usually 3.5—3.6, but quite different values such as 4.0 or 6.7 (*15*) have been reported). In view of this, studies on the physico-chemical properties of troponin are being re-investigated under precise control of Ca ion concentration; the results obtained will be reported later.

** We initially reported this ratio to be around 0.33 (*2*) and later to be 0.5 (*15*). Thus the ratio appeared to have increased with the progress of our study, probably reflecting an improvement of extraction technique. It now seems to have reached a plateau value, *viz.*, 0.6—0.65. In any case, the amino acid composition of native tropomyosin could be expressed by the linear combination of those of troponin and tropomyosin.

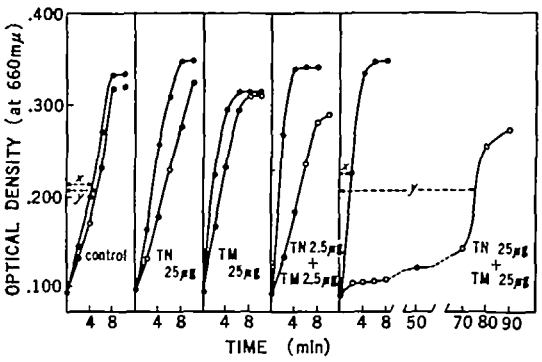

FIG. 1. Effects of troponin, tropomyosin, and their complex on the superprecipitation of native tropomyosin-free myosin B.

Reaction mixtures contained in final concentrations: 0.01 M KCl, 4 mM $MgCl_2$, 0.02 M Tris-maleate buffer (pH 6.8), 0.5 mM ATP, 0.7 mg/ml native tropomyosin-free myosin B, either 0.01 mM $CaCl_2$ or 0.1 mM GEDTA, and specified concentrations of troponin in μg/ml (designated as TN in this figure), tropomyosin (designated as TM in this figure) or their mixture. Temperature, 18°C.

—○— GEDTA; estimated free Ca ion concentration, 1×10^{-7} M.

—●— $CaCl_2$; estimated free Ca ion concentration, 1.3×10^{-5} M.

Concerning *x* and *y* see the legends for Figs. 2 and 3.

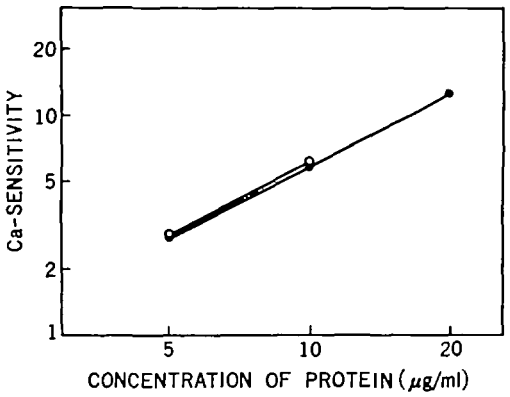

FIG. 2. Comparison of Ca-sensitizing activity of native tropomyosin and of a complex of troponin-tropomyosin.

The sensitivity to Ca ion was determined by the method illustrated in Fig. 1. The ordinate indicates the ratio between the times required for the increase in the turbidity of the reaction mixture to reach the half maximum values after addition of ATP in the presence of low (1×10^{-7} M) and high concentrations (1.3×10^{-5} M) of Ca ion, *viz.*, the ratio of "*y*" to "*x*" shown in Fig. 1. The abscissa indicates the concentration of native tropomyosin or of the troponin-tropomyosin complex added to actomyosin.

—○— Native tropomyosin.

—●— Troponin-tropomyosin complex.

The ratio of troponin to tropomyosin in this complex was 2 to 3. Other experimental conditions were essentially the same as those described for Fig. 1 except that 0.65 mg/ml native tropomyosin-free myosin B was used and the reactions were performed at 20°C.

The data showing the effect of varying the amounts and ratios of the complex of troponin and tropomyosin on native tropomyosin-free myosin B and synthetic actomyosin are demonstrated in Figs. 3 and 4, respectively. While the sensitivity of native tropomyosin-free myosin B to Ca ions was completely restored to its original level by adding appropriate amounts of the troponin-tropomyosin complex, synthetic actomyosin was much less sensitized even by addition of much greater amounts of the complex. A similar observation was previously made in the case of native tropomyosin applied to synthetic actomyosin and trypsin [EC 3.4.4.4]-treated myosin B (*cf.* Figs. 5 and 11 in reference (*2*)). The reason for incomplete restoration of the sensitivity of synthetic actomyosin is not yet clear.

The sensitizing effect of troponin in the presence of tropomyosin on native tropomyosin-free myosin B increased fairly linearly with the increase in tropomyosin concentration up to 60 to 70 per cent of the concentration of troponin and then tended to reach a plateau (Fig. 3). Thus the ratio of troponin to tropomyosin which gave the maximum effect was around 1.5.

It was rather difficult to estimate this ratio in the case of synthetic actomyosin (Fig. 4), since relatively greater experimental errors were involved in this experiment, but as a whole the ratio seemed to be not less than unity. Thus the ratio of troponin to tropomyosin which appeared to be the most favorable for the sensitization of the actomyosin system seems to be higher than the ratio of

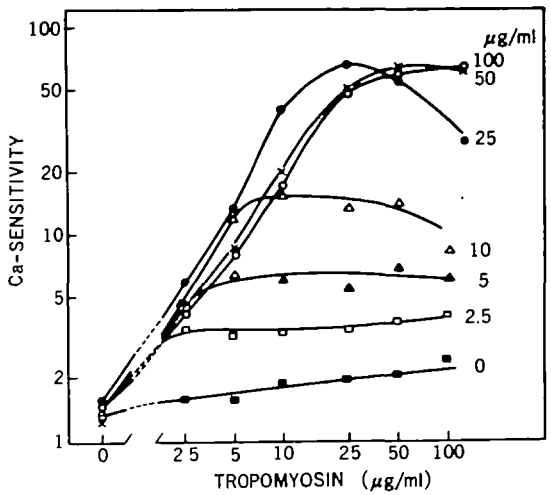

FIG. 3. Effect of varying concentrations of troponin and tropomyosin on Ca-sensitivity of native tropomyosin-free myosin B.

Numerals given to the right of the curves indicate the corresponding concentrations of troponin. Experimental conditions were essentially the same as those described for Fig. 1. As for other details, see the legend for Fig. 2.

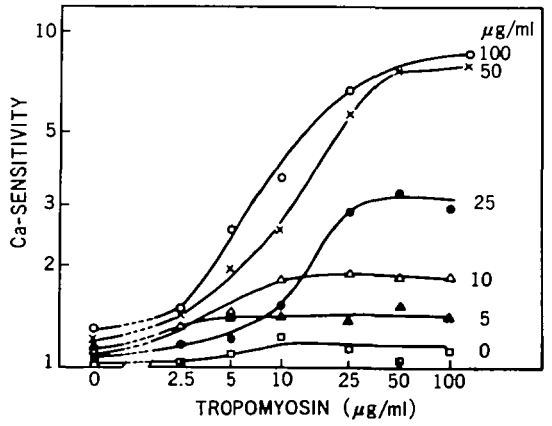

FIG. 4. Effect of varying concentrations of troponin and tropomyosin on the Ca-sensitivity of synthetic actomyosin.

Experimental conditions were essentially the same as those in Fig. 3 except that synthetic actomyosin, 0.7 mg/ml (myosin : actin, 3 : 1), was used in place of native tropomyosin-free myosin B.

about 0.6 obtained with native tropomyosin. Whether or not this discrepancy implies some physiological significance has not yet been clarified.

The maximum sensitization of 0.7 mg/ml of native tropomyosin-free myosin B was obtained at 15 μg/ml of tropomyosin (Fig. 3) and that of 0.6 mg/ml of synthetic actomyosin at 40 μg/ml (Fig. 4). Since the ratio of tropomyosin to actomyosin in myofibrils is estimated to be around 0.05, the above values are not far from those expected from physiological considerations.

Troponin as the Primary Protein in Sensitizing Actomyosin to Ca ion—It is now clear that the elementary process of physiological contraction is under the control of Ca ion and dependent on the interaction of at least four kinds of protein, *i.e.*, actin, myosin, tropomyosin and troponin. The next step of our investigation was to identify the particular protein among the four functioning as the receptor of contraction-triggering action of Ca ion. As will be described in the next section, troponin has been shown to possess a unique Ca-binding capacity. However, this fact alone cannot be taken as definitive evidence for the Ca-receptive role of troponin. Since contraction is based on the dynamic interaction of the above proteins in the presence of ATP, conclusive evidence should be derived from investigations dealing with the interaction itself. This was achieved by an experiment which was intended to clarify the mechanisms of pharmacological actions of alkaline-earth metal ions on cardiac and smooth muscles (*6*).

It is well known that Sr ion can replace Ca ion in most of the biological functions of the latter. This is also true for the regulatory effect of Ca ion on contractile systems. It was found, however, that the sensitivity of cardiac myosin B to Sr ion was quantitatively several-fold greater than that of skeletal muscle (Table II). This finding provided a key avenue of approach to the elucidation of the problem as to which protein was primarily responsible as the Ca-receptor.

Tabel III gives a detailed description of the previous preliminary report (*6*). It is clear that the response of reconstituted systems to Sr ion can be divided into two distinct groups, cardiac and skeletal types with no intermediate type being demonstrated. The type of response of the reconstituted system

is decided only by the source of troponin, *i.e.*, if troponin is derived from cardiac muscle, the sensitivity of the reconstituted system is always high to Sr ion irrespective of the source of other three kinds of protein. This strongly indicates that the regulatory action of Ca ion on actin-myosin interaction is mediated only through troponin molecules and that the other three protein components have no direct interaction with Ca ion.

TABLE II

Sensitivities of myosin B preparations from skeletal and cardiac muscle to Sr and Ba ion in comparison with those to Ca ion.

The figures in the columns represent the reciprocal ratios of that concentration of specified ions to 1.8×10^{-6} M which induces the same degree of superprecipitation as that induced by 1.8×10^{-6} M free Ca ion. The concentration of free Ca ion was determined by using GEDTA-Ca buffer consisting of 0.05 mM GEDTA and 0.024 mM Ca ion (including estimated amount of contaminating Ca). As for other details see the legend for Fig. 1.

	Ca^{++}	Sr^{++}	Ba^{++}
Skeletal	(1)	1/34	<1/600
Cardiac	(1)	1/5	1/32

Ca Ion Bound to Troponin—Troponin has been shown to bind Ca ion fairly strongly (*6*, *7*, *18*, *19*). The results presented in the previous section have strongly suggested that this Ca-binding property of troponin may have a cardinal importance in the physiological function of this protein.

As shown in Table IV, troponin contained about 4 moles of Ca per 100,000 g protein, of which most were exchangeable. The binding constant of the bound Ca was reported to be about $6\times10^{5}\ M^{-1}$ (*6*). However, more careful analysis has revealed that the binding of Ca to troponin cannot be ac-

TABLE III

Comparison of relative sensitivities to Sr and Ca ion of contractile systems reconstituted from protein components of skeletal and cardiac muscle.

Myosin B				Sensitivity to Sr^{++} [1] relative to that to Ca^{++}	Type of response (skeletal type or cardiac type)
Native tropomyosin-free myosin B		Native tropomyosin			
Myosin	Actin	Tropomyosin	Troponin		
S				1/20	(S)
C				1/3	(C)
C		S		1/20	S
C		C		1/4	C
S		S		1/23	S
S		C		1/3	C
C		S	S	1/19	S
S		S	C	1/4	C
S	S	S	S	1/24	S
S	S	S	C	1/4	C
S	S	C	S	1/24	S
S	S	C	C	1/4	C

1) The concentration of Ca ion which induced the same degree of superprecipitation as that induced by 9.4×10^{-5} M free Sr ion (if sensitive to Sr ion) or 4.6×10^{-4} M free Sr ion (if insensitive to Sr ion) was determined and the ratio of this Ca ion concentration to the corresponding Sr ion concentration was considered as the sensitivity to Sr ion. Actually, the concentration of Ca ion ranged from 2.1 to 2.7×10^{-6} M. All experiments were carried out in 0.1mM GEDTA. Temperature, 21—24°C. As for other details see the legend for Fig. 1.

C : cardiac, S : skeletal.

TABLE IV

Amount of Ca bound to troponin and native tropomyosin.

Figures in parentheses indicate the number of experiments performed. The amount of non-exchangeable Ca was corrected taking the remaining radioactivity of Ca^{45} into account (see "METHODS"). The smallest value for non-exchangeable Ca without correction was 0.18 mole per 100,000 g. ±x indicates standard error. Note: The amount of total Ca of troponin determined in earlier experiments (1965—1966) was 5.10±0.32 moles per 100,000 g (8 determinations) and was definitely higher than the value below. The reason for this discrepancy is not clear, but it is certain that the preparations used in recent experiments such as presented in this Table show much higher activities than the old ones.

	Troponin	Native tropomyosin
	(moles/100,000 g)	
Total	3.78±0.38 (4)	1.72±0.09 (4)
Non-exchangeable	0.11±0.05 (3)	0.17±0.04 (3)
Exchangeable	3.67±0.39	1.55±0.10

counted for by assuming a single binding constant for all binding sites. The solid line shown in Fig. 5 represents the values calculated on the assumption that the binding constant of half of the sites were $1.3\times10^{6}\ M^{-1}$ and that of the remaining half $5\times10^{4}\ M^{-1}$.* Since the line seems to fit fairly well with the experimental results, the assumption of heterogeneous Ca-binding sites in one molecule may not be untenable.

Table V shows the affinity of various protein components in the contractile system for Ca ion. Although both myosin and actin contain tightly bound, non-exchangeable Ca, their binding capacity of exchangeable Ca is rather low (*20*). Thus, among the various structural proteins, troponin appears to be the only protein having a strong capacity for exchangeable Ca. This result is in good agreement with that of FUCHS and BRIGGS (*18a*).

* This is, of course, not the only possibility for explaining the results presented in Fig. 5. Various kinds of other combinations could also be postulated.

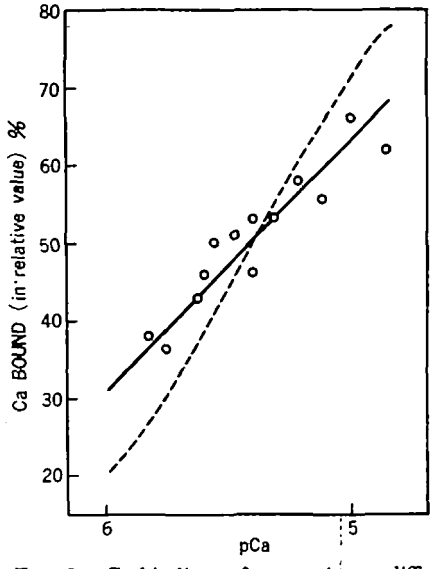

FIG. 5. Ca-binding of troponin at different pCa.

The ordinate indicates the amount of Ca bound to troponin in relative value assuming 4 moles of Ca-binding sites per 100,000 g protein. The pCa plots on the abscissa shows the free Ca ion concentration in the medium, in which the Ca-binding of troponin was determined. Solid line was drawn by calculation assuming a binding constant, $1.3\times10^{6}\ M^{-1}$, for a half of the binding sites and another one, $0.5\times10^{5}\ M^{-1}$, for the remaining half. Dashed line was obtained by assuming a single binding constant, $2.5\times10^{5}\ M^{-1}$, for all binding sites.

TABLE V

The amount of exchangeable Ca ion bound to structural proteins of muscle.

Concentration of Ca ions equilibrated with proteins	2×10^{-5} M	3×10^{-8} M
	(μmoles/g)	
Myosin B	1.03	0.86
Synthetic actomyosin	0.20	0.09
F-actin	0.15	——
Myosin	0.19	0.06
Tropomyosin	——	0.04
α-Actinin (6S component)	——	0.01
Troponin	——	17.8[1)]

1) Derived from the data in Fig. 5.

Treatment of myosin B or myofibrils with a weak alkaline solution can eliminate the troponin-tropomyosin complex (*10*). Con-

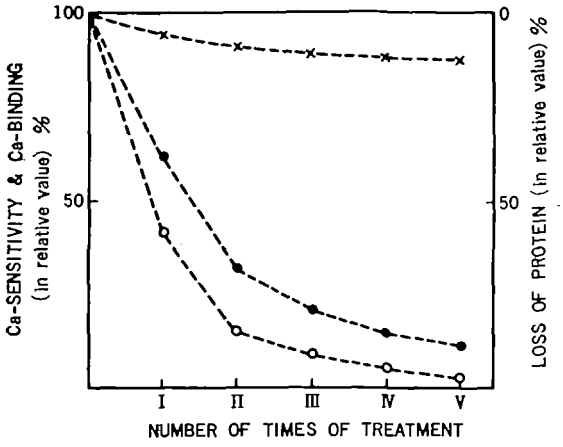

FIG. 6. Effect of repeated washing of myosin B with a weak alkaline solution on Ca-sensitivity and Ca-binding.

Myosin B suspended in a small volume of low ionic strength solution ($\mu=0.02$) was mixed well with 2 mM $NaHCO_3$ to make a final suspension of about 0.7 mg/ml. After 15 min, the solution was centrifuged down at 12,000×g for 20 min. A small part of the resulting precipitate was suspended in a small volume of 0.02 M KCl and was tested for Ca-sensitivity and Ca-binding. The amount of protein in the supernatant was determined to estimate the loss of protein due to the washing. The results obtained with the once washed preparation are designated as " I " in this figure. The remaining larger part of the sedimented material was suspended in 2 mM $NaHCO_3$ and was treated further in the same way as described above. Thus, the washing was repeated five times. Each time, a small part of the myosin B preparation was removed to determine its Ca-sensitivity and Ca-binding. The supernatant was also tested for loss of protein.

—○— Ca-sensitivity plotted on logarithmic scale; determined by the method illustrated in Fig. 1 (see also the legend for Fig. 2).

—●— Exchangeable Ca bound to myosin B; determined by the centrifugation method.

—×— The amount of protein lost due to the washing calculated from the protein content in the supernatants.

currently with the removal of this complex, the Ca-binding capacity shows a decrease accompanied by a parallel decrease in sensitivity of the system to Ca ion (Fig. 6). Mild trypsin-treatment preferentially removes troponin from

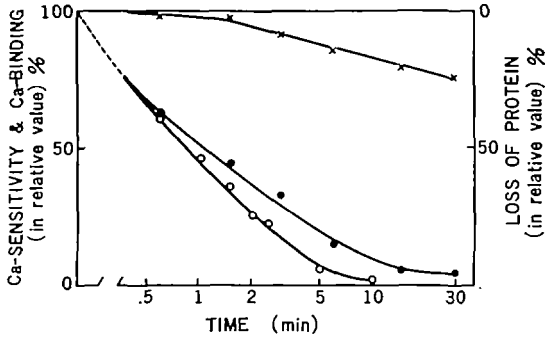

FIG. 7. Effect of trypsin treatment on the Ca-sensitivity and Ca-binding capacity of myosin B.

Myosin B suspended in 0.1 M KCl containing 1 mM $NaHCO_3$ was treated with trypsin at various concentrations noted on the abscissa. The reaction was stopped by addition of twice the weight of trypsin inhibitor.

—○—, —●— See the legend for Fig. 7.

—×— The amount of protein lost due to trypsin treatment.

myosin B with a simultaneous lowering of its Ca-binding capacity as well as Ca-sensitivity (Fig. 7). Thus, removal of troponin from the contractile system is always accompanied by a disappearance of exchangeable Ca-binding capacity.

It was also found* that the Ca-binding capacity of (i) troponin, (ii) the troponin-tropomyosin complex and (iii) the troponin-tropomyosin-F-actin complex was not modified by the presence of (i) tropomyosin, (ii) F-actin and (iii) myosin, respectively. These facts indicate that none of the other contractile proteins, although they have some direct or indirect interaction with troponin, can exert an influence on the Ca-binding property of troponin.

Fig. 8 illustrates the mode of Ca-binding of myosin B at a low Ca ion concentration. The apparent binding constant, $1.5\times10^6\ M^{-1}$, estimated from this curve, is in good agreement with the binding constant of troponin

* Different from the case of (i) or (iii), where either the chelating resin method or the centrifugation method was exclusively used, the comparison of the values derived from both methods must be made in (ii). Hence, in the case of (ii), the above conclusion should perhaps be accepted with some reservation.

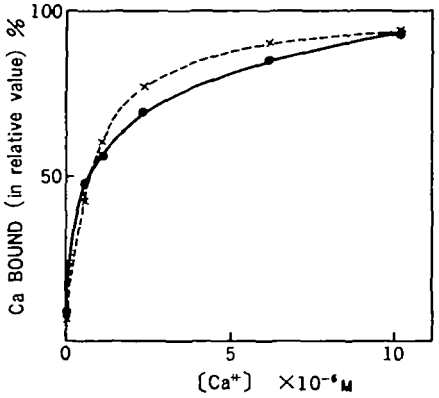

FIG. 8. Ca-binding of myosin B at varying concentrations of Ca ion.

Experiments were carried out at 0-4°C. Free Ca ion concentration was determined by the use of GEDTA-Ca buffer consisting of 0.05 mM GEDTA and appropriate concentrations of Ca ion including Ca^{45}. The dashed line was obtained by calculation assuming the binding constant of 1.5×10^6 M^{-1}. It should be mentioned that the deviation of the experimental curve for myosin B from the calculated curve was significant, indicating heterogeneity among the Ca-binding sites in agreement with the results presented in Fig. 5.

mentioned before.

All the findings presented above indicate strongly that the binding capacity of exchangeable Ca in the contractile system is exclusively due to troponin. As emphasized in the previous section, however, the effect of Ca ion is related to the dynamic phase of the interaction of structural proteins. Therefore, it seemed necessary to investigate whether the Ca-binding capacity of troponin alone or as part of the whole contractile system would be modified in the presence of ATP. All the experiments along this line* showed that the Ca-binding capacity was not modified by the presence of ATP. These results together with those shown in Table III have completely excluded the possility that the Ca-binding property of troponin might be transferred to other protein(s) or that one of the contractile proteins might acquire the ability to bind Ca ion at a crucial stage of interaction.

* The effect of ATP on the Ca-binding capacity of i) troponin, ii) troponin-tropomyosin complex, iii) troponin-tropomyosin-F-actin complex, iv) troponin-tropomyosin-F-actin-myosin complex and v) myosin B were examined. In the case of iv) and v), the comparison was made under three different conditions: without ATP, in the clearing state and in the super-precipitated state.

TABLE VI

Comparative affinities of skeletal and cardiac troponin for Sr and Ca ion.

	Binding constants[1] (M^{-1})	
	Ca^{++}	Sr^{++}
Skeletal	9.3×10^5	3.5×10^4 (1/27)
Cardiac	3.4×10^5	1.2×10^5 (1/3)

1) Figures in parentheses are the ratios of the binding constant of each troponin for Sr ion to that for Ca ion. The binding constants were calculated under the assumption that the sites for Ca ion or Sr ion of skeletal and cardiac troponins were 2 moles and 1 mole per 100,000 g respectively, since the results presented in Fig. 5 indicated that only half of 4 moles of Ca per 100,000 g of skeletal troponin showed a higher binding constant, around 10^6 M^{-1}, and the amount of chemically determined Ca bound to cardiac troponin was half of that bound to skeletal troponin.

Affinities of Skeletal and Cardiac Troponins for Sr Ion—All the results presented in the previous sections support the conclusion that the physiological function of troponin is based solely on its remarkable Ca binding characteristics. From this it would be expected that the differences in sensitivity to Sr ion between cardiac and skeletal myosin B reflect differences in affinity between cardiac and skeletal troponins for Sr ion.

In Table VI, the binding constants of skeletal and cardiac troponins for Sr ion are compared with those for Ca ion. The ratio of the affinity of cardiac troponin for Sr ion to that for Ca ion was found to be about 1/3, whereas the corresponding ratio in the case of skeletal troponin was 1/27. Thus the ratio of the affinity for Sr ion of skeletal troponin to that of cardiac troponin is about 1/9 of that of the corresponding affinity ratio

for Ca ion. These ratios, *viz.*, 1/3, 1/27 and 1/9 based on the property of the single protein, troponin, were in excellent agreement with those given in Table II, *viz.*, 1/5, 1/34 and 1/7 and in Table III, *viz.*, 1/4, 1/22 and 1/6, which had been derived from the experiments involving the complicated interaction of the various contractile proteins. This finding has substantiated further the idea that the affinity of troponin for Ca ion is underlying basis of the mechanism of the contraction-triggering action of Ca ion.

It should be pointed out that the binding constant of cardiac troponin for Ca ion is considerably lower than that of skeletal troponin. This is in consistence with the observation made in a preliminary experiment that the cardiac contractile system has lower sensitivity to Ca ion.

Effects of Sulfhydryl-Blocking Agents on Physiological Function of Troponin—Troponin has been shown to contain about 5 moles of sulfhydryl groups per 100,000 g, 1.8—2.5 moles of which do not react with NEM. This is in good agreement with the results of STAPRANS *et al.* (*21*), who have shown that 1.3 moles per 100,000 g of "relaxing protein", *viz.*, native tropomyosin, are not blocked by NEM. Since metal binding by myosin and actin is greatly affected by sulfhydryl-blocking agents, it was of interest to investigate their effects on the functions of troponin.

It was unexpectedly found that NEM did not exert an influence either on the Ca-binding capacity or the Ca-sensitizing action of troponin. This is in accord with the result of STAPRANS *et al.* (*21*) showing little effect of NEM on the Ca-sensitizing action of native tropomyosin. PCMB also did not show any effect on the Ca-binding activity, but did exert a slight but significant effect on the Ca-sensitizing action* (Fig. 9). Sulfhydryl-protecting agents such as dithiothreitol did not show any effect on these activities.

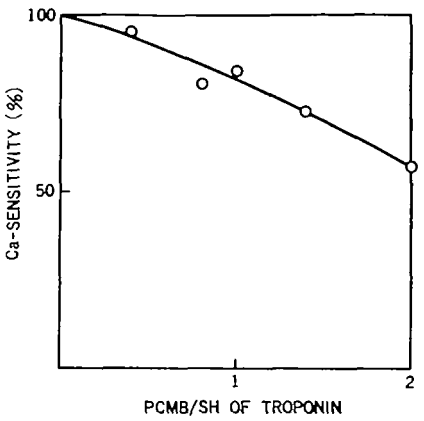

FIG. 9. Effect of PCMB on Ca-sensitizing action of troponin.

Ordinate: the relative value of Ca-sensitivity on logarithmic scale (see the legends for Figs. 1 and 2). Abscissa: molar ratio of PCMB to sulfhydryl groups of troponin, the number of which was assumed to be 5 moles per 100,000 *g*. Troponin incubated with specified amounts of PCMB was added to the suspension of the actomyosin system just prior to the addition of ATP. Final concentrations: native tropomyosin-free myosin B, 0.65 mg/ml; tropomyosin 0.031 mg/ml; troponin 0.031 mg/ml. For other details see the legend for Figs. 1 and 2.

Generally speaking, sulfhydryl groups seem to have no substantial role in the function of troponin. At least, it is reasonably certain that these groups are not involved in the Ca-binding activity. However, we must consider the fact that about two moles of sulfhydryl groups remain unaffected by NEM with the possibility that PCMB once attached to these sulfhydryl groups would migrate to other protein(s), possibly to myosin, during experimental examination of the Ca-sensitivity. Therefore, before excluding the involvement of sulfhydryl group in the function of troponin, it is essential that experiments be carried out with a sulfhydryl-blocking agent which can bind to all sulfhydryl groups with covalent linkage.

* Sulfhydryl agents, particularly PCMB, modify the interaction of actin and myosin in such a way that the response corresponding to the relaxation is inhibited (*22*, *23*). This should be kept in mind when the sensitivity of actomyosin systems to Ca ion is examined in the presence of PCMB; otherwise, one could mistakenly conclude that PCMB abolishes the Ca-sensitizing activity of troponin. The inhibitory effect of PCMB shown in Fig. 9 might be explained in this manner.

DISCUSSION

The results presented in this article further support the postulation that troponin is the Ca-receptive protein of the contractile system, *i.e.*, the contraction-triggering action of Ca ion becomes effective only when it binds to troponin (*6*, *7*). No other protein seems to show any particular Ca-binding activity during the entire course of interaction of myosin, actin and ATP. In other words, the affinity of the contractile system for Ca ion is always and almost exclusively due to the Ca-binding capacity of troponin.

The question which naturally arises next is how Ca ion exerts its influence on the interaction of myosin and actin through troponin. In dealing with this question, it seems pertinent to summarize our previous findings concerning troponin:

1) Troponin has a strong affinity for tropomyosin and promotes aggregation of the latter (*3*).

2) Troponin exerts a pronounced effect on the physico-chemical properties of F-actin in the presence of tropomyosin (*24*). Some differences in electron microscopic profile between F-actin preparations with and without the troponin-tropomyosin complex have also been noticed (*cf.* (*7*)).

3) Troponin can be easily removed from myosin B as well as from myofibrils by mild trypsin-treatment (*2*, *4*). Tropomyosin, which in solution is as sensitive to trypsin as troponin, is more resistant to trypsin when it is associated with F-actin (*4*).

4) Troponin can bind to trypsin-treated thin filaments when tropomyosin is left unaffected. Further digestion, which eventually removes tropomyosin from the thin filaments, abolishes the binding of troponin to the thin filament. This binding is restored when the digested myofibrils are pretreated with tropomyosin (*25*)*.

5) Troponin is distributed along the entire thin filament of myofibrils at approximately 400 Å periodicity (*26*) (tropomyosin also exists along the entire thin filament, but no peridicity has so far been demonstrated).

6) The weight ratio of troponin to tropomyosin in native tropomyosin, which may reflect the situation *in vivo*, is about 0.6. The content of tropomyosin in muscle is estimated to be 5—6 mg/ml and that of troponin about 3 mg/ml. If troponin has a molecular weight of 50,000 as it has been suggested by a preliminary experiment**, it is very plausible to assume that two troponin molecules and two tropomyosin molecules exist per pitch of 400 Å.

7) Troponin does not bind to myosin (*25*, *28*).

In view of these findings, it may be concluded that the association of troponin with the thin filament is made through its binding to tropomyosin which in turn can directly bind to F-actin, and that 400 Å periodicity due to the presence of troponin is based upon the periodic distribution of tropomyosin at the same interval. Thus it appears that troponin can modify the structure of actin not directly, but only indirectly through mediation of tropomyosin. On the basis of these facts and considerations, one might propose the following two possible explanations:

a) The influence of a conformational change of troponin due to Ca ion is mediated through tropomyosin to F-actin, inducing a change in the structure of F-actin. As a result, the interaction of actin and myosin is modified. In this mechanism each actin molecule has the same importance, whether it is located near the troponin molecule or not.

b) Troponin, although not directly bound to actin molecule, is located near the site of interaction of actin with myosin head so that a change in structure of troponin due

* Drabikowski and Nonomura (*27*) have made the interesting observation that troponin and F-actin at fairly high concentrations and equivalent ratio give rise to an aggregate precipitable in 0.1 M KCl. This fact, however, may not be considered as evidence for direct binding of troponin and F-actin *in vivo*, since troponin cannot bind to the thin filament once tropomyosin has been removed (*25*). It should also be noticed that F-actin can make such aggregates with various kinds of non-muscle proteins (H.Asai, personal communication).

** Determined by the light scattering method (T. Wakabayashi, personal communication).

to Ca ion can modify the interaction. In this case we must assume that the actin molecules near the troponin molecule would be in a special position. The interaction of the other actin molecules with myosin should be inhibited more or less by a mechanism not necessarily under the influence of Ca ion. Tropomyosin may play an important role in the latter mechanism.

The mechanism described under (b) seems —because of the rather arbitrary nature of the assumptions made in it—to be less plausible than that under (a). However, it is tempting to assume that the 400 Å periodicity has a particular significance in the physiological process of muscle contraction. For the present, we cannot decide between the two alternatives.

It should be emphasized here that, whichever explanation we may choose, the essential nature of Ca ion as the trigger of contraction should be considered as a releasing factor of an existing repression, *i.e.*, in the absence of Ca ion troponin exerts a depressing action on the interaction of actin and myosin and this inhibition is abolished by the action of Ca ion on troponin. This kind of explanation might appear to be strange in view of the dramatic activating effect of Ca ion on the contractile system. However, if we inquire into mechanisms of various physiological phenomena, it is rather common to find that the activation of a biological system is induced through the release of a preexisting inhibition. For example, activation of the excitation process of the surface membrane could be interpreted in this way.

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