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Ca^{2+}-BINDING AND Ca^{2+}-SENSITIZING FUNCTIONS OF CARDIAC NATIVE TROPOMYOSIN, TROPONIN, AND TROPOMYOSIN

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SUMMARY

Procedures are described by which troponin and tropomyosin can be isolated from cardiac muscle rapidly, with minimal damage by oxidation. Cardiac relaxing proteins inhibit actomyosin ATPase activity in the presence of ethyleneglycoltetraacetic acid (EGTA), and permit graded stimulation by Ca^{2+}. This stimulation is independent of preexisting inhibition, and greater than that obtained with skeletal proteins. Characteristics of Scatchard plots for Ca^{2+} binding suggest that troponin contains one class of sites which interact at high fractional occupancy. Interaction appears to be enhanced by tropomyosin. Mean values for the estimated maximum affinity and capacity of six canine cardiac troponin preparations were: $4.92 \cdot 10^6$ M^{-1}, and $21.58 \cdot 10^{-6}$ moles $\cdot g^{-1}$. Values for skeletal troponin were not significantly different. Native tropomyosin bound about half as much Ca^{2+} per g, with maximum affinity the same as troponin. Pure tropomyosin bound no Ca^{2+}. Cardiac and skeletal proteins differ in that the former are much more labile, and more readily influenced by ions and drugs.

INTRODUCTION

Cardiac contraction is graded by chemical processes within the individual muscle cells rather than, as in skeletal muscle, by summation of contraction of motor units[1]. It is therefore particularly important to determine how cardiac actomyosin ATPase activity is controlled by Ca^{2+}. Actomyosin from skeletal muscle is Ca^{2+}-insensitive; Ca^{2+} control is conferred upon it by a protein complex termed native tropomyosin[2]. Recent studies indicate that the complex consists of at least 4 distinct proteins [3–5]. This paper describes the Ca^{2+}-binding and Ca^{2+}-sensitizing functions of homologous cardiac proteins, isolated with minimal damage by oxidation and catheptic activity.

METHODS

Preparation of proteins

Myosin B. Hearts were removed from dogs anesthetized with sodium pento-

Abbreviation: EGTA, ethyleneglycoltetraacetic acid.

barbital, and immediately immersed in 10 mM imidazole, 0 °C. Animals were not ventilated after thoracotomy, in order to lower myocardial tissue pO_2. Muscle was immediately minced in the cold room, and thoroughly rinsed to remove hemoglobin. It was homogenized 1:10 (w/v) in N_2-equilibrated Weber–Edsall solution containing 4 mM dithiothreitol. This initial homogenate has high catheptic activity, and contains high concentrations of potential oxidants. To minimize exposure to these substances, extraction was limited to the time required to transfer the homogenate to the Sorvall centrifuge (less than 10 min). Brief extraction largely eliminates mitochondrial proteins, which are much less soluble than actomyosin at high ionic strength[6]. The extract was centrifuged for 10 min and immediately diluted with 10 vol. of cold glass-distilled water containing 0.5 mM dithiothreitol. The first dilution precipitate was used as Ca^{2+}-sensitive myosin B, or as the source for native tropomyosin. At this stage most of the oxidants and cathepsins have been removed.

Native tropomyosin. All the following steps were performed in the presence of 2 mM dithiothreitol. Myosin B was washed twice with ethanol and twice with diethyl ether, and dried for 30 min. The powder was suspended in 1 M KCl, using a loose Dounce homogenizer. It was found that 30 min is sufficient to quantitatively extract native tropomyosin from muscle powder. In 7 preparations the average yield was 44 mg/100 g trimmed muscle. Assuming mitochondria comprise 1/3–1/2 of cardiac wt[1] this yield is the same as that observed by ourselves and others for skeletal muscle. Native tropomyosin was collected by $(NH_4)_2SO_4$ fractionation between 40 and 65 % saturation, and dissolved in water. Residual $(NH_4)_2SO_4$ could be completely eliminated by a 6-h dialysis. (It was necessary to remove oxidants and Ca^{2+} from the dialysis tubing by boiling it in 1 mM ethyleneglycoltetraacetic acid (EGTA), then in several changes of glass-distilled water). Starting from the time of cardiectomy, 16–18 h were required to obtain native tropomyosin. When labile properties of this protein were to be studied, the experiments were performed immediately after isolation.

Troponin and tropomyosin. Starting from native tropomyosin, procedures were identical to those of Hartshorne and Mueller[7]. As in the case of skeletal muscle, the optimum pH for isoelectric fractionation was 4.6, and the optimum protein concentration 1 mg/ml. In early experiments proteins were decalcified on a Chelex 100 column suitably equilibrated with Mg^{2+}. More recently, they were stirred at 6 °C for several h in a resin slurry. The slurry procedure is faster, and eliminates freeze–drying to concentrate the eluate. From 60–90 % of protein-bound Ca^{2+} was removed.

Ca^{2+}-desensitized actomyosin. Ca^{2+}-desensitized actomyosin was prepared by washing myosin B in 2 mM Tris–HCl, pH 7.6; see Fig. 3. If the washing is carried out in the presence of dithiothreitol, the protein becomes excessively hydrated. Dithiothreitol was therefore omitted from the wash solution. The desensitized actomyosin was suspended in reaction mixture containing 1 mM dithiothreitol about 1 h before use to restore those sulfhydryl groups of myosin which might have been oxidized.

Standard procedures

Ca^{2+} binding was measured by procedures identical in every respect to those of Fuchs and Briggs[8]. Binding parameters were calculated from least-squares regression lines for Scatchard plots.

Contaminant Ca^{2+} in proteins and solutions was measured in every experiment

by a modification of the method of Kepner and Hercules[9]. Total contaminant Ca^{2+} in proteins and solutions averaged $5 \cdot 10^{-6}$ M.

ATPase activity was measured as appearance of inorganic phosphate as previously described[6], except that in the present experiments temperature was 37 °C.

Protein concentration was measured by the Lowry method, standardized with bovine serum albumin.

Disc electrophoresis was performed as described by Yasui *et al.*[10] except that the urea concentration was 8 M.

Solutions and reagents

All solutions were prepared in doubly glass-distilled, deionized water. Reagent grade inorganic salts were products of J. T. Baker; ATP (disodium salt) was obtained from Sigma Chemical Co., and dithiothreitol from Calbiochem.

Ca^{2+} binding was conducted in 10 mM KCl, 0.5 mM $MgCl_2$, 10 mM imidazole pH 7.0. In these experiments disposable plastic ware was used where possible; all glassware was acid-rinsed. For ATPase activity, reaction mixture consisted of 10 or 70 mM KCl, 20 mM imidazole, 0.5 mM $MgCl_2$, 1 mM ATP, 5 mM azide and 3 mM Ca^{2+}–EGTA "buffer". Values for pCa^{2+} were estimated from the ratio of EGTA to total Ca^{2+}, assuming the stability constant for EGTA, pH 7.0, is $1.18 \cdot 10^6$ M^{-1} (ref. 11).

RESULTS

Gel electrophoresis

Polyacrylamide gels from a representative cardiac preparation are shown in Fig. 1. Gels for native tropomyosin and troponin were loaded with 50 μg. Since tropomyosin stains much more readily with aniline black than does troponin, 100 μg were loaded onto tropomyosin gels in order to identify contaminant troponin. The three gels shown in the figure were given equal photographic exposure.

Since the gels were run in 8 M urea, native tropomyosin (filled circle) was dissociated into its constituent proteins. Despite presence of 2 mM dithiothreitol, much of the tropomyosin appears to have been oxidized; the darkly-stained bands are due to S–S tropomyosin[10]. The troponin complex appears as a lightly-stained, fast-moving band, and three additional light bands. In most gels of native tropomyosin at least one of the slower troponin bands was obscured by tropomyosin.

After one cycle of isoelectric fractionation at pH 4.65 (triangle), the tropomyosin bands are much less prominant, despite the high affinity of tropomyosin for aniline black. Correspondingly, there is a great increase in width and intensity of the fast troponin band. It is this band which correlates with Ca^{2+} binding. The slower troponin bands are also more prominent.

Tropomyosin collected from the initial $(NH_4)_2SO_4$ fractionation between 40 and 65 % saturation always contained a small amount of contaminant troponin, as judged by the presence of a fast-moving band, and by the ability to bind a small amount of Ca^{2+}. Further fractionation between 50 and 65 % saturation at low protein concentration eliminated the fast-moving band (open circle). Recall that twice as much material was chromatographed, compared with the other two gels. The foregoing properties of cardiac proteins are the same as those of proteins isolated, by identical procedures, from canine limb skeletal muscle.

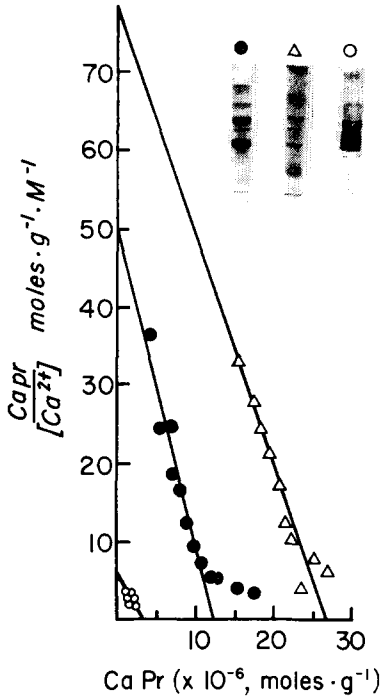

Fig. 1. Polyacrylamide gels of troponin (△), tropomyosin (○) and the parent native tropomyosin (●), together with Scatchard plots of Ca^{2+} binding by the three proteins. Values for Ca^{2+} affinity ($\times 10^6$, M^{-1}): troponin, 2.9; native tropomyosin, 3.6; tropomyosin, 1.9. Values for binding capacity ($\times 10^{-6}$, moles·g^{-1}): troponin, 26.5; native tropomyosin, 13.0; tropomyosin, 2.7. Conditions: 10 mM KCl, 0.5 mM $MgCl_2$, 10 mM imidazole, pH 7.0. Gels of troponin and native tropomyosin were loaded with 50 μg, tropomyosin gels with 100 μg.

Ca^{2+} binding

Troponin. The first data point on each Scatchard plot in Fig. 1 was calculated from the partition of $^{45}Ca^{2+}$ between protein and Chelex 100 in the presence of contaminant Ca^{2+}. The remaining points were obtained by adding increments of $^{40}Ca^{2+}$. Though the plot for troponin is linear, this is not acceptable evidence that only one class of independent binding sites exists. This is true because concentrations of $^{40}Ca^{2+}$ greater than 100 μM could not be explored for technical reasons[8]. It is these high Ca^{2+} concentrations which would be expected to reveal non-linearity[12]. Moreover, in three of six cardiac and one of two skeletal troponin preparations, data points between 70 and 100 μM Ca^{2+} did, in fact, fall off the line defined by the other data points. We attempted to account for this non-linearity by assuming that two independent classes of non-interacting binding sites exist, with binding capacities N_1 and N_2, and affinities K_1 and K_2[12]. In Eqn 1, CaPr denotes protein-bound calcium.

$$\mathrm{CaPr} = \frac{N_1K_1[Ca^{2+}]}{1 + K_1[Ca^{2+}]} + \frac{N_2K_2[Ca^{2+}]}{1 + K_2[Ca^{2+}]} \qquad (1)$$

It was possible to accurately replicate each non-linear Scatchard plot of troponin by applying curve-fitting techniques to Eqn 1. In the case of troponin, the second term

in the equation contributed less than 10 % to the first four or five data points. It is for this reason that simple linear extrapolation and conventional curve fitting[4,8] yield similar results for the supposed high-affinity sites of troponin. For reasons cited in Discussion, Eqn 1 does not adequately describe the binding data. Since we believe that one class of interacting sites exists, rather than two classes of independent sites, only values based on extrapolation of the initial, roughly linear portion of the plots are shown in Table I. Assuming negative interaction occurs, the constants so estimated approximate maximum values.

Native tropomyosin. Scatchard plots for all eighteen preparations of cardiac native tropomyosin were obviously non-linear, and in almost every case the slope of the terminal portion of the plots was much lower than for troponin; see Fig. 1. In eleven preparations, however, at least four data points fell on a straight line which could be extrapolated to the coordinates. Binding parameters based on such extrapolation are shown in Table I. Binding capacity so estimated is about half that of troponin ($P < 0.001$) but the estimated affinity constants for the two proteins are not significantly different.

TABLE I

ESTIMATED MAXIMUM VALUES OF Ca^{2+}-BINDING CONSTANTS FOR CARDIAC AND SKELETAL PROTEINS

Protein	*Number of preparations*	*Affinity* ($\times 10^6$, M^{-1}) $\pm$ S.E.	*Capacity* ($\times 10^{-6}$, $moles \cdot g^{-1}$) $\pm$ S.E.
Cardiac troponin	6	4.92 $\pm$ 0.45	21.58 $\pm$ 2.92
Cardiac native tropomyosin	18	4.21 $\pm$ 1.47	10.17 $\pm$ 1.97
Cardiac tropomyosin	4	3.72 $\pm$ 0.98	3.29 $\pm$ 0.84
Skeletal troponin	2	3.80	25.64
Skeletal native tropomyosin	3	3.17	13.1

Tropomyosin. The low binding activity of tropomyosin is shown in Fig. 1. When plotted on an expanded scale constants could be estimated by extrapolation in four of six preparations (see Table I). Since binding capacity decreased with repeated cycles of $(NH_4)_2SO_4$fractionation, it is likely that Ca^{2+} binding is due entirely to contamination with troponin. The fact that the affinity constant is not significantly different from that of troponin is consistent with this view, which has been previously expressed by Fuchs and Briggs[8].

Stability of binding parameters: Observations on five preparations of cardiac native tropomyosin were made repeatedly over a 3-week period, during which the proteins were stored at -15 °C (see Fig. 2). (Freezing and thawing on the day of isolation did not affect binding parameters.) Least squares regression lines and 95 % confidence bands are shown. The regression predicts that binding capacity, but not affinity, would fall significantly below the initial value about 1 month after isolation. Those portions of the protein complex responsible for Ca^{2+} control are much more

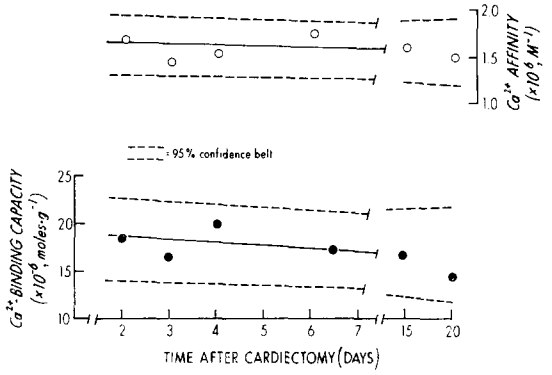

Fig. 2. Mean values for Ca^{2+}-binding capacity and Ca^{2+} affinity among five preparations of native tropomyosin, as a function of time after isolation. Least-squares regression lines and 95 % confidence intervals are indicated.

labile; see below. Consequently, preparations aged 2–3 weeks were incapable of influencing actomyosin, despite normal Ca^{2+}-binding activity.

Control of ATPase activity

Desensitization and resensitization. A typical Ca^{2+} concentration–effect curve for fresh cardiac myosin B is shown in open circles, Fig. 3. The experiment is one of

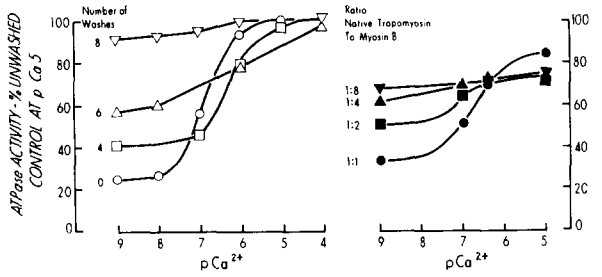

Fig. 3. Desensitization of cardiac myosin B by washing in 2 mM Tris, pH 7.6, and partial restoration of Ca^{2+} control by native tropomyosin. Conditions: 70 mM KCl, 0.5 mM $MgCl_2$, 20 mM imidazole, pH 7.0, 1 mM ATP, 5 mM azide, 3 mM Ca^{2+}–EGTA buffer, 1 mg/ml myosin B. 100 % = 0.03 μmole P_i/mg per min at 37 °C.

seven performed in reaction medium containing 70 mM KCl, ($I \simeq 0.12$). Responsiveness to Ca^{2+} was progressively reduced by washing in Tris–HCl, pH 7.6. In most cases 6–8 washes resulted in fully desensitized actomyosin, which could then be used to assay for the Ca^{2+}-control functions of the regulator proteins[5]. As shown in Fig. 3, filled symbols, addition of progressively larger amounts of native tropomyosin resulted in progressively greater restoration of Ca^{2+} control. Maximum effect at this ionic strength was attained at a 1:1 ratio of native tropomyosin to Ca^{2+}-desensitized actomyosin. The pCa^{2+} for half-maximum activation is the same for fresh and reconstituted myosin B (open and filled circles). Maximum stimulation by Ca^{2+} and inhibition by EGTA, however, were always greater for fresh myosin B when the experiment

was performed at physiological ionic strength. In contrast, when the same experiment was performed in reaction mixture containing 10 mM KCl, restoration of Ca^{2+} control was more complete, and much less native tropomyosin was required. In six such experiments a ratio of native tropomyosin to Ca^{2+}-desensitized actomyosin of only 1:5–1:8 was sufficient for maximum response. Results entirely comparable to the foregoing were obtained with troponin and tropomyosin, if these proteins were present in optimum ratio to each other (see below).

Troponin and tropomyosin. Effects of troponin and tropomyosin alone, and combined in various ratios, are shown in Fig. 4. The experiment, one of four, was

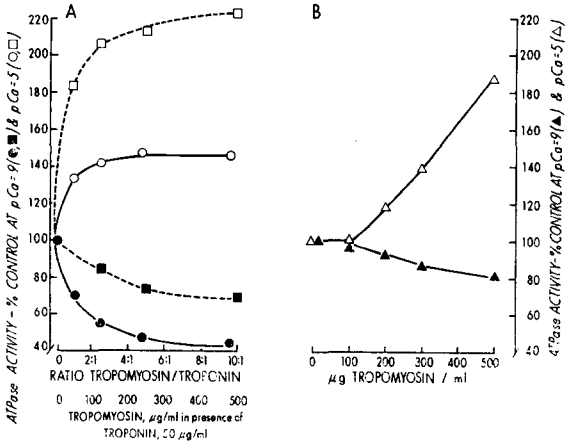

Fig. 4. Restoration of Ca^{2+} sensitivity of Ca^{2+}-desensitized actomyosin by various ratios of tropomyosin/troponin (A) and by tropomyosin contaminated by a small amount of troponin (B). Circles and squares denote two preparations of troponin, tested with the same preparation of tropomyosin on the same desensitized actomyosin. Conditions: 10 mM KCl, 0.5 mM $MgCl_2$, 20 mM imidazole, pH 7.0, 1 mM ATP, 5 mM azide, 3 mM Ca^{2+}–EGTA buffer, 0.5 mg/ml Ca^{2+}-desensitized actomyosin. 100% = 0.04 µmole P_i/mg per min at 37 °C. Troponin denoted by square symbols is the same as that shown in Fig. 1.

performed in reaction medium containing 10 mM KCl, and 0.5 mg Ca^{2+}-desensitized actomyosin per ml. Filled symbols denote observations in the presence of 3 mM EGTA, open symbols indicate observations at pCa^{2+} 5. Tropomyosin alone had no effect at concentrations less than 100 µg/ml. Higher concentrations behaved like native tropomyosin, *i.e.* inhibition at pCa^{2+} 9 (filled triangles) and stimulation at pCa^{2+} 5 (open triangles). This is to be expected, since tropomyosin was always contaminated by a small amount of troponin, as shown in Fig. 1.

The circles and squares in Fig. 4A represent two preparations of troponin. Both were without effect on ATPase activity in the absence of added tropomyosin. Both were tested on the same Ca^{2+}-desensitized actomyosin, when combined with the tropomyosin preparation whose properties are shown in Fig. 4B. To determine the optimum tropomyosin/troponin ratio, the troponin concentration was held constant at 50 µg/ml and the tropomyosin concentration was varied. At a ratio of 2:1, stimulation by Ca^{2+} and inhibition by EGTA are almost maximal with both troponin preparations. The additional small effect at higher ratios (tropomyosin concentrations) could

be due to troponin which accompanied tropomyosin as a contaminant. With skeletal muscle proteins, maximal effect was attained at a ratio of only 1:1.

Note that the two troponin preparations yield quantitatively different effects, though combined with the same preparation of tropomyosin. One produces greater inhibition in the presence of EGTA, whereas the other produces greater stimulation in the presence of Ca^{2+}. It is likely that these differences reflect yet another protein ratio, namely that of troponin A, the Ca^{2+}-binding moiety, to troponin B, the ATPase-inhibiting component or components of troponin[4,5,13].

Components of troponin. The activity of troponins A and B varied markedly among preparations of native tropomyosin. Extreme examples of this are shown in Fig. 5. Open and closed circles denote two preparations; the dashed line indicates

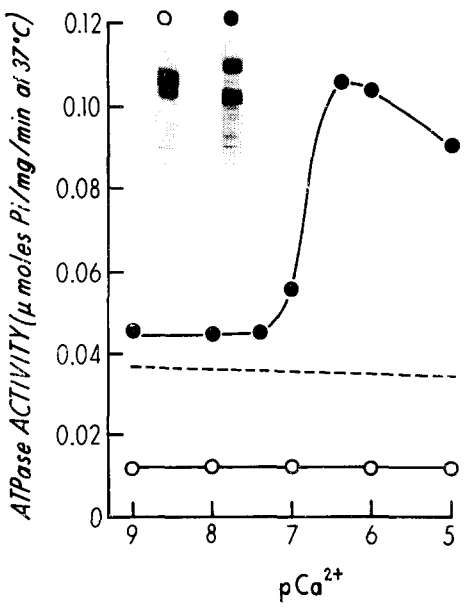

Fig. 5. Dashed line indicates ATPase activity of Ca^{2+}-desensitized actomyosin alone. ○ and ● show changes produced by addition of two preparations of cardiac native tropomyosin. ○, failure of Ca^{2+} to release inhibition indicates absence of troponin A activity. Note absence of fast-moving band thought to be troponin A from polyacrylamide gel. ●, stimulation of ATPase activity at pCa^{2+} 9 indicates lack of troponin B activity. Nevertheless, the preparation permits graded stimulation by Ca^{2+}. The corresponding gel contains a fast-moving band consistent with troponin A. Gels were loaded with 50 μg. Other conditions as for Fig. 4.

ATPase activity of Ca^{2+}-desensitized actomyosin. One produced strong inhibition, which was not released by Ca^{2+}. The Ca^{2+}-binding activity of this protein was too low to evaluate by use of the $^{45}Ca^{2+}$-partition technique. A polyacrylamide gel loaded with 50 μg of the material contained bands consistent with tropomyosin and troponin B, but the fast-moving component of troponin was absent. We have no explanation for this selective loss of troponin A; the preparation was the only one of eighteen to behave in this fashion. In contrast, the preparation shown in filled circles not only failed to inhibit the ATPase activity of Ca^{2+}-desensitized actomyosin in the presence

of EGTA, but actually produced slight stimulation. Also, ATPase activity more than doubled in the presence of Ca^{2+}. Since the polyacrylamide gel of the material contains tropomyosin and slow as well as fast-moving components of troponin, it is likely that absence of inhibition by EGTA is due to inactivation rather than absence of troponin B. This is consistent with the fact that inhibition of ATPase activity was always the most labile property of cardiac native tropomyosin. Most preparations lost the ability to inhibit ATPase activity within 3–4 days of cardiectomy, and in all cases within a week. The ability of cardiac native tropomyosin to permit Ca to stimulate ATP hydrolysis usually persisted for several weeks, so that aged preparations tended to behave like the one shown in filled circles in Fig. 5. Preparations which had lost all potency for Ca^{2+} control of actomyosin still bound Ca^{2+}, often with estimated values for binding constants unchanged over those observed when the material was freshly isolated.

DISCUSSION

Problems of isolation

Troponin and tropomyosin possess oxygen-labile SH groups, and are highly susceptible to tryptic digestion[1,5,10,14]. Cardiac muscle homogenates contain exceptionally high concentrations of myoglobin and cytochrome[1], as well as trypsin-like cathepsins. The presence of these potentially damaging substances during the initial stages of isolation appears to account for the difficulty experienced heretofore in preparing cardiac relaxing proteins. The use of dithiothreitol to protect SH groups activates cathepsins (A. Dounce, personal communication), but was found necessary empirically. The most effective measure to control both oxidation and digestion was to minimize exposure time. The shortest practical time in Weber–Edsall solution was 10 min. The amount of native tropomyosin obtained per g wet wt of muscle with this short extraction time was the same as when the extraction continued for several hours. Since native tropomyosin is a component of the thin filament, brief extraction after thorough homogenization does not appear to result in actin-deficient myosin B. Subsequent isolation steps require several days to complete using conventional procedures, but certain functions of cardiac proteins deteriorate on a time base of days or even hours[15,16]. This was not true of skeletal muscle proteins. In searching for time-saving steps it was found that native tropomyosin could be quantitatively extracted from myosin B powder within 30 min; indeed longer extraction times result in contamination with other proteins. It was usually possible to isolate native tropomyosin within 18 h, and to fully characterize it with respect to Ca^{2+} binding and ATPase activity within 24–30 h of cardiectomy.

Control of ATPase activity

The chief difference between cardiac and skeletal regulatory proteins is that the former are far more labile. It is therefore likely that most or all other differences are artifacts of isolation. For example, the fact that twice as much cardiac as skeletal tropomyosin must be added to cardiac troponin to fully restore Ca^{2+} control probably reflects the fact that cardiac tropomyosin was partly oxidized, as judged by gel electrophoresis. Oxidation of tropomyosin could also account for the ineffectiveness of cardiac proteins at 70 mM KCl, for one of the functions of tropomyosin is to permit Ca^{2+} control at physiological ionic strength[13].

We have not fractionated cardiac troponin, but one preparation of what should have been native tropomyosin contained no troponin A, as judged by gel electrophoresis and Ca^{2+} binding, and caused a Ca^{2+}-insensitive inhibition of Ca^{2+}-desensitized actomyosin typical of troponin B. Another preparation failed to inhibit ATPase activity at high pCa^{2+}, but permitted a graded increase in ATPase activity when pCa^{2+} was lowered. These fortuitous results indicate that cardiac troponin also consists of at least two functional subunits. Oxidation of skeletal troponin selectively reduces troponin B activity[10]. Perhaps because of this susceptibility to oxidation the ability of fresh cardiac native tropomyosin to inhibit ATPase activity usually disappears within a few days of isolation. Unlike the skeletal protein, the activity of cardiac troponin B cannot be restored by incubation in dithiothreitol.

Others have found that skeletal troponin A releases inhibition by troponin B, but seldom increases ATPase activity appreciably above the control level[4,5]. In contrast, cardiac proteins regularly double control ATPase activity at low pCa^{2+} (see Figs 4 and 5). The greater capacity for Ca stimulation is of interest in view of the greater importance of intracellular chemical processes for regulation of cardiac function. The difference between heart and skeletal proteins does not appear to be due to the desensitized protein used for assay, for it was true of heart and skeletal native tropomyosins from the same animal, tested on the same cardiac desensitized protein. Since stimulation by cardiac proteins occurs even in the absence of demonstrable troponin B activity, stimulation and inhibition are clearly independent functions of troponin.

Perhaps the most interesting difference between cardiac and skeletal regulatory proteins is that the former are far more susceptible to influence by chemical agents, including cardio-active drugs. Both the Ca^{2+}-binding[15] and Ca^{2+}-sensitizing functions[15,16] can be modified. Responsiveness to drugs is the most labile property of all, and must be sought immediately after isolation.

Ca^{2+} binding

Binding parameters for troponin. As shown in Table I, estimated binding constants for canine cardiac and skeletal troponins are identical. Values are the same as those reported by Fuchs and Briggs[8] and Hartshorne and Pyun[4] for troponin from rabbit psoas. Since the methods used in all three laboratories are identical, it seems likely that the binding constants of troponin are the same among muscle groups, and for at least two animal species. Ebashi *et al.*[2] reported the affinity constant for skeletal troponin as $0.93 \cdot 10^6\ M^{-1}$ for experiments conducted at pH 6.8. This figure is also the same as ours, when the effect of pH on Ca^{2+} affinity[17] is considered. Their figure for cardiac troponin, however, is less than $1/4$ that which we observe at the same pH. Presumably, the difference is attributable to the preparative techniques described in this paper.

Non-linearity of Scatchard plots. Two interpretations of the non-linearity of Scatchard plots for troponin and native tropomyosin are possible. Curves fitted by two regression lines may denote two classes of independent non-interacting sites[12]. This model is the one currently in use[2,4,8]. The fact that a curve can be "fitted", as judged visually, does not prove that two classes of sites do, in fact, exist[18]. Similar curves are generated by a single class of sites which interact[12]. If the two-class model is correct, it should lead to a unique solution; only one set of values for N_1, N_2, K_1

and K_2 should fit the data. However, a computerized search of parameter space revealed that in every case numerous combinations of N and K values provided a good visual "fit" to the data; some of the values differed by three orders of magnitude. Application of the method of non-linear least squares revealed that only some data sets could be fitted at least squares minimum for the four parameters in Eqn I. Moreover, for those data sets for which one or more least-squares minima could be found, the set of parameters with the lowest least squares sum was frequently uninterpretable. We consider the foregoing conclusive evidence that the two-class model does not fit the data. A detailed description of the computations has been submitted[18] and an alternative model based on one class of interacting sites is in preparation. For purposes of this report, however, the values obtained by linear extrapolation approximate maximum values observed at low fractional occupancy, and permit comparison with the data of others.

As shown in Table I, tropomyosin does not alter the affinity constant of troponin, as estimated by extrapolation. The striking increase in non-linearity of Scatchard plots when tropomyosin is present, however, indicates that tropomyosin increases interaction of troponin's binding sites. Consequently, the affinity constant for native tropomyosin, averaged over the entire range of Ca^{2+} concentrations, is reduced. A further reduction in mean affinity is observed in the presence of actomyosin (unpublished observations). The estimated maximum values for binding parameters shown in Table I must therefore be cautiously interpreted. In particular, quantitative comparison of Ca^{2+} binding by troponin and sarcoplasmic reticulum must await further analysis of the binding functions of both.

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