

The Effects of Partial Extraction of TnC upon the Tension-pCa Relationship in Rabbit Skinned Skeletal Muscle Fibers

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ABSTRACT The activation of contraction in vertebrate skeletal muscle involves the binding of Ca^{2+} to low-affinity binding sites on the troponin C (TnC) subunit of the regulatory protein troponin. The present study is an investigation of possible cooperative interactions between adjacent functional groups, composed of seven actin monomers, one tropomyosin, and one troponin, along the same thin filament. Single skinned fibers were obtained from rabbit psoas muscles and were then placed in an experimental chamber containing relaxing solution maintained at 15°C . Isometric tension was measured in solutions containing maximally and submaximally activating levels of free Ca^{2+} (*a*) in control fiber segments, (*b*) in the same segments after partial extraction of TnC, and finally (*c*) after recombination of TnC into the segments. The extraction was done at $11\text{--}13^{\circ}\text{C}$ in 20 mM Tris, 5 mM EDTA, pH 7.85 or 8.3, a procedure derived from that of Cox et al. (1981. *Biochem. J.* 195:205). Extraction of TnC was quantitated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the control and experimental samples. Partial extraction of TnC resulted in reductions in tension during maximal Ca activation and in a shift of the relative tension-pCa (i.e., $-\log[\text{Ca}^{2+}]$) relationship to lower pCa's. The readdition of TnC to the extracted fiber segments resulted in a recovery of tension to near-control levels and in the return of the tension-pCa relation to its original position. On the basis of these findings, we conclude that the sensitivity to Ca^{2+} of a functional group within the thin filament may vary depending upon the state of activation of immediately adjacent groups.

INTRODUCTION

The regulation of contraction in vertebrate skeletal muscle involves the binding of calcium to troponin (Ebashi and Endo, 1968), a regulatory protein associated with the actin-containing thin filaments. Activation is thought to result from the binding of calcium to two low-affinity sites located on the troponin C (TnC) subunit of troponin, which through a series of events involving troponin I (TnI),

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troponin T (TnT), and tropomyosin results in the disinhibition of the thin filament. The molecular mechanism for activation of the thin filament may involve either a movement of tropomyosin from its relaxed position in which the interaction of myosin with actin is blocked (Haselgrove, 1972; Huxley, 1972) or the release of inhibition by troponin-tropomyosin of a kinetic step in the cross-bridge interaction cycle (Chalovich and Eisenberg, 1982). Calcium thus has a switch-like role in muscle contraction. In perhaps the simplest model of this action of calcium, the occupation of the binding sites on TnC activates a group of seven actin monomers that are overlapped by the tropomyosin molecule associated with a particular troponin complex. It is likely, however, that such a model does not fully represent the molecular events of thin-filament activation. Bremel and Weber (1972) have performed experiments involving myosin and regulated actin in solution, the results of which suggest that at low levels of MgATP ($\sim 10^{-6}$ M) there are cooperative effects caused by attached rigor cross-bridges within one functional group of seven actin monomers to allow attachment of nucleotide-bound cross-bridges to other actin monomers within that functional group. Measurements of tension as a function of MgATP concentration in crayfish skinned muscle fibers (Reuben et al., 1971) have demonstrated that tension development is indeed possible at low MgATP in the absence of activating calcium. Findings in this laboratory (Moss and Haworth, 1984) have indicated that in the same range of MgATP concentrations, significant amounts of muscle shortening occur. A possible interpretation of these results, consistent with the scheme proposed by Bremel and Weber, is that the formation of cross-bridge rigor bonds within a functional group of seven actins allowed cross-bridge cycling, albeit at a slow rate because of lowered MgATP, within the functional group. Brandt et al. (1980, 1982) have suggested further that in order to explain the steepness of the tension-pCa relationship at low calcium concentrations, many functional units on the thin filament may remain activated because of the presence of attached cross-bridges, even though calcium, after an initial period of binding, is no longer bound to the TnC associated with those functional groups. Alternatively, the steepness may be the result of an additional Ca-regulatory mechanism, possibly involving a Ca activation of the cross-bridges (Moss et al., 1983*b*). The calcium dependence of the MgATPase activity of myosin and regulated actin in solution is qualitatively different from that of tension development in skinned fibers in that the former relationship is steeper at high concentrations of calcium (Murray and Weber, 1980). In order to explain this phenomenon, Murray and Weber proposed that the number of actin monomers that are disinhibited per functional group is smaller at low than at high calcium concentrations.

In the present study, we have investigated further the molecular mechanism of thin-filament activation in mammalian skeletal muscle. Partial extraction of TnC from skinned single fibers resulted in marked rightward shifts of the tension-pCa relationship that were reversed by recombination of TnC into the fiber segments. This reduction in the Ca sensitivity of tension development after the extraction of part of the TnC content of these fibers suggests that there are physiologically relevant, cooperative interactions between adjacent functional groups along the thin filaments.

A report of this work was made at the Annual Meeting of the Biophysical Society (Greaser and Moss, 1984).

METHODS

Psoas muscles were obtained from adult male New Zealand rabbits. Bundles of ~50 fibers were stripped free while in relaxing solution (Moss, 1979) and were then tied with surgical silk to glass capillary tubes. The bundles were stored at -22°C in relaxing solution containing 50% (vol/vol) glycerol for 3–14 d before use. Individual fibers of up to 1.5 cm in length were then pulled from each bundle and mounted in the experimental chamber containing relaxing solution. The sarcomere length in the relaxed fiber segments was adjusted to $2.5\ \mu\text{m}$ by changing the overall segment length. The force transducer, motor, and solution-changing device were as previously described (Moss et al., 1983a). The fiber segments were activated in solutions containing various concentrations of free calcium between 0.1 and $10\ \mu\text{M}$, which are expressed as pCa's (i.e., $-\log[\text{Ca}^{2+}]$) in the present report. The relaxing and activating solutions were in most cases identical to those of Julian (1971). In some cases, noted in the text, solutions were used that contained 7 mM EGTA, 1 mM free Mg^{2+} , 20 mM imidazole, 6.28 mM total ATP, 14.5 mM creatine phosphate, various free Ca^{2+} concentrations, and sufficient KCl to yield an ionic strength of 180 mM. For the latter solutions, the computer program of Fabiato and Fabiato (1979) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex, based on the stability constants listed by Godt and Lindley (1982). The apparent stability constant for Ca-EGTA was corrected to 15°C (Fabiato and Fabiato, 1979) and for the effects of ionic strength (Martell and Smith, 1974). At any given pCa, a steady tension was allowed to develop, at which time the segment was rapidly (i.e., within 1 ms) slackened and subsequently relaxed. The difference between the steady developed tension and the tension baseline obtained immediately after the slack step was measured as total tension. Active tension was calculated as the difference between the total tension and the resting tension (usually $<1\ \text{mg-wt}$) measured in the same segment while in relaxing solution. Tensions (P) at submaximally activating levels of calcium were expressed as a fraction of P_0 , the tension obtained during maximal activation at pCa 5.0 (or pCa 4.5 in the solutions containing 7 mM EGTA). Every third or fourth contraction was performed at pCa 5.0 in order to assess any decline in fiber performance (Moss, 1979). In the experiments in which alterations in the tension-pCa relation after partial TnC extraction were measured, P_0 after extraction was found to decline by $\geq 50\%$. Submaximal tensions measured after the extraction of TnC were expressed as a fraction of P_0 obtained under the same conditions.

The relationship between relative isometric tension and pCa was measured in any one fiber segment first before any treatment (i.e., control), then in the same segment after partial extraction of TnC, and finally after recombination of TnC into the segment. Because of the large number of contractions involved in such an experimental protocol and the tendency for skinned fibers to deteriorate after repeated activations (Moss, 1979), we were not always able to obtain tension data under all three experimental conditions in a given fiber segment. The partial extraction of TnC was done by bathing the fiber segments in a solution containing 20 mM Tris, 5 mM EDTA, pH 7.85 (Cox et al., 1981), at $11\text{--}13^{\circ}\text{C}$ for ~60 min (i.e., standard extraction conditions). TnC was recombined into the fiber segments by bathing the segments in relaxing solution containing ~1 mg/ml purified TnC (prepared by the method of Greaser and Gergely, 1971). This soak was continued for 40 min at 15°C (Moss et al., 1982) and was followed by two successive 5-min washes in relaxing solution in order to remove excess TnC. In most fiber preparations, TnC extraction and recombination were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Giulian et al., 1983), with a

single modification. Rather than a 30-min fixation of the gel in a solution containing glutaraldehyde, the fixation was continued for 12 h before silver staining to improve retention of LC₃ (Kushmerick, M., personal communication) and TnC within the gel matrix. In those instances in which SDS-PAGE was done, the fiber segment was divided into three pieces of approximately equal lengths. The first of these was dissolved in SDS-containing sample buffer (Giulian et al., 1983). The other two pieces were tied into the experimental chamber; one was between the force transducer and motor and was used for physiological measurements; the other was tied at both ends to the motor arm so that it was exposed to the same solutions as the experimental segment. After the treatment to partially extract TnC, the segment tied only to the motor arm was removed and dissolved in sample buffer. Finally, after the recombination of TnC and the subsequent determination of the relative tension-pCa relation, the experimental segment was dissolved in sample buffer.

Since we had no means to measure absolute amounts of protein within the fiber segments, it was necessary to express TnC as a fraction of other myofibrillar proteins thought to be unaffected by the extraction conditions, as we have done previously for subunits of other contractile proteins (Moss et al., 1982, 1983a). After the staining and gel-drying procedures, the gels were scanned using a laser-light scanning densitometer, described previously (Giulian et al., 1983). The relative amount of TnC present in any given fiber segment was taken as the ratio of the area of the peak corresponding to TnC to that corresponding to TnI. TnI was selected for normalization so that there was virtually no change in tension in the relaxing solution after the extraction procedure, which indicates that TnI was not removed during this procedure. Had TnI been extracted, tension in the relaxed segment would have been expected to increase because of disinhibition of the thin filament (see Greaser and Gergely, 1981). Additional evidence that the TnI content is unaffected by the extraction procedure was obtained by SDS-PAGE of the extracting solution that bathed several fiber segments during the extraction procedure, which is described in the Results. There was no evidence of TnI in the extractant after the extraction procedure. Quantitatively similar results in terms of TnC content were obtained by using LC₁ or total light chains rather than TnI. After extraction, the ratio of the area of the peak corresponding to LC₂ to the sum of the areas corresponding to LC₁ and LC₃ was always within 10% of control values. This finding provides further evidence that the contents of the other myofibrillar proteins were minimally affected by the extraction procedure.

RESULTS

SDS-PAGE of Single Fiber Segments

Extraction of TnC was verified by SDS-PAGE of segments of the same skinned fiber before treatment, after partial extraction of TnC, and in many cases after recombination of TnC into the fiber (Fig. 1, lanes 1-3). The gels were subsequently stained using an ultrasensitive silver stain (Giulian et al., 1983). A previous report from this laboratory (Giulian et al., 1983) indicated that staining of skeletal muscle TnC with silver was not linear; however, linear staining of TnC was achieved in the present study through modification of the gel fixation procedure (described in the Methods). With this protocol, linear staining of purified samples of skeletal (or cardiac; not shown) TnC is possible in amounts between 5 and 40 ng per sample (Fig. 2). The amount of TnC in our fiber samples was ~27 ng, if it is assumed that myofibrillar protein constitutes 12% of

fiber weight and that TnC accounts for 1.3% of total myofibrillar protein (Yates and Greaser, 1983*a, b*). This amount of TnC is well within the range of linear staining, which provides a firm basis for correlating decreases in TnC content and maximum tension development in a given fiber segment.

To determine whether the fiber contents of proteins other than TnC were affected by the extraction procedure, SDS-PAGE was also performed on the solution that bathed the pooled fiber segments during the extraction (Fig. 1,

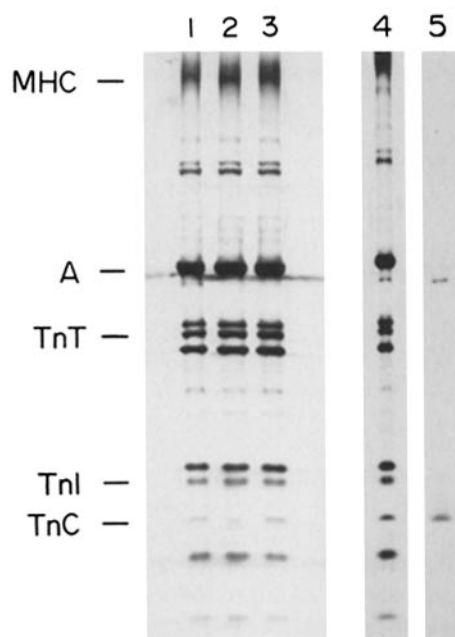


FIGURE 1. Lanes 1–3: silver-stained, SDS-polyacrylamide gel of segments of the same single fiber before the extraction procedure (lane 1), after partial extraction of TnC (lane 2), and after recombination of TnC (lane 3). The segment length in each case was ~ 3 mm. Lanes 4 and 5: SDS-polyacrylamide gel demonstrating the protein content of the extracting solution after the extraction procedure. A control fiber segment 3.0 mm in length was run in lane 4. Four fiber segments totalling 12 mm in length were then bathed in $24 \mu\text{l}$ of extracting solution for 2 h as described in the Methods. $6 \mu\text{l}$ of the extracting solution was then diluted with $6 \mu\text{l}$ of sample buffer and run in lane 5. Abbreviations used were: MHC, myosin heavy chain; A, actin; TnT, troponin T; TnI, troponin I; TnC, troponin C. Lanes 1–3 and lanes 4 and 5, respectively, were run on two different gels.

lanes 4 and 5). To ensure that the amount of extracted protein would be within the range of detection of our staining procedure, these segments were extracted in microcentrifuge tubes (0.5 ml total volume) containing $6 \mu\text{l}$ of extracting solution for each 3-mm length of fiber in the tube. Thus, after extraction, $6 \mu\text{l}$ of the extracting solution should contain an amount of protein equivalent to that extracted from the 3-mm lengths of fiber that were used in the mechanical experiments. From these gels, it is apparent that TnC is the only major myofi-

brillar protein (except for soluble myoplasmic proteins) that is extracted from the fibers by this procedure. An additional, unidentified protein was also extracted by this procedure; however, it is unlikely that this protein was involved in the effects on tension reported in this paper, since these effects were entirely reversed by recombination of TnC into the fiber segments (described below).

Effect of Partial Extraction of TnC upon Tension Development

Tension traces obtained during maximum Ca^{2+} activation are shown for a single fiber segment in Fig. 3. In this particular case, the steady isometric tension under control conditions was 1.65 kg/cm^2 , and it declined to 0.543 kg/cm^2 after the standard procedure for TnC extraction. Tension recovered to 1.53 kg/cm^2 after the soak in TnC-containing relaxing solution. Tension data from several fiber

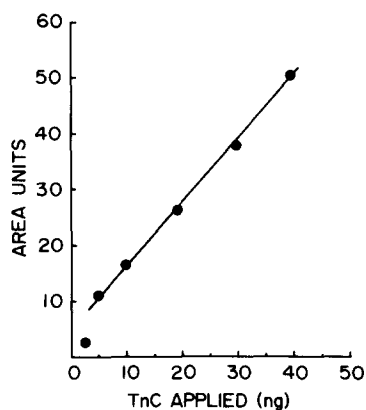


FIGURE 2. Plot of intensity of silver stain as a function of the amount of TnC applied to an SDS-polyacrylamide gel. Intensity is expressed in arbitrary units measured as the areas under the peaks on the corresponding densitometric scans. The straight line was fitted to the data points between 5 and 40 ng inclusive by the least-squares error method.

segments during maximal Ca^{2+} activation indicated that tension at $\text{pCa } 5.49$, as a percent of the control P_o , declined by $\sim 68\%$ after the standard extraction procedure. The recovery of tension after TnC recombination occurred on the average to within 10% of the control P_o value. It was possible to vary the percent reduction in tension by changing the duration of the extraction, with greater reductions occurring after longer extraction times (Fig. 4). It was possible to accelerate the extraction of TnC greatly by raising the pH of the extracting solution to 8.5 (Fig. 4). However, fibers that were bathed in this solution were not as mechanically robust during subsequent activations as those subjected to pH 7.85 during the extracting procedure. Thus, pH 8.5 was used only infrequently. In all of the experiments in which alterations in the tension-pCa relation were investigated, an extraction time of ~ 60 min was used.

In the several fibers in which the tension-pCa relationship was studied, the maximum Ca-activated tension was found to decrease to 41.9% ($N = 12$; $SD = 7.7$) of control P_o after the extraction procedure, whereas the TnC content

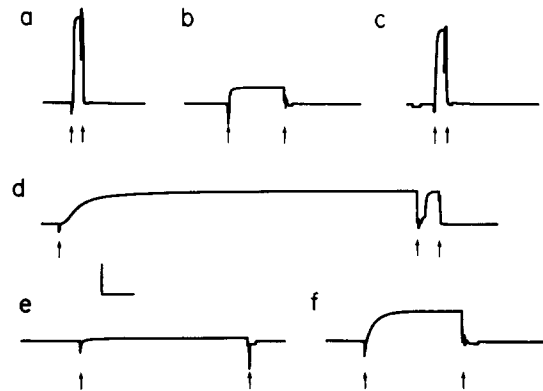


FIGURE 3. Original records of isometric tension developed by one fiber segment before and after partial extraction of TnC and after readdition of TnC. Steady tensions as a fraction of the maximum Ca-activated tension in the control segment were as follows:

	pCa 5.49	pCa 6.51	P6.51/P5.49
Control	1.00 (a)	0.40 (d)	0.40
TnC extracted	0.32 (b)	0.06 (e)	0.19
TnC re-added	0.93 (c)	0.41 (f)	0.44

decreased to 57.8% (SD = 13.1) of the value obtained from the control pieces of the corresponding segments. Examples of tension traces for a single fiber segment activated in solutions of pCa 6.51 are shown in Fig. 3. In this particular instance, P/P_0 was 0.40 under control conditions, but declined to 0.19 after partial extraction of TnC. Recombination of TnC into the fiber segment resulted in a reversal in P/P_0 to nearly the control value. The relative tension-pCa data from all the fiber segments are shown in Fig. 5. The tension-pCa relationship under control conditions is similar in form to that obtained previously in this laboratory (Moss et al., 1983b). The Hill plot transformation of these data (Moss

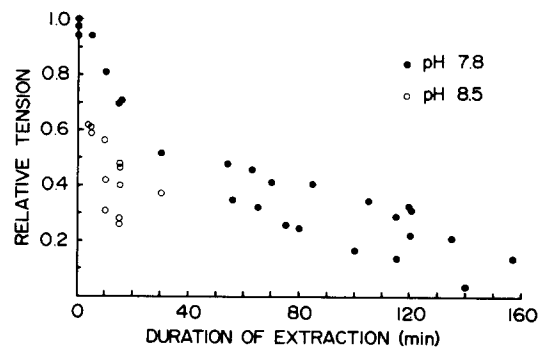


FIGURE 4. Plot of steady isometric tension as a function of the duration of extraction at two different pH's. Tension in each case was measured in solution of pCa 5.49. In a given fiber, tension was expressed as a fraction of the tension developed in the same fiber before extraction (i.e., duration of 0 min).

et al., 1983b) resulted in a plot that could conveniently be treated as two straight lines. The Hill coefficient (n) for $P/P_o > 0.5$ was 1.73, which agrees well with our previously reported value in rabbit psoas muscle (Moss et al., 1983b); for $P/P_o < 0.5$, n was 5.43. After the partial extraction of TnC from these fiber segments, the tension-pCa relation underwent a rightward shift by ~ 0.3 pCa unit, assessed at $P/P_o = 0.50$. The shape of the relationship for $P/P_o > 0.5$ was little changed after the extraction, the value of n being 1.67. However, for $P/P_o < 0.5$, the relationship had a distinctly lower slope ($n = 3.59$) after the extraction procedure.

Reversibility of the Effects of Partial TnC Extraction on the Tension-pCa Relation

The degree of reversibility of the effects of TnC extraction was investigated in several fiber segments by recombining TnC into previously extracted fibers. The example shown in Fig. 6 is typical of the results that were obtained. The

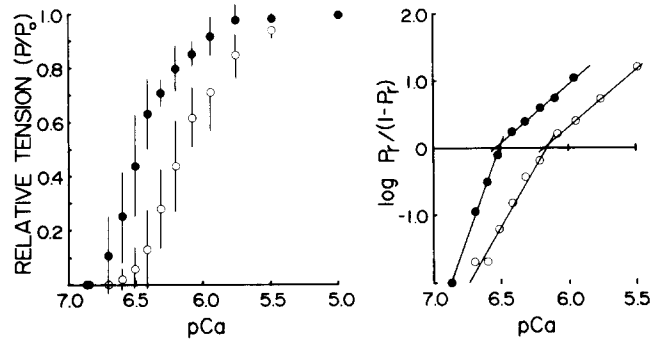


FIGURE 5. Plots of the dependence of tension on pCa before (●) and after (○) partial extraction of TnC. In A, tension at each pCa is expressed as a fraction of the tension developed by the same fiber at pCa 5.0. Error bars indicate 1 SD. In B, the relative tension-pCa data have been transformed to yield a Hill plot.

extraction procedure resulted in a rightward shift of the tension-pCa relation, as well as a reduction in the steepness of the relationship at high pCa's. The readdition of TnC to the fiber segments, which was confirmed by SDS-PAGE (Fig. 1), resulted in a leftward shift of the tension-pCa relation to approximately the control position and recovery of the steepness seen in the control data. Thus, the effects of the extraction procedure on this relationship appear to be a highly specific result of the loss of TnC from the fiber segments.

A notable feature of the tension-pCa data obtained after TnC recombination is the tendency of many of the relative tensions at pCa 6.51 to lie slightly above the corresponding control data point. This phenomenon was highly variable in terms of the magnitudes of the observed differences, ranging from no difference in most fibers to substantial differences in a small number of fibers. In the latter case, the recombination of TnC was accompanied by an increase in the maximum Ca-activated tension relative to the control value in the same fiber segment. A likely explanation for these results is that the TnC content of some fiber segments was partially depleted. An immediately apparent mechanism for such a depletion

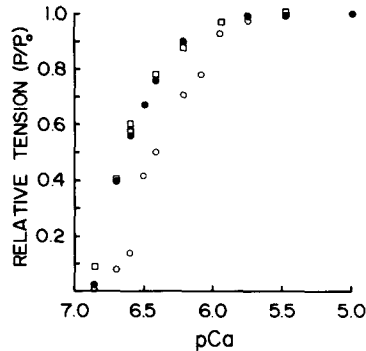


FIGURE 6. Relative tension-pCa data from a single fiber segment demonstrating the reversibility of the effects on tension development caused by partial extraction of TnC. Data were obtained before treatment (●), after the extraction procedure (○), and after recombination of TnC into the fiber segment (□). Tensions measured under each condition are expressed as a fraction of that developed at pCa 5.0 under the same condition.

would be a dissociation of TnC from the fiber segments during storage in the glycerol-containing relaxing solution. Once we became aware of the apparent TnC depletion, control fibers were routinely bathed in the TnC-containing relaxing solution before obtaining a baseline tension-pCa relationship. The data of Fig. 7 were obtained from a fiber segment that demonstrated a 20% increase in P_0 after the addition of TnC to the otherwise untreated fiber segment. This was the largest such increase that was observed and it was accompanied by a small leftward shift of the tension-pCa relation relative to control. A subsequent partial extraction of TnC resulted in a shift of the relation that was substantially to the right of that obtained in the untreated control segment. The occurrence of partially TnC-deficient segments such as this did not correlate well with time in storage, which argues against a slow, continual dissociation of TnC or proteo-

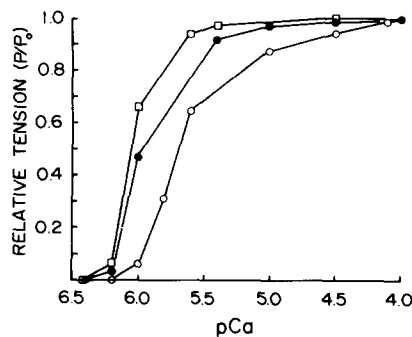


FIGURE 7. Relative tension-pCa data from a single fiber segment demonstrating a likely TnC deficiency under control conditions. Data were first obtained before treatment (●), then after TnC addition (□), and finally after partial TnC extraction (○).

lytic degradation under these conditions. Rather, the incidence of the phenomenon was high in certain rabbits and was virtually zero in most rabbits. While we have been unable to determine the cause of TnC depletion, its undetected occurrence may at least in part explain the variability in P_o values and relative tension-pCa relationships obtained in different fibers and by different investigators.

Tension-pCa Relationships Obtained in Solutions with Increased Ca-buffering Capacity

Although the majority of the measurements performed in this study involved the use of solutions containing 4 mM EGTA (Julian, 1971), as has been routine practice in this laboratory (e.g., Moss et al., 1983a, b), several experiments of a

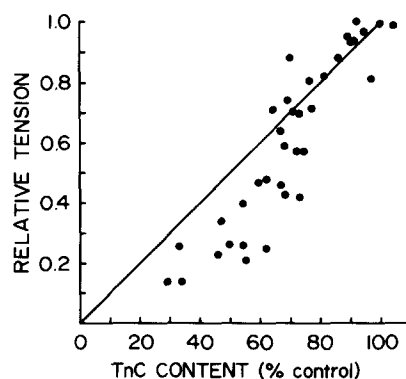


FIGURE 8. Maximum Ca-activated tension and TnC content measured in the same fiber segments after partial extraction of TnC. In each case, tension has been expressed as a fraction of the maximum Ca-activated tension developed by the same segment before extraction. In similar fashion, TnC content has been referenced to the TnC content before extraction. The latter was done by forming the quotient of the TnC/TnI ratios under the two conditions and multiplying the result by 100. The straight line is the predicted relationship between these variables, assuming that adjacent functional groups are independently activated by Ca^{2+} .

confirmatory nature were done using solutions containing 7 mM EGTA and an ATP-regenerating system, as described in the Methods. The data in Fig. 7 were obtained in these solutions and demonstrate the basic features of the effects of partial TnC extraction seen previously in Figs. 5 and 6 in terms of both the rightward shift of the tension-pCa relation and the decrease in the steepness of the relationship at high pCa's.

Relationship Between Maximum Ca-activated Tension and TnC Content After Partial Extraction

If Ca^{2+} binding to TnC serves as a simple switch to activate a functional group of seven adjacent actins associated with a single troponin-tropomyosin complex, the fall in P_o following the TnC extraction procedure should be proportional to the reduction in TnC content. Alternatively, there may be interactions between

adjacent or overlapping functional groups, as suggested by Murray and Weber (1980). In such a case, the number of actins within a functional group that are made available for interaction with myosin will vary depending on the state of activation of immediately adjacent functional groups. In this situation, it is unlikely that the relationship between the maximum Ca-activated tension and the TnC content would be linear. Experiments were done in an attempt to distinguish between these possibilities.

The maximum Ca-activated tension was measured in 36 fiber segments before and after extraction for periods of between 0 and 160 min in solutions of pH 7.85. The TnC content was measured in each of these same fibers by SDS-PAGE of an untreated segment of fiber and of the extracted segment after the tension measurement. A plot of tension vs. TnC content is shown in Fig. 8, in which both variables have been normalized to control values obtained in the same fiber segments. The relationship appears to be nearly linear for a TnC content between ~70 and 100% of control. However, for a TnC content between ~70 and 30% of control, all of the data points lay below the linear relationship. Thus, in this region, tension dropped to a greater extent than did the content of TnC.

DISCUSSION

The main findings of the present study are that partial extraction of TnC from mammalian skinned skeletal muscle results in a reduction in the maximum Ca-activated tension, and that the relationship between tension and pCa at submaximally activating levels of calcium is shifted to lower pCa's, i.e., the muscle fiber apparently becomes less sensitive to activating calcium. Both of these effects are reversed by the recombination of TnC into the fiber segments. Although the reductions in P_o might be explained at least in part on the basis of a simple reduction in the number of available actin sites after extraction, the observed decreases in tension at low levels of calcium were disproportionately much greater than those for P_o , which suggests that tension development is not a linear function of the number of functional groups that are activated.

Murray and Weber (1980) have suggested, based on the slow initial rise of the relationship between actin-activated MgATPase activity and $[Ca^{2+}]$ in solutions of myosin S-1 and regulated actin, that the number of actin binding sites that are disinhibited is smaller per functional group at low than at high $[Ca^{2+}]$. This is presumably a result of interactions between adjacent functional groups along the thin filaments, possibly as a result of the overlap of the end regions of adjacent tropomyosins. Thus, at low $[Ca^{2+}]$, the activation of a single functional group would result in disinhibition of perhaps four or five actin monomers, out of a possible seven, because of a failure of the end portions of the associated tropomyosin to move from their relaxed, or inhibiting, positions. Only when adjacent functional units are activated and their tropomyosins undergo the appropriate change in conformation will the endmost monomers of the first functional group be activated. The likelihood that adjacent groups will simultaneously be Ca-activated would of course be increased at higher $[Ca^{2+}]$. A prediction of such a model is that the upper portion of the tension-pCa relation should be steeper than the lower portion, which would reflect a greater number of actin monomer disinhibitions per increment of $[Ca^{2+}]$. Our own data (Figs. 5–

7) indicate that in rabbit fast-twitch skeletal muscle, the tension-pCa relationship is steeper at lower levels of calcium, corresponding to a P/P_o of less than ~ 0.5 , which suggests in fact that the number of actin monomers disinhibited per functional group is greatest at low levels of Ca^{2+} . The greater steepness of the tension-pCa relation vs. the ATPase-pCa relation is apparent in the data of Levy et al. (1977), who measured tension and ATPase activity in the same skinned skeletal muscle fibers. Shiner and Solaro (1982) have suggested a model for Ca^{2+} regulation that qualitatively predicts these differing dependences of ATPase and tension on Ca^{2+} , which in their scheme result from cross-bridge binding within one functional group altering the Ca^{2+} binding by neighboring TnC's. Hill's (1983) recent model for Ca^{2+} regulation of contraction incorporated cooperativity in cross-bridge binding as a result of interactions between the ends of neighboring tropomyosin molecules. Consistent with our results, this model predicts greater steepness in the tension-pCa relation at low $[\text{Ca}^{2+}]$ when it is assumed that tropomyosin does not move at all until Ca^{2+} is bound at low-affinity sites on the associated TnC.

In considering comparisons of mechanical and biochemical data, a necessary caution is that measurements of actomyosin ATPase activity and tension do not necessarily yield information about exactly the same step(s) in the cross-bridge cycle. On the basis of earlier work (Gordon et al., 1966), tension provides a measure of the numbers of attached cross-bridges, whereas actomyosin ATPase activity is more a reflection of the rate of cross-bridge turnover (see, for example, Barany, 1967). Another difference is that mechanical measurements are done on muscle preparations that have intact filament lattices, such that cross-bridges are held in a preferred orientation with respect to thin-filament binding sites, and the cross-bridges are under mechanical load. Both of these factors might be expected to affect the rates of cross-bridge attachment and detachment. On the other hand, in *in vitro* measurements of ATPase activity, the orientation of cross-bridge heads with respect to actin would be expected to be random in solutions of acto-S1 or acto-heavy meromyosin. Myofibrillar ATPase activities are perhaps more physiological with regard to subunit orientation, although in this case the myofibrils would be expected to be greatly shortened so that overlap of thin filaments within a sarcomere would interfere with cross-bridge formation (see, for example, Gordon et al., 1966). Thus, it is by no means certain that absolute agreement between the two sets of studies can be expected.

It is interesting that the relationship between maximum Ca-activated tension and TnC content (Fig. 8), at least to the extent that the latter value reflects the degree of activation, has a shape that is similar to that which would be predicted by the model of Weber and Murray. The data of Fig. 8 were obtained under maximal Ca activation, unlike the tension-pCa data. According to our above hypothesis, the relationship shown in Fig. 8 should actually be steeper at low levels of TnC content than at high. The possibility exists, therefore, that the relationships between tension and pCa and between tension and TnC content reflect two separate underlying phenomena. An appealing conclusion, though at this point tentative, is that in the intact filament lattice, Ca^{2+} is involved in the activation of a process in addition to the disinhibition of the thin filament,

possibly involving a Ca activation of the cross-bridges, as we have suggested previously (Moss et al., 1983*b*). Such a process would not be apparent in the solution biochemical measurements in which the collisions of actin and myosin are random as a result of diffusion. In the experiments in which the TnC content was reduced (Fig. 8), Ca^{2+} was maintained at maximal levels and would thus be expected to be well above threshold for any secondary Ca-activated processes. If an additional Ca-modulated process is operative in intact muscle, then it could very well be that the model of Murray and Weber (1980) for thin-filament activation is correct for dissociated systems of contractile proteins, but that the tension-pCa relation represents the superimposition of the second process on thin-filament regulation.

The partial extraction of TnC resulted in a rightward shift in the tension-pCa relation, giving the appearance that the contractile system is less sensitive to Ca^{2+} under these conditions. Brandt et al. (1984) reported that extraction of 40–50% of the total TnC from rabbit psoas fibers resulted in a decrease in the Hill coefficient from 5–8 to ~ 2 . In the present study, there was no change in the Hill coefficient for $P/P_0 > 0.5$. The steepness of the tension-pCa relation in this range of relative tensions appears to be directly related to the number of low-affinity Ca^{2+} binding sites on TnC, since the substitution of slow muscle TnC into fast-twitch skeletal muscle fibers halves the Hill coefficient in this region (Moss, R. L., M. R. Lauer, G. G. Giulian, and M. L. Greaser, manuscript submitted for publication). This suggests that, at least for $P/P_0 > 0.5$, there is maximum cooperativity between the low-affinity sites in fast-twitch muscle, which is consistent with the idea that Ca^{2+} must be bound at both sites in order to induce movements of tropomyosin. For $P/P_0 < 0.5$, there was a decrease in the Hill coefficient after the extraction procedure, which indicates a decrease in cooperativity in this range of relative tensions. As suggested by Brandt et al. (1984), this may result from a simple blockade of the spread of cooperativity along the thin filament caused by the presence of permanently inactive functional groups. Consistent with this point of view, a recent report (Grabarek et al., 1983) has indicated that there is a high degree of cooperativity in Ca^{2+} binding at the low-affinity sites on TnC. Since in the presence of myosin the Hill coefficient for Ca binding becomes > 2.0 , these authors suggest that more than two Ca^{2+} binding sites are involved under these conditions and that these sites are distributed among troponins on the same thin filament and interact via intervening tropomyosins. Under no circumstances did Grabarek et al. observe the cooperativity of calcium binding to be as great as the cooperativity observed in the Ca regulation of MgATPase activity in the presence of regulated actin ($n = \sim 4$), which leads to the conclusion that there seems to be no direct correlation between the binding of Ca^{2+} to the low-affinity sites of TnC and Ca activation of ATPase activity (also see Shiner and Solaro, 1982).

Calcium activation of tension development in skinned skeletal muscle fibers is still more cooperative than ATPase activity (Levy et al., 1976). The involvement of an additional Ca-regulatory process (or processes) could help to explain these differences and could be envisioned to entail cooperativity of cross-bridge attachment (Brandt et al., 1980, 1982), as described earlier, or possibly a direct calcium

activation of cross-bridges, as hypothesized by Moss et al. (1983*b*). Brandt et al. (1980, 1982) have suggested an alternative mechanism to explain the relative steepness of the tension-pCa relation at low levels of Ca^{2+} . Assuming that cyclic binding and release of Ca^{2+} at the low-affinity sites on TnC occur more rapidly than the dissociation of attached cross-bridges from actin, these investigators concluded that a precise relationship between tension and pCa might not be expected, since some functional groups would contain bound myosin even though both low-affinity Ca-binding sites may not always be occupied. In particular, they hypothesized that cross-bridge stress decreases the rate of cross-bridge cycling, so that at high forces there would be more force produced in proportion to Ca^{2+} than at low forces, thereby making the tension-pCa relation appear quite steep. While the results of the present study do not distinguish between these mechanisms, it is unlikely that the rightward shift in the tension-pCa relation after partial TnC extraction is the result of an increase in cross-bridge cycling rate, as would be deduced from the model of Brandt et al. (1982), since in preliminary studies (unpublished) we have found that the velocities of shortening at intermediate pCa's are actually reduced after extraction.

The authors would like to thank Drs. A. M. Gordon, X. Grabarek, and P. J. Reiser for their helpful comments on an earlier version of the manuscript. We are grateful to Carrie Poole for preparation of the manuscript.

This work was supported by grants from the National Institutes of Health (HL25861 and AM31806), and the School of Medicine and the College of Agriculture and Life Sciences of the University of Wisconsin. This work was done during the tenure of an Established Investigatorship (to R.L.M.) from the American Heart Association and with funds contributed in part by the Wisconsin affiliate.

Original version received 8 January 1985 and accepted version received 22 April 1985.

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