Inhibition of Contraction of Cultured Muscle Fibers Results in Increased Turnover of Myofibrillar Proteins But Not of Intermediate-Filament Proteins

NANCY J. CRISONA and RICHARD C. STROHMAN
Department of Zoology, University of California, Berkeley, California 94720.

ABSTRACT Muscle fibers are maintained in culture in a fully contractile state and are relaxed by the addition of 10^{-7} M tetrodotoxin (TTX). This toxin binds to muscle membrane Na^+-channels, abolishes spontaneous contractions and causes failure of the fiber to accumulate myosin heavy chains. These effects are reversible on removal of TTX. Synthesis and accumulation kinetics have been obtained for myofibrillar and for cytoplasmic filament proteins in normal, active muscle and in TTX-relaxed muscle fibers in culture. In relaxed fibers the synthesis of most proteins remained normal or slightly elevated. However, the accumulation of all myofibrillar proteins examined was markedly inhibited in TTX-treated cultures, whereas the accumulation of cytoplasmic filament proteins was normal or slightly elevated. Myofibrillar proteins examined were α-actin, troponin-C, myosin fast light chain 1, myosin fast light chain 2, α,β-tropomyosins and the phosphorylated forms of tropomyosin and fast light chain 2. Cytoplasmic filament proteins studied were vimentin, α,β-desmin and β,α-actin. We also examined the synthesis and accumulation of six unidentified muscle-specific proteins and nine unidentified nonmuscle-specific proteins. Most of these proteins showed a normal accumulation pattern in TTX-relaxed fibers. We concluded that muscle fibers made inactive by TTX display an increased instability of all myofibrillar proteins while cytoplasmic filament proteins and cytoplasmic proteins in general are relatively unaffected. We suggest that TTX interferes, in a manner as yet unidentified, with assembly and normal stability of myofibrils. Decreased assembly and/or increased instability of myofibrils would lead to increased rates of myofibrillar protein degradation.

Differentiated skeletal muscle fibers in vitro can respond to varying activity demands placed upon them. Cultured muscle fibers will exhibit hypertrophy as measured by myosin heavy chain accumulation if the fibers are electrically stimulated (1). Hypertrophy will also be produced by mechanical stretch of fibers cultured on elastic membranes (2); this effect now appears to be mediated by a mechanical activation of plasma membrane sodium pumps (3). Work from this laboratory has shown a strong correlation between cultured fiber inactivity and decreased accumulation of myosin heavy chains (MHC). For example, if normal fibers are depolarized with 12 mM external K^+ they will not contract spontaneously and they will fail to accumulate myosin (4). We have demonstrated the same hypotrophic effect for cultured muscle fibers treated with tetrodotoxin (TTX). TTX and high $[K^+]$ do not interfere with myogenic cell fusion or with myofibrillar protein synthesis but produce three- to fivefold increases in myosin heavy chain turnover (5, 6). Thus, two distinctly different reagents, high external $[K^+]$ and TTX, both of which act on the muscle plasma membrane, produce instability in myofibrillar peptides. TTX acts by binding to muscle membrane Na^+-channels (7) while high external $[K^+]$ will depolarizes the fiber membrane preventing action potentials (8). Both treatments produce fiber relaxation and both are reversible within 24–48 h. Relaxation-induced MHC instability appeared to be relatively specific since general protein synthesis and degradation rates were only marginally affected by the two treatments (4, 6).

We were interested therefore to see whether TTX-induced relaxation affected other myofibrillar peptides. Using a two-dimensional gel electrophoresis system, we have now examined

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the synthesis and accumulation of 40 different muscle peptides in control and in TTX-treated muscle cultures. Our results show that relaxation by TTX produces a specific and coordinated increased turnover of all contractile proteins examined, with little or no effect on cytoskeletal or nonmuscle specific proteins. We speculate that inhibition of unidentified plasma membrane events interferes with normal myofibril assembly and that, consequently, newly synthesized but unassembled myofibrillar peptides exhibit increased rates of degradation.

MATERIALS AND METHODS

Myogenic Cultures: Myoblasts were isolated from breast tissue of 11.5-d chicken embryos, and cultures were prepared as previously described (6). After 2 d in culture, cytosine arabinoside (araC) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) was added to the culture medium at a final concentration of 5×10^{-6} M. Tetrodotoxin (TTX, Calbiochem-Behring Corp.) was added at the same time at a final concentration of 1.6×10^{-7} M.

For protein accumulation experiments, cultures were plated as usual. 24 h after plating, the medium was removed and replaced with medium containing radio-labeled leucine and 10% or 20% of the usual concentration of cold leucine (4×10^{-4} M) in the modified Eagle's medium (MEM). The decreased cold leucine had no effect on the viability or development of the cells and served to increase the specific activity of the radio-label in the cell lysates. Control cultures were usually labeled with [^{3}H]leucine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY; 60 Ci/mmol) at a final concentration of 10 μCi/ml and TTX cultures with [^{14}C]leucine (Amersham Corp., Arlington Heights, IL; L-[U-^{14}C]-leucine, 339 mCi/mmol) at a final concentration of 2.5 μCi/ml. Reversing the radiolabels or using different concentrations of cold leucine had no effect on the results. 2 d after plating, araC was added to all dishes and TTX to half of them. 3 d after plating and every 2 d thereafter, the cells were re-fed with the same medium without araC. We determined that it takes 2 d for the specific activity of total protein to reach equilibrium under these labeling conditions and that this specific activity remains constant with refeeding every 2 d.

For protein synthesis experiments, cells were grown in the usual complete medium. AraC and TTX were added at day 2. The cells were re-fed at day 3 and every 2 d thereafter with complete medium without araC and with TTX where appropriate. For protein synthesis measurement the cells were pulse labeled for 1 h in leucine-free MEM (2 ml for 100-mm dishes, 1 ml for 60-mm dishes) containing TTX where appropriate. Control cells were labeled with 100 μCi/ml of [^{3}H]leucine and TTX cells with 30 μCi/ml of ^{14}C-leucine. Reversal of the labels did not affect the results.

Preparation of Cell Lysates: Total cell lysates were prepared by the method of Garrels and Gibson (9). Volumes are for 100-mm culture dishes. Cultures were placed on ice, the medium was removed, and the dishes were washed with 3 × 10 ml of cold PBS (8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4 \cdot 12 H_2O$, 0.2 g KH_2PO_4/l solution). Cells were scraped from the dish in 200 μl of staphylococcal nuclease (50 μg/mml in 2 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.8) and collected at the edge of the dish. SDS and 2-mercaptoethanol were added to final concentrations of 0.2% and 2.0%, respectively. The lysate was homogenized by pipetting up and down in a Pipetteman. Then 20 μl of DNAse-RNAse mix were added (1 mg/ml DNAse I, 0.5 mg/ml RNAse A, 50 mM $MgCl_2$, 0.5 M Tris-HCl, pH 7.0). The lysate was left on ice for 5 min and then homogenized again. The lysates were dried in a vacuum centrifuge and immediately resuspended in lysis buffer A of O'Farrell (10). The lysates were stored in aliquots at −70°C.

Determination of Total Protein Concentration and Specific Activity: Total protein in the lysates was determined by the method of Bradford (11). Samples were first diluted 1:10 with 5M urea to reduce the concentration of interfering reagents in the lysis buffer.

For determination of radioactivity, 2-μl aliquots of the lysates were spotted on Whatman 3MM filter paper disks, precipitated batchwise in cold 10% trichloroacetic acid (TCA), washed three times with cold 7.5% TCA, rinsed with cold 100% ethanol, and dried. The filters were counted in 10 ml of cocktail (15.16 g Omnifluor per gallon of toluene) plus 0.75 ml of 90% NCS (Amersham Corp., Arlington Heights, IL).

Specific activity of total protein was determined to verify constant labeling conditions for the accumulation experiments.

Two-dimensional Gel Electrophoresis: The two-dimensional gel electrophoresis system combines features of the methods of O'Farrell (10) and Garrels (12).

Isoelectric focusing (IEF) was carried out in 17 cm × 1.5 mm I.D. glass tubes prepared according to Garrels (12). The first-dimension IEF gel consisted of 2.7% acrylamide-*bis* acrylamide, 9.5 M urea, 2% Nonidet P-40 and 2% ampholytes, pH 5–8 (LKB Instruments, Broma, Sweden). IEF gel solutions were prepared according to Garrels (12), frozen at −70°C, and used within 2 wk. All other solutions used were those described by O'Farrell (10). 15-cm IEF gels were routinely run at 400 V for 20 h and 800 V for 2 h after prefocussing. The gels were removed from the tubes by means of an air jet and kept at −70°C in 0 buffer (O'Farrell [10]) until use.

The second dimension was carried out on a 12% SDS polyacrylamide slab gel (20 cm × 23 cm × 1.5 mm) without a stacking gel. The IEF gel was thawed, equilibrated for 5 min in fresh 0 buffer, loaded onto the slab gel, and overlayed with 0.9% agarose in 0 buffer containing bromphenol blue. Electrophoresis was carried out at 3 W/gel until the dye marker reached the end of the gel (~7 h). Gels were stained overnight in 0.01% Coomassie Blue, 40% methanol, 5% acetic acid. After destaining, the gels were washed well in water and prepared for fluorography by soaking for 30 min in fresh 1 M sodium salicylate according to Chamberlain (13).

The gels were dried, fluorograms prepared, and protein spots of interest excised from the gels. The gel pieces were solubilized in 0.75 ml of 90% NCS and heated in an oven for 2 h at 50°C. 10 ml of cocktail (15.16 g Omnifluor per gallon of toluene) were added to each vial, the vials left overnight, and ^{3}H and ^{14}C radioactivity counted.

300 μg of protein were routinely loaded onto first-dimension gels, 150 μg each from control and TTX cultures. In synthesis experiments, 150 μg of protein represented ~300,000–400,000 cpm each of ^{3}H and ^{14}C. In accumulation experiments, 150 μg of protein was equivalent to $\sim 1 \times 10^6$ cpm each of ^{3}H and ^{14}C.

RESULTS

Accumulation of Proteins in Control and TTX-treated Cultures

Previous experiments in this laboratory demonstrated that inhibition of contraction in cultured muscle fibers caused by addition of TTX to the culture medium resulted in a significant decrease in the accumulation of MHC without a concomitant decrease in its synthesis (5, 6). We wanted to determine whether the accumulation of other proteins was also affected by chronic relaxation of the muscle fibers. Although we had found that there was only a slight increase in the rate of degradation of total protein in TTX-treated cultures, it was possible nevertheless that there was a significant effect on the degradation of other myofibrillar proteins in addition to MHC. The identification of such proteins could provide an insight into the mechanism by which specific protein degradation is controlled.

To quantify the accumulation of specific proteins, we selected approximately 40 protein spots on two-dimensional (2-D) polyacrylamide gels of muscle cell lysates for analysis and grouped them into three classes. The first class consists of nine peptides known to comprise part of the myofibrillar structure and to play a role in the contraction process itself. This class will be referred to as the "Contractile" proteins and includes α-actin, the α- and β-subunits of tropomyosin (α-TM and β-TM) and the phosphorylated forms of each (α-TM-P and β-TM-P), fast myosin light chains 1 and 2 (LCF1 and LCF2) and the phosphorylated form of 2 (LCF2-P), and the C subunit of troponin (TnC).

The second class consists of four proteins which will be referred to as "Filament" proteins. These include three protein subunits of 10-nm intermediate filaments: vimentin, which forms intermediate filament in cells of mesenchymal origin, and α- and β-desmin which comprise intermediate filaments in muscle cells (14, 15, 16). It has been demonstrated that vimentin and desmin form part of the structure of Z disks of myofibrils (15), and Holtzer et al. (see reference 16 for most recent summary) has shown that antibodies directed against muscle-specific 10-nm filament protein also localize to the Z-I region in mature myotubes. The Lazarides group (14, 15, 17) proposes that the intermediate filaments function to align and/or stabilize myofibrils. Holtzer et al. (16), while finding intermediate filament protein localization in Z-I areas of myofibrils,

does not find evidence for such an aligning function for intermediate filaments. Whatever their role, the intermediate filament proteins do form part of the myofibrillar structure. Unlike the contractile proteins, they are not thought to be involved in force generation. The fourth protein of this class is β-actin, the nonmyofibril actin species which forms microfilaments in muscle (18) and nonmuscle (9) cells.

The third group of proteins analyzed consists of approximately 25 proteins selected on the basis of relative abundance and good resolution on the 2-D gels. Of these proteins, six are found in muscle cells but not in fibroblasts and are therefore termed muscle-specific; we have not determined the identity or function of these proteins. The remaining proteins are common to fibroblasts and muscle cells and have been termed nonmuscle-specific proteins. We have established the identity of only one of these, calmodulin. This third class of proteins will be referred to as "Others".

To measure protein accumulation, we maintained control and TTX treated cultures throughout the course of the experiment in medium containing [^{3}H]leucine and ^{14}C-leucine, respectively, as described in Materials and Methods. With this labeling procedure, the specific activity of total protein remained constant during the time in culture. At selected time points, ranging from 3 to 10 d after cell plating, total cell lysates of control and TTX cultures were prepared. The lysates were assayed for total protein content, and equal amounts of protein from the control and TTX cultures were mixed and subjected to electrophoresis on the same 2-D gel. The gels were stained and fluorograms prepared from them in order to locate protein spots of interest not visible on the gels by Coomassie Blue staining. A typical stained gel and fluorogram are shown in Fig. 1. The spots of interest were then excised from the gel and counted in order to determine the radioactivity due to ^{3}H and ^{14}C. Since constant specific activity was maintained in each set of control and TTX cultures, the amount of ^{3}H and ^{14}C radiolabel in each protein spot will be a measure of the amount of that protein accumulated in control and TTX cells, respectively. Therefore the ^{14}C/^{3}H ratio for each protein indicates the relative abundance of that protein in TTX cultures as compared to controls.

Preliminary examination of the data revealed that the TTX/control ratios showed little variability within the class of Other proteins described above. The Contractile proteins, however, showed considerably reduced ratios. Due to differences in the incorporation of label and total counts applied to the 2-D gels, the absolute values of the TTX/control ratios varied in different experiments. To compare the results from different experiments, we normalized the data using the following method. Since the TTX/control ratios for the Other proteins were fairly similar, indicating no effect of TTX treatment on these proteins, we calculated the mean TTX/control ratio for the proteins in this class. This mean ratio was then set equal to 1.00, and the ratio for each individual protein spot was normalized to this mean.

Data from such accumulation experiments enabled us to analyze the effect of long-term inhibition of contraction on the three classes of proteins. Fig. 2 shows accumulation data for a number of nonmuscle-specific proteins from the class of Other proteins. While there is some variability within this class, with some proteins exhibiting slight increases or decreases in accumulation with time, the linear regression lines calculated from the data points exhibit two characteristics: (*a*) there is little, if any, change in the accumulation of these proteins in TTX as compared to control cells with increasing time in culture, and (*b*) the absolute values of the TTX/control ratios cluster close to the mean value of 1.00. A similar graph of the data for the Contractile proteins presents a different picture. As shown in Fig. 3, all nine Contractile proteins show decreasing TTX/control ratios with increasing time in TTX. In addition, ratios for individual proteins deviate greatly from the mean of 1.00, decreasing to as low as 0.40. While the accumulation of all nine contractile proteins examined decreased in TTX-treated cells, there was a differential effect on individual proteins. Subunits of the same protein were similarly affected: α- and β-TM showed the greatest final decrease in accumulation, and the data points for the two myosin light chains generated closely parallel lines. α-Actin was the least affected of the major contractile proteins. An interesting result from these experi-

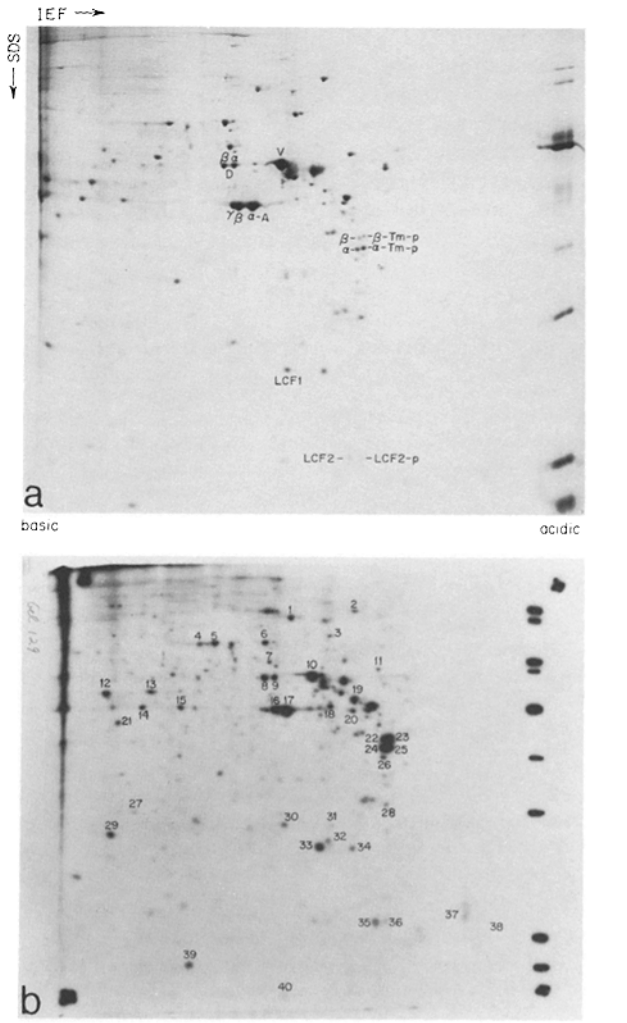

FIGURE 1 Two-dimensional PAGE of lysates of cultured muscle cells. (*a*) Coomassie-Blue-stained gel. (*b*) Fluorogram of a gel from a synthesis experiment. The first dimension consisted of IEF from the basic to the acidic end using ampholytes of pH 5 to 8. The second dimension was carried out on 12% SDS polyacrylamide slab gels. The numbered spots refer to members of classes of proteins identified in the text and in Figs. 2-9. Certain spots have been identified by co-migration with marker proteins as follows: 8, β-desmin; 9, α-desmin; 10, vimentin; 16, β-actin; 17, α-actin; 22, β-TM; 23, β-Tropomyosin-phosphate; 24, α-TM; 25, α-Tropomyosin-phosphate; 33, myosin fast light chain 1; 35, myosin fast light chain 2; 36, myosin fast light chain 2-phosphate; 37, troponin C; 38, calmodulin (not visible on this gel).

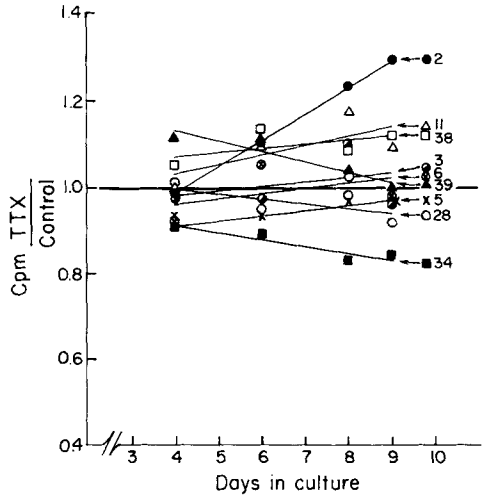

FIGURE 2 Accumulation data for nonmuscle-specific proteins. The ordinate is the normalized ratio of cpm in TTX cells compared to cpm in control cells for each protein spot. The abscissa indicates days after cell plating. TTX is added to experimental cultures at day 2. The data points represent the average of two experiments. The lines are the calculated linear regression for the data points. The proteins indicated are common to both fibroblasts and muscle cells. We have identified only protein 38 which is calmodulin.

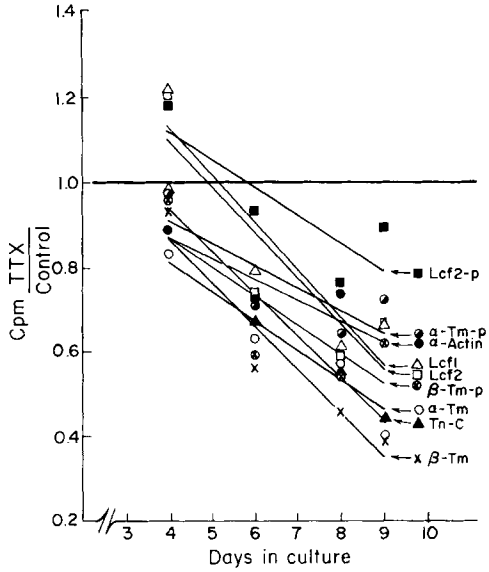

FIGURE 3 Accumulation data for contractile proteins. See legend to Fig. 2. Abbreviations for Figs. 3–9: α and β D, desmins; $\alpha\beta$A, actins; α-, β-, α-P-, and β-P TM, nonphosphorylated and phosphorylated tropomyosin subunits; *Lcf1*, myosin fast light chain 1; *Lcf2*, myosin fast light chain 2; *Lcf2-p*, phosphorylated myosin fast light chain 2; *Tn C*; *Cam*, calmodulin.

ments is that the phosphorylated forms of α- and β-TM and of myosin light chain 2 are not reduced in accumulation to the same degree as the nonphosphorylated forms. Since the specific functions of these phosphorylated subunits is not understood it is difficult to speculate on the implications of the apparent increased stability of these proteins during prolonged muscle inactivity.

A graph of the accumulation data for the four Filament proteins (Fig. 4) appears similar to the results for the nonmuscle-specific proteins (Fig. 2). The lines show little deviation from the mean value of 1.00 and, with the possible exception of vimentin, show no change with increasing time in culture.

These data demonstrate that there is a concerted decrease in the accumulation of all the contractile proteins we examined. None of the filament proteins and none of the proteins common to both muscle cells and fibroblasts were similarly affected by TTX treatment. However, among the approximately forty proteins analyzed, six were muscle-specific but of unknown function. The data for these six proteins are shown in Fig. 5. Two of the proteins, 18 and 20, show a slight increase in accumulation, and three proteins, 32, 51, 56, show a slight decrease. The absolute values of the TTX/control ratios for these proteins fall within the standard deviation from the mean found for the nonmuscle-specific proteins (Fig. 2). While the lines indicate a greater change over time than is seen for most of the other proteins in Fig. 2, our conclusion is that there is no significant effect on the accumulation of these five proteins. By contrast, the sixth protein of this group, protein 30, undergoes a decrease in accumulation to 30% of control levels in cells treated with TTX for 7 d. We had at first believed that this

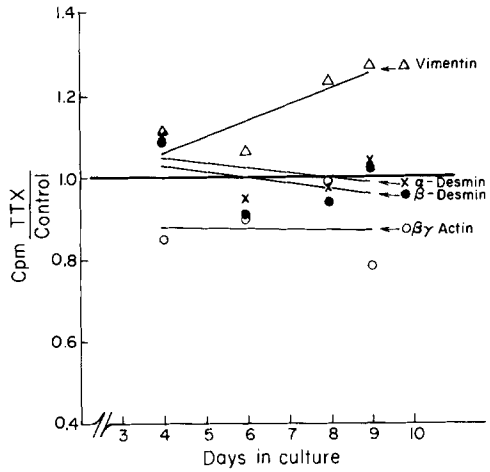

FIGURE 4 Accumulation data for filament proteins. See legend to Fig. 2.

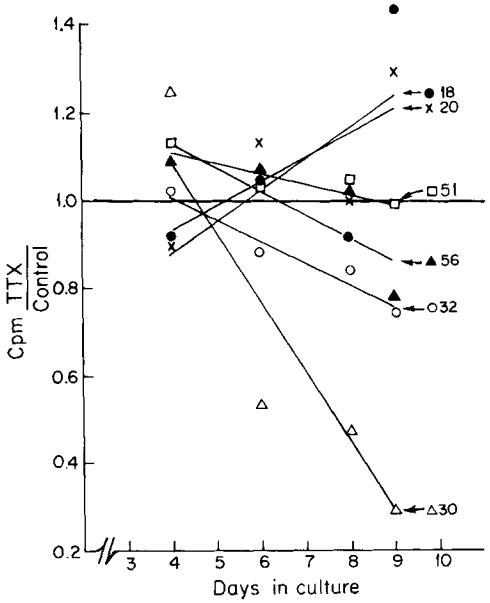

FIGURE 5 Accumulation data for muscle-specific proteins. See legend to Fig. 2. The proteins indicated are found in muscle cells but not in fibroblasts.

protein was slow myosin light chain 1 which has been reported to be made by cultured muscle cells derived from embryonic breast, a fast muscle (19). However, after repeated coelectrophoresis of slow myosin light chain markers with these labeled cell lysates, we observed that protein 30 did not comigrate with the LCS1 marker prepared either from adult ALD muscle, a slow muscle, or from embryonic ALD. We have therefore concluded that protein 30 is not slow myosin light chain 1 nor an embryonic form of it. Since it is a muscle-specific protein and is the only protein out of approximately forty analyzed (other than known contractile proteins) that decreases as a consequence of relaxation, we believe it is likely that this protein functions somehow in contraction or possibly in myofibrillar assembly (see Discussion).

To verify that the changes observed in the accumulation of the Contractile proteins were significant, we analyzed the data using the Student's *t* test. These results are shown in Table I. The mean TTX/control ratio was calculated for each class of proteins: Contractile, Filament, and Others. The *t* test was used to compare the mean for the Contractile proteins with the mean for the Others at each time point in the experiment. After 2 d in TTX there is no significant difference between the means of the Contractile proteins and of the class of Other proteins. Similarly, there is no difference between the means of the Filament proteins and of the Others. However, by 4 d in TTX, the mean ratio for the Contractile proteins had dropped to 0.70, a highly significant difference from a mean of 1.00 for the Others. The Contractile proteins' mean TTX/control ratio remains significantly lower than the mean for the Other proteins throughout the experiment. By contrast, there is no significant change with increasing days in culture in the mean ratio for the group of Filament proteins nor any difference between the filament protein mean and the mean of the Others.

In summary, when muscle cultures are prevented from contracting by treatment with TTX, there is a significant, coordinated, and highly specific decrease in the accumulation of proteins known to function in mediating contraction. Out of at least thirty proteins, including muscle-specific proteins of unknown function and proteins serving a structural but not functional role in the contractile apparatus, only one other muscle-specific protein undergoes a decrease in accumulation in TTX cells comparable to that seen for the Contractile proteins.

TABLE I

Accumulation of Three Classes of Proteins in TTX Compared with Normal Muscle Fiber Cultures

Days in culture	Protein class*	n‡	TTX/control $\bar{X} \pm$ S.D.§	SEM‖	P¶
4	Contractile	9	1.02 ± 0.14	0.05	NS
	Filament	4	1.04 ± 0.12	0.06	NS
	Other	29	1.00 ± 0.09	0.02	
6	Contractile	9	0.70 ± 0.11	0.04	<0.001
	Filament	4	0.96 ± 0.07	0.04	NS
	Other	29	1.00 ± 0.13	0.02	
8	Contractile	9	0.61 ± 0.10	0.03	<0.001
	Filament	4	1.03 ± 0.13	0.07	NS
	Other	29	1.00 ± 0.14	0.03	
9	Contractile	9	0.60 ± 0.16	0.05	<0.001
	Filament	4	1.03 ± 0.10	0.10	NS
	Other	29	1.00 ± 0.20	0.04	

TTX was added on day 2 of culture. See Materials and Methods and text for description of labeling and for normalization procedure. The mean TTX/Control ratio for the class of proteins indicated as "Other" was calculated and set to a value of 1.00. The ratio for each of the other peptide spots from the 2-D gel was normalized to this mean.

* See text.

‡ Number of proteins in each class.

§ Mean ratio of cpm in TTX/Control per spot ± the standard deviation.

‖ Standard error of the mean.

¶ The Student's *t* test was used to determine the significance of the difference between the means for the Contractile proteins as compared to Other proteins and for the Filament proteins compared to Other proteins. *NS* represents a *P* value of >0.02. A *P* value of <0.001 indicates a highly significant difference between the means.

Measurement of Protein Synthesis

We next investigated whether prolonged relaxation of the muscle cells resulted in a decrease in the synthesis of any of the forty proteins. Protein synthesis was measured by pulse labeling control and TTX cultures with [^{3}H]leucine and [^{14}C]leucine, respectively, for 1 h as described in Materials and Methods. It had been established that under these pulsing conditions the leucine pools in control and TTX cultures reach comparable specific activity by the end of a 1-h labeling period (8). The cells were harvested at the end of the labeling period and total cell lysates prepared. The lysates were run together on 2-D gels and the gels analyzed exactly as in the protein accumulation experiments. A ratio of radioactivity per protein spot in TTX cultures as compared to controls was obtained and the ratios were normalized as described above.

A graph of the data for the synthesis of a number of nonmuscle-specific proteins is shown in Fig. 6. Like the accumulation data for these same proteins (Fig. 2), the linear regression lines indicate little deviation from the mean of 1.00 and show no significant change in the synthesis of these proteins with increasing time of culture in TTX.

For the nine contractile proteins, however, the synthesis data (Fig. 7) are strikingly different from the accumulation data (Fig. 3). Those proteins whose accumulation was most reduced in TTX cultures—α-TM, β-TM, LCF1, LCF2, and Tn C-show no reduction in synthesis in TTX cells nor any change in synthesis over time. The four contractile proteins which showed less decrease in accumulation—α-actin, α-TM-P, β-TM-P, and LCF2-P-show a slight decrease in synthesis with increasing time in TTX. It is not clear whether these increases are significant.

A graph of the data for the synthesis of the Filament proteins (Fig. 8) indicates no effect of TTX treatment on these proteins. Fig. 9 shows the data for the six unknown muscle-specific

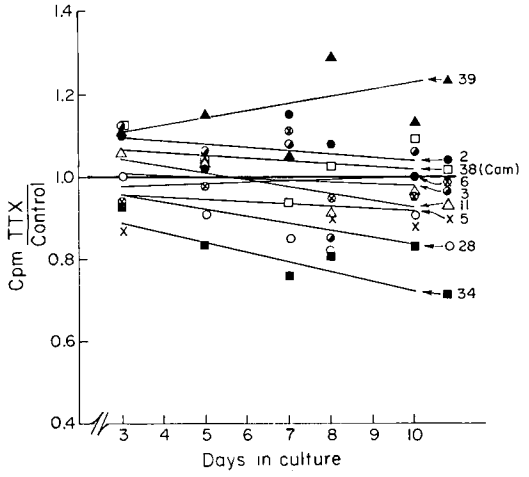

FIGURE 6 Synthesis data for nonmuscle-specific proteins. See legend to Fig. 2.

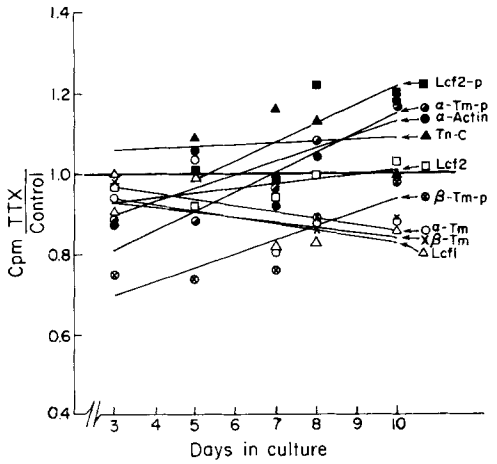

FIGURE 7 Synthesis data for contractile proteins. See legend to Fig. 2.

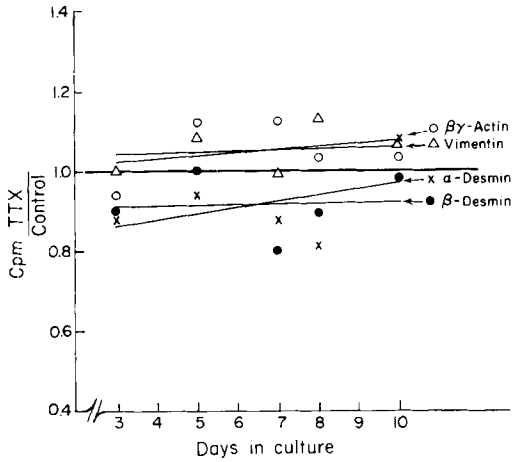

FIGURE 8 Synthesis data for filament proteins. See legend to Fig. 2.

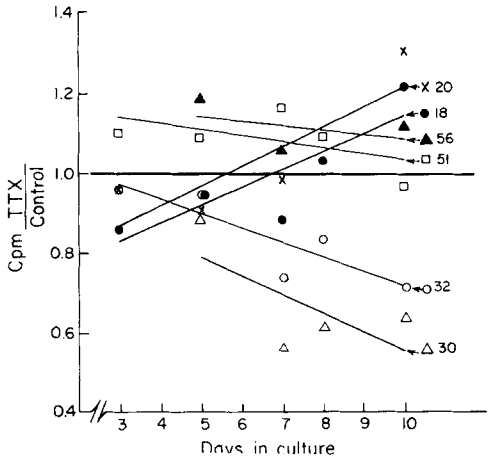

FIGURE 9 Synthesis data for muscle-specific proteins. See legend to Fig. 2.

proteins; the synthesis and accumulation graphs for these proteins are quite parallel (Fig. 4). The two proteins showing an increase in accumulation over time, proteins 18 and 20, show a similar increase in synthesis. Proteins 51 and 56, which had a slight decrease in accumulation, show no effect on synthesis. There is some decrease in both the synthesis and accumulation of protein 32. Protein 30, whose accumulation was decreased more than that of any other protein examined, also shows the greatest decrease in synthesis.

A statistical analysis of these synthesis data for the three classes of proteins, Contractile, Filament, and Other, is presented in Table II. The Student's t test gives no significant difference between the means for the TTX/control ratios of the Contractile proteins or of the Filament proteins as compared to the mean of the Other proteins. As seen in Table I, the accumulation of the Contractile proteins was significantly decreased after maintenance of cultures in TTX medium for 4 d. However, there is no reduction in the synthesis of the Contractile proteins as a group, even after eight days in TTX.

Measurement of Synthesis and Accumulation in Parallel Cultures

The synthesis and accumulation experiments described above were carried out separately on different sets of cultures. Although the results of such experiments were always qualitatively the same, showing a significant decrease specifically in the accumulation of the Contractile proteins with no corresponding decrease in synthesis, we wanted to verify these results by measuring synthesis and accumulation in the same set of cultures. Muscle cell cultures were grown and labeled as usual. On days 6 and 7 after plating, one control and one TTX dish were pulse-labeled to determine protein synthesis, and one control and one TTX dish grown continuously in label were harvested to measure accumulation. The results of these experiments are shown in Table III. At days 6 and 7 there is a significant decrease in the accumulation of the Contractile protein group to ~70% of control levels. Synthesis of these proteins, however, is reduced by only 10%. As in the other experiments there is no effect on either the synthesis or accumulation of the Filament protein class in these experiments.

DISCUSSION

Using coelectrophoresis on 2-D gels of labeled cell lysates, we have been able to quantify the relative synthesis and accumulation of at least forty proteins in control and contraction-inhibited muscle cultures. TTX, which blocks the sodium influx

TABLE II

Synthesis of Three Classes of Proteins in TTX Compared with Normal Muscle Fiber Cultures

Days in culture	Protein class	n	TTX/control $\bar{X} \pm SD$	SEM	P
4	Contractile	8	0.91 ± 0.08	0.03	NS
	Filament	4	0.93 ± 0.05	0.03	NS
	Other	24	1.00 ± 0.08		
5	Contractile	9	0.96 ± 0.11	0.04	NS
	Filament	4	1.04 ± 0.08	0.04	NS
	Other	29	1.00 ± 0.09	0.02	
7	Contractile	9	0.91 ± 0.13	0.04	NS
	Filament	4	0.95 ± 0.14	0.07	NS
	Other	29	1.00 ± 0.14	0.03	
8	Contractile	9	0.99 ± 0.14	0.05	NS
	Filament	4	0.97 ± 0.14	0.07	NS
	Other	28	1.00 ± 0.14	0.03	
10	Contractile	9	1.02 ± 0.13	0.04	NS
	Filament	4	1.04 ± 0.04	0.02	NS
	Other	28	1.00 ± 0.15	0.03	

TTX was added on day 2 of culture. See Table I for explanation of figure symbols.

TABLE III

Synthesis and Accumulation of Three Classes of Proteins from Parallel Cultures in TTX and in Control Media

Days in culture	Type of data	Protein class	*n*	TTX/Control $\bar{X} \pm SD$	SEM	*P*
6	Synthesis	Contractile	9	0.88 ± 0.11	0.04	0.01
		Filament	4	0.88 ± 0.19	0.10	NS
		Other	28	1.00 ± 0.12	0.02	
6	Accumulation	Contractile	9	0.71 ± 0.09	0.03	<0.001
		Filament	4	0.91 ± 0.20	0.10	NS
		Other	28	1.00 ± 0.12	0.02	
7	Synthesis	Contractile	9	0.91 ± 0.11	0.04	0.003
		Filament	4	1.01 ± 0.04	0.02	NS
		Other	28	1.00 ± 0.06	0.01	
7	Accumulation	Contractile	9	0.72 ± 0.14	0.05	<0.001
		Filament	4	1.00 ± 0.18	0.19	NS
		Other	28	1.00 ± 0.13	0.03	

TTX was added on day 2 of culture. See Table 1 for details.

required for muscle contraction, was used to abolish the spontaneous contractions exhibited by normal muscle fibers in culture. The long-term application of TTX has been shown to have no effect on cell viability, myoblast fusion, or initial synthesis of muscle-specific proteins by myotubes (5, 6). Prolonged relaxation of muscle fibers in culture does, however, result in considerable cell atrophy. We have attempted in these studies to determine the biochemical basis for this hypotrophy. By first describing the effect of prolonged relaxation on muscle fibers, we may then begin to elucidate the mechanism by which contractile activity acts to regulate muscle mass.

Of the forty proteins we examined, approximately half were common to fibroblasts and muscle cells. These proteins presumably include metabolic enzymes and regulatory proteins unrelated to specific muscle cell phenotype or uniquely to the process of contraction. When we measured the relative abundance of these proteins in TTX and control muscle cultures, we found little variability in the TTX/control ratio among the different proteins and little change in the ratio for a particular protein with increased time of culture in TTX. As seen in Fig. 2, the TTX/control ratios for all proteins of this class, except for protein 2, lie within the values of 0.8 to 1.2. For any given protein, the change in the ratio over the course of the experiment was generally <0.1. Similar results were obtained for the synthesis of these proteins. Fig. 6 shows that, although there is a slightly larger spread in the values, the TTX/control ratios for the different proteins also lie approximately between the values of 0.8 and 1.2. The change in individual protein ratios is again generally <0.1. Since this class of proteins shows little deviation of the TTX/control ratios from the mean value of 1.00, we concluded that neither the synthesis nor the accumulation of nonmuscle-specific proteins was affected by inhibiting contraction in the muscle cultures. Using these proteins, we have been able to establish a baseline level of variability in the relative synthesis and accumulation of proteins we believe to be unaffected by TTX treatment. We have subsequently used two criteria to judge whether other proteins of known function were affected by contraction inhibition. First, if the synthesis or accumulation of a protein is indeed affected, we should see a change in the TTX/control ratio of >0.1 with increasing time in TTX. Second, the TTX/control ratio at the end of the experiment should lie outside the values of 0.8 to 1.2 observed for the nonmuscle-specific proteins.

With these two criteria, there is clearly a significant and concerted decrease in the accumulation of all the major contractile proteins we examined: α-actin, α- and β-TM, fast myosin light chains 1 and 2, and Tn C. The TTX/control ratios for accumulation of these proteins after 7 d of culture in TTX range between 0.4 and 0.6 (Fig. 3). Although all these proteins are decreased in TTX cultures, their accumulation is affected to different degrees. α-Actin, for example, is affected the least, decreasing to 60% of controls in TTX cultures and showing a change over time of approximately 0.3. β-TM decreases to 40% of controls with a change in its TTX/control ratio of 0.5 between 3 and 9 d in culture. This decrease in accumulation could have been due either to decreased synthesis of these proteins and/or to increased degradation. However, as seen in Fig. 7, the synthesis data for these proteins do not parallel those for accumulation. For the four proteins most decreased in accumulation in TTX cultures, α- and β-TM, LCF1 and 2, and Tn C, there is no effect on synthesis. The TTX/control ratios for synthesis of these proteins lie between 0.8 and 1.1, with a change for any protein of <0.1 between three and ten days in culture. Clearly, the decreased accumulation of these proteins is not due to a turn-off of synthesis and must instead be caused by an increased rate of degradation.

The contractile proteins appear to fall into two classes on the basis of these synthesis and accumulation data. As described, α- and β-TM, LCF1 and -2, and Tn C are drastically reduced in accumulation, with no accompanying decrease in synthesis. The other four proteins, α-actin and the phosphorylated forms of α- and β-TM and of LCF2, also show a decrease in accumulation, though to a lesser degree than the other four proteins (Fig. 3). As judged by the second criterion described above, we would conclude that there is also no significant effect on the synthesis of these proteins since the TTX/control ratios lie approximately between the values of 0.8 and 1.2 observed for nonmuscle-specific proteins. However, judging by the first criterion of change in the TTX/control ratio over time in TTX, they appear to show a slight increase in synthesis over the changes measured for the non-muscle-specific proteins, with differences in the TTX/control ratios of 0.2 to 0.3 from day 3 to day 10 in culture (Fig. 7). Whether or not these data reflect a significant increase in the synthesis of these four proteins is unclear. It is clear, however, for all nine Contractile proteins, that the observed decrease in accumulation is not due to decreased synthesis but rather to increased degradation.

Our hypothesis is that those proteins directly involved in the

contraction process are specifically degraded as a consequence of loss of contractile function. Therefore α- and β-TM, TnC, LCF1, LCF2, and MHC (5, 6) are all subject to increased degradation in TX-treated cultures. α-Actin, a major contractile protein, may be affected less because it functions both as a contractile protein and as a cytoskeletal protein. Gard et al. (20) have reported, and we have corroborated, that α-actin is the major form of actin isolated in cytoskeletal preparations from cultured striated muscle fibers. Therefore the fraction of the cellular α-actin that is assembled into cytoskeletal elements may be protected from the degradation mechanism that appears to act selectively on Contractile proteins. The lessened decrease in the accumulation of the phosphorylated forms of α- and β-TM and of LCF2-P may also reflect other roles for these proteins in muscle cells. Alternatively, these phosphorylated forms may simply be less susceptible to proteolysis than the nonphosphorylated forms.

The results described thus far show a clear difference between the effect of prolonged muscle relaxation on a class of nonmuscle-specific proteins and the effect on a class of muscle-specific proteins, the Contractile proteins. These Contractile proteins are all assembled into the thick and thin filaments that comprise the functional elements of the Contractile apparatus, the myofibrils. There are, however, other proteins which form part of the myofibrillar structure but do not function in force generation during contraction. Included in these proteins are α- and β-desmin and vimentin which are located in the Z disks of the sarcomeres (16, 20, 21). The fate of these proteins in contraction-inhibited muscle cells allows us to more precisely define the mechanism underlying hypotrophy and its extent of action. Fig. 8 shows that there is no effect on the synthesis of these proteins in TTX-treated cells. Fig. 4 indicates no change in the accumulation of the desmin subunits with increasing time of culture in TTX, and a slight increase in the accumulation of vimentin. Clearly, these myofibrillar but noncontractile proteins are not subject to the degradative process that acts on the known contractile proteins. On the basis of experiments measuring the synthesis and accumulation of MHC in TTX-treated cultures, Strohman et al. (6) postulated that MHC, though synthesized at a normal rate, is not assembled into myofibrils and that this newly synthesized MHC is therefore increasingly susceptible to degradation. The data presented here on the decreased accumulation of nine other contractile protein subunits in addition to MHC support this hypothesis that myofibrillar assembly does not proceed normally in TTX-treated cells, rendering the unassembled proteins subject to degradation.

If new myofibrils are not assembled in contraction-inhibited cells, we might then expect structural proteins of the myofibrils, such as desmin and vimentin, also to display high degradation rates. Our data show clearly, however, that they do not. A similar situation has been reported by Croop et al. (22) in which myofibril disassembly and myosin degradation are increased sharply by incubation of cultured myotubes with a phorbol ester. Under these conditions, however, there is extensive accumulation of muscle-type 10-nm filaments that may consist of vimentin and desmin. The simplest explanation for the stability of desmin and vimentin is that they are not suitable substrates for the protease(s) responsible for contractile protein degradation. Ishiura et al. (23) have described a calcium-activated protease that is specific for a number of contractile proteins including myosin and tropomyosin. Chronic relaxation of muscle cells may result in an elevated intracellular calcium level, by leakage of calcium from the sarcoplasmic reticulum, for example, which would activate such a protease (24). Other myofibrillar proteins, such as desmin and vimentin, might not be degraded by this protease. Alternatively, desmin and vimentin, normally associated with myofibrillar structure, may polymerize or remain in 10-nm filaments if their usual assembly into Z-disks is aborted by inhibited myofibril assembly. In the polymerized form, desmin and vimentin might be expected to be resistant to attack by proteolytic enzymes.

The final group of proteins we examined consisted of six muscle-specific proteins of unidentified function. These proteins constitute the most heterogeneous class. Two of the six, 51 and 56, appear to be unaffected in both synthesis and accumulation by TTX treatment (Figs. 5 and 9). Two others, 18 and 20, show an increase in both synthesis and accumulation. Several proteins have been reported to be regulated by contractile activity and to show increased accumulation when contraction is inhibited. These include the acetylcholine receptor and acetylcholine esterase (25, 26). The most interesting protein in relation to muscle hypotrophy is protein 30. This protein undergoes the greatest reduction in accumulation of any protein examined, dropping to 30% of control levels after 7 d of culture in TTX. Unlike any of the contractile proteins, it also shows a significant decrease in synthesis. As mentioned earlier, this protein migrates quite close to slow myosin light chain 1 from either adult or embryonic slow muscle. It is possible that this protein plays a regulatory role in myofibrillar assembly and/or constitutes part of the pathway that links muscle activity with protein accumulation.

By manipulating the level of contractile activity of striated muscle fibers in culture, we have been able to elucidate several features of a pathway by which muscle cells regulate their mass in response to functional need. Our results have shown that muscle hypotrophy involves the specific regulation of the accumulation of the proteins that mediate contraction. This regulation does not act at the level of transcription since the all nine contractile proteins examined appeared to be synthesized at near-normal levels. The specific decrease in the accumulation of these proteins must instead be regulated at a posttranslational level by a specific degradation process that does not act on other proteins sharing properties with Contractile proteins. Thus other proteins located in the myofibrillar structure but not involved in contraction, such as desmin and vimentin, are found at control levels in TTX-treated cells. Calmodulin, a calcium-binding protein that exhibits extensive structural and functional homology with Tn C (27, 28), remains at normal levels in TTX cultures (Fig. 2) while Tn C decreases to 40% of controls.

We do not currently understand the mechanism by which TTX brings about this concerted degradation of myofibrillar proteins. The only known direct effect of TTX is to block Na^+-channels (see reference 7 for a recent review of TTX effects on chick embryo muscle in culture). The indirect effects of TTX are beginning to be more widely described and are quite interesting. Steinbach (8) has shown that TTX will block spontaneous contractions in embryonic muscle fibers. A number of authors including Cohen and Fischbach (26), Walker and Wilson (25), Rieger et al. (29) and Rubin et al. (30) have demonstrated a TTX-induced inhibition of normal cholinergic system development that approximately works through an increased synthesis of AChR. Our own studies have clearly shown a TTX-induced increased degradation of myofibrillar proteins. It is also true that TTX prevents the transition of α-

actinin from cytoplasmic stress fibers to myofibril Z-disks or at least prevents the development of a staining pattern using anti-α-actinin that reflects the incorporation of α-actinin into Z-disks during myotube maturation. These observations by Jockusch and Jockusch (31) are the first to link TTX effects to cytoplasmic filaments. Finally, TTX apparently will increase dissociation of AChR binding to the muscle membrane cell surface or to the underlying cytoskeletal component (J. Prives, personal communication).

It may be useful to speculate on these diverse aspects of TTX on embryonic muscle fibers. It appears that perturbation of cell surface plasma membrane or T-tubule Na^+-channels by TTX has a far-reaching effect extending to destabilization of AChR-anchoring sites on cytoskeletal units, prevention of α-actinin association with Z-disks, and destabilization or inhibition of assembly of myofibrils. These diverse effects could possibly be explained by a common mechanism by which perturbation of plasma membrane/T-tubule membrane Na^+-channels produces some distortion in binding of submembrane protein or cytoplasmic filament protein to myofibrils. These putative associations between plasma membrane or cytoplasmic filament protein and myofibrils are imagined to be necessary for nascent myofibril formation and for the stability of maturing fibrils. There is morphological evidence going back to Heidenhain (32, 33), Holtzer (34), Fellini and Holtzer (35), and Fischman (36) that newly synthesized myofibrils are first assembled just below the cell cortex in embryonic muscle fibers. The latest morphological evidence for a relationship between the plasma membrane and myofibril assembly sites comes from the work of Peng et al. (37) suggesting assembly just under the plasma membrane followed by translocation of fibrils into the interior of the fiber.

Our working hypothesis is that myofibril assembly and stability requires association with cytoskeletal components including those in close association with plasma membrane and T-tubule membrane sites. If these sites are perturbed by agents such as TTX, or depolarization by high K^+ (4, 5, 6), assembly and stability of myofibrils will be reduced and myofibrillar peptides will be increasingly susceptible to the action of muscle cell proteolytic processes.

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