Regulation of Myofibrillar Accumulation in Chick Muscle Cultures: Evidence for the Involvement of Calcium and Lysosomes in Non-uniform Turnover of Contractile Proteins

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ABSTRACT The effect of calcium on myofibrillar turnover in primary chick leg skeletal muscle cultures was examined. Addition of the calcium ionophore A23187 at subcontraction threshold levels (0.38 μM) increased significantly rates of efflux of preloaded $^{45}Ca^{+2}$ but had no effect on total protein accumulation. However, A23187 as well as ionomycin caused decreased accumulation of the myofibrillar proteins, myosin heavy chain (MHC), myosin light chain 1f (LC1f), 2f (LC2f), α-actin (Ac), and tropomyosin (TM). A23187 increased the degradation rate of LC1f, LC2f, and TM after 24 h. In contrast, the calcium ionophore caused decreased degradation of Ac and troponin-C and had no effect on the degradation of MHC, troponin-T, troponin-I, or α,β-desmin (Dm). In addition, A23187 did not alter degradation of total myotube protein. The ionophore had little or no effect on the synthesis of total myotube proteins, but caused a marked decrease in the synthesis of MHC, LC1f, LC2f, Ac, TM, and Dm after 48 h.

The mechanisms involved in calcium-stimulated degradation of the myofibrillar proteins were also investigated. Increased proteolysis appeared to involve a lysosomal pathway, since the effect of the Ca^{++} ionophore could be blocked by the protease inhibitor leupeptin and the lysosomotropic agents methylamine and chloroquine. The effects of A23187 occur in the presence of serum, a condition in which no lysosomal component of overall protein degradation is detected. The differential effect of A23187 on the degradative rates of the myofibrillar proteins suggests a dynamic structure for the contractile apparatus.

Continuous intracellular protein degradation is a characteristic of all cell types, and regulation of this process can be as important as protein synthesis in determining cellular protein content and growth rates (14, 17). In muscle, growth protein degradation can be affected by insulin, insulin-like factors, glucocorticoids, activity, and nutrition (9, 14, 17, 20, 31, 41). Despite the importance of intracellular proteolysis, the responsible proteolytic mechanisms are not clearly defined. The turnover of the myofibrillar proteins includes an extra element of complexity, since these proteins are assembled into thick and thin filaments, which are further arranged into myofibrils (12). In embryonic muscle the polymerization of nascent contractile proteins into thick and thin filaments is followed by the longitudinal and lateral association of these filaments into myofibrils (12). In postnatal muscles, addition of newly synthesized contractile proteins occurs primarily at the periphery of the myofibrils (32). The individual contractile protein subunits are now believed to have heterogeneous rates of turnover (46), which is difficult to reconcile with a degradative mechanism involving the bulk destruction of the organelle. Possibly, degradation of the contractile apparatus involves either localized disassembly and/or the selective proteolysis of the myofibrillar proteins (43, 46).

Several proteolytic activities that can degrade myofibrillar proteins in vitro have been described. These include acid proteases of the lysosome such as cathepsins B, L, and H (3). The contractile proteins are also substrates for the nonlysosomal calcium-activated neutral proteases, which have been shown to remove Z-lines in muscle (19). In addition, the smooth muscle trypsin-like serine proteinases can also degrade myofibrillar proteins (22). Also, muscle cells contain nonlysosomal ATP-dependent proteolytic activities, but the susceptibility of the myofibrillar proteins to these proteases has not been determined (10, 11). The roles that any of these proteases

play in the in vivo degradation of the contractile proteins and the regulation and control of such proteolytic activities are still unclear.

Calcium has been shown to be important in the regulation of many cellular activities. It is known to mediate stimulus-response coupling in a wide variety of biological systems, most notably excitation and contraction in muscle (8). It has also been implicated as a second messenger in certain hormone actions (35, 39) and in the secretion of products from both endocrine and exocrine glands (35). Calcium is required for membrane fusion during myotube formation (7) and has been shown to enhance prostaglandin production in various tissues, including skeletal muscle (36). Calcium can stimulate overall rates of proteolysis in muscle and in hepatocytes (9, 15, 21, 25, 36, 40, 47). However, the effect of calcium on protein synthesis is more controversial; this cation has been shown to stimulate (21), inhibit (25), or have no effect (36) on this process in skeletal muscle.

In the present study we have examined the ability of altered calcium to affect the degradation as well as the synthesis of individual myofibrillar proteins. These studies have revealed that the turnover of individual contractile proteins is regulated nonuniformly by a calcium-dependent mechanism involving lysosomes. The results support the hypothesis that contractile proteins are released from the myofibril before their breakdown to amino acids.

MATERIALS AND METHODS

Myotube Cultures: Primary cultures of chick embryo skeletal muscle were prepared as described previously (20). The cells were plated on gelatin-coated dishes at a density of 3×10^6 cells/100-mm plate and grown in complete medium consisting of 40:60 Hank's balanced salt solution (HBSS): Waymouth's medium, 15% selected horse serum, 2% chick serum, and 1% antibiotic mixture. The HCO_3^- concentration was adjusted to 25 mM. The Ca^{++} concentration in the medium was 1 mM.

Measurement of Calcium Efflux: 7-d-old cultures were labeled for 18 h with $^{45}Ca^{++}$ (5 μCi/ml). The cultures were then washed five times with serum-free medium and transferred to complete medium in the presence and absence of A23187 (0.38 μM, dissolved in 100% ethanol). The final ethanol concentration in both control and A23187-treated cultures was adjusted to 0.1%. The release of radioactivity was measured by sampling the culture medium at the indicated times and counting the samples in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Protein content of the cultures was measured by the Bradford assay (4).

Preparation of Subcellular Fractions: The cells were washed with ice-cold HBSS containing HCO_3^- (25 mM). Subsequent procedures were carried out at 4°C. Cells were harvested with the use of a Teflon-coated razor blade in the presence of 0.75 ml/plate of low salt buffer (0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol, 0.01 M Tris-maleate buffer, pH 7.0) containing the protease inhibitors leupeptin (5 μg/ml) and pepstatin (5 μg/ml). Cells were homogenized using a Dounce homogenizer with 25 strokes of a tight fitting B pestle. The homogenates were centrifuged at 10,000 *g* for 15 min. The supernatant was then centrifuged at 100,000 *g* for 1 h. The high speed supernatant (soluble fraction) was concentrated by means of acetone precipitation and solubilized in lysis buffer (9.5 M urea, 2% [wt/vol] Nonidet P-40, 2% Ampholines, consisting of 1.6% pH range 5–7 and 0.4% pH range 3–10, and 5% β-mercaptoethanol) (34). The low speed pellet was suspended in low salt buffer plus Triton X-100 (1%) and centrifuged at 10,000 *g*. The Triton supernatant (membrane fraction) was concentrated as described above. The remaining pellet (myofibrillar fraction) was washed twice with low salt buffer plus protease inhibitors and solubilized in lysis buffer.

Isolation and Separation of Proteins: Troponin was partially purified according to the method of Matsuda, Obinata, and Shimada (30), with modifications. Cells were harvested on ice, as described previously, in the presence of 0.90 ml/plate Guba-Straub's buffer (0.3 M KCl, 0.09 M KH_2PO_4, 0.06 M K_2HPO_4, pH 6.3) containing 0.5% Triton X-100, pepstatin (5 μg/ml), and leupeptin (5 μg/ml). Cells were homogenized at 0°C using a Dounce homogenizer with 25 strokes of a tight fitting pestle. The homogenates were centrifuged at 20,000 *g* for 30 min. The pellet was then extracted overnight in 10 × vol of 0.4 M LiCl, 2 mM EDTA, 1 mM $NaHCO_3$, 0.05% NaN_3, 5 μg/ml pepstatin, and 5 μg/ml leupeptin. The extract was then centrifuged at 20,000 *g* as before. The supernatant was concentrated by means of acetone precipitation and solubilized in lysis buffer.

One-dimensional gel electrophoresis was performed according to Laemmli and King (24). A 10% separating gel was used to resolve the troponin components. Myosin heavy chain (MHC) was resolved from total cellular protein using a 7.5% gel. Two-dimensional gel electrophoresis was performed according to O'Farrell (34), except that the second dimension separating gel consisted of 10% acrylamide.

Measurement of Protein Accumulation: Myotube cultures were labeled on day 7 with fresh growth medium containing [^{35}S]methionine (5 μCi/ml), in the presence and absence of either A23187 or ionomycin (both at 0.38 μM). After various incubation times, the labeling medium was removed, and the cells were washed with ice-cold HBSS containing HCO_3^- (25 mM). Cells were harvested as described previously in the presence of 100 μl/plate lysis buffer. The cells were homogenized in a Dounce homogenizer with 25 strokes of a tight-fitting pestle. The urea concentration was readjusted to 9.5 M with solid urea. Cell homogenates were stored frozen (−20°C) until use. Protein spots were identified by co-electrophoresis on one- and two-dimensional gels with purified marker proteins and were excised from the gel using a hollow stainless steel rod. Gel slices were incubated in 1 ml NCS (Amersham Corp., Arlington Heights, IL) (90%) for 2 h at 50°C, and then counted in a toluene-based fluor in a liquid scintillation counter. Equal amounts of protein were loaded onto each gel as determined by the Bradford assay (4). The effect of the calcium ionophores on protein accumulation was determined as the percent difference between cpm remaining in specific proteins in control and ionophore-treated cultures. The significance of the differences between the groups was determined using the Student's *t*-test.

Incorporation of isotope into total myotube proteins and subcellular fractions was determined after trichloroacetic acid (TCA) precipitation of cell homogenates. Aliquots containing equal amounts of protein (100 μl) were precipitated with 2 ml cold TCA (10%). The precipitates were collected on Whatman glass microfiber filters (GF/B), pre-wetted with ice-cold TCA (10%), using a vacuum manifold. Filters were washed with cold 10% and 5% TCA and dried in an oven (80°C for 1 h). Protein was digested with NCS (90%) and counted in a liquid scintillation counter as described previously.

Measurement of Protein Degradation: Myotube cultures were labeled on day 7 with fresh medium containing [^{35}S]methionine (20 μCi/ml). After 24 h, this medium was removed, and the cells were rinsed three times with HBSS containing 25 mM HCO_3^- and 10-fold excess cold methionine (2 mM). Cells were then transferred to the experimental medium (containing 2 mM methionine) in the presence and absence of A23187 (0.38 μM). After chase periods of varying times, cell protein was harvested, and one- and two-dimensional gel electrophoreses were performed as described above.

Measurement of Protein Synthesis: Myotube cultures were fed on day 7 with fresh medium in the presence and absence of A23187 (0.38 μM). After various incubation times, fresh medium containing [^{35}S]methionine (100 μCi/ml) was added to the cultures. After 1 h of incubation, cell protein was harvested and incorporation of isotope was determined as described previously.

Materials: Leupeptin was a generous gift from Dr. Alfred Stracher (Downstate Medical Center, Brooklyn, NY). Chloroquine and methylamine were obtained from Sigma Chemical Co. (St. Louis, MO). A23187 and ionomycin were obtained from Calbiochem-Behring Corp. (La Jolla, CA). All tissue culture plastics were obtained from Falcon Labware (Oxnard, CA). Waymouth's medium, HBSS, horse and chick serum, and antibiotics were from Gibco (Grand Island, NY). L-[^{35}S]Methionine (specific activity 1,100–1,400 Ci/mmol) and $^{45}Ca^{++}$ (specific activity 10–40 mCi/mg calcium) were obtained from Amersham Corp. All chemicals for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA).

RESULTS

Effect of Calcium Ionophore A23187 on Calcium Efflux

Primary chick leg skeletal muscle cultures were grown in medium containing 15% horse serum and 2% chicken serum. Myoblasts fused to form multinucleated myotubes after 48–

[1] *Abbreviations used in this paper:* Ac, α-actin; Dm, α,β-desmin; HBSS, Hank's balanced salt solution; LC1f and LC2f, myosin light chains 1f and 2f; MHC, myosin heavy chain; TCA, trichloroacetic acid; TM, tropomyosin.

72 h in culture. The myotubes exhibited cross-striations by 5 d, but only rarely exhibited spontaneous contractions.

To determine the effects of the calcium ionophore A23187 on skeletal myotubes, we incubated 7-d-old cultures in the presence and absence of different concentrations of A23187 (0–3.8 μM). At a concentration of 0.38 μM, the ionophore was found to be below the threshold level needed to cause contraction of the myotubes and did not cause any detectable alterations in the morphology of the cultures, as judged by light microscopy (data not shown). However, this low dose of A23187 stimulated movement of intracellular calcium in the myotube cultures; there was increased efflux of $^{45}Ca^{++}$ from previously labeled cells in the presence of the ionophore, as compared with controls (Fig. 1). Similar results were obtained if cells were exposed to A23187 for 24 h before the efflux measurement (data not shown). Higher doses of A23187 (3.8 μM) caused contraction and subsequent detachment of the myotubes from the culture dish. Additional dose-response experiments were performed as described below.

Calcium-dependent Regulation of Protein Accumulation

To determine the effect of calcium on protein metabolism in muscle, we incubated 7-d-old myotube cultures in the presence and absence of A23187. After various times, cells were harvested and specific proteins were resolved by one- and two-dimensional polyacrylamide gel electrophoresis; a typical gel is shown in Fig. 2. By 72 h in the presence of A23187, there was decreased accumulation of the myofibrillar proteins, myosin light chain 1f (LC1f), and myosin light chain 2f (LC2f), as determined visually by decreased Coomassie Blue staining (Fig. 3). Indeed, all of the myofibrillar proteins appear to display decreased accumulation in A23187-treated cultures, although this effect was particularly evident for the myosin light chains. These effects appear to be selective, since no differences were detected in total protein content between control and 96-h A23187-treated cultures.

To better quantify the effects of calcium on protein accumulation, we chose to use an isotope labeling technique rather than measurement of Coomassie Blue staining. In the following studies continuous labeling of myotube cultures with

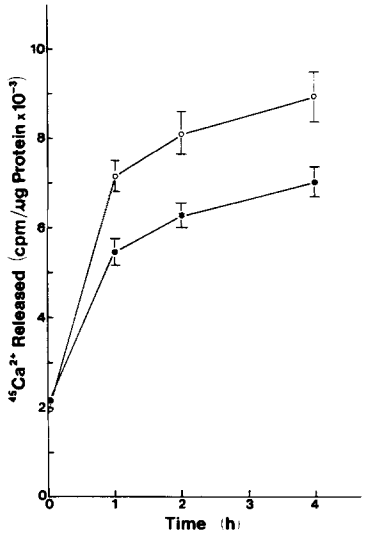

FIGURE 1 Effect of A23187 on $^{45}Ca^{++}$ efflux in myotube cultures. Cells were labeled for 18 h with $^{45}Ca^{++}$ (5 μCi/ml), washed five times in serum-free medium, and transferred to complete medium either in the presence (○) or absence (●) of A23187 (0.38 μM). The release of radioactivity was determined by sampling the culture medium at the indicated times. $n = 6$ for each point. Similar results were obtained in at least two independent experiments.

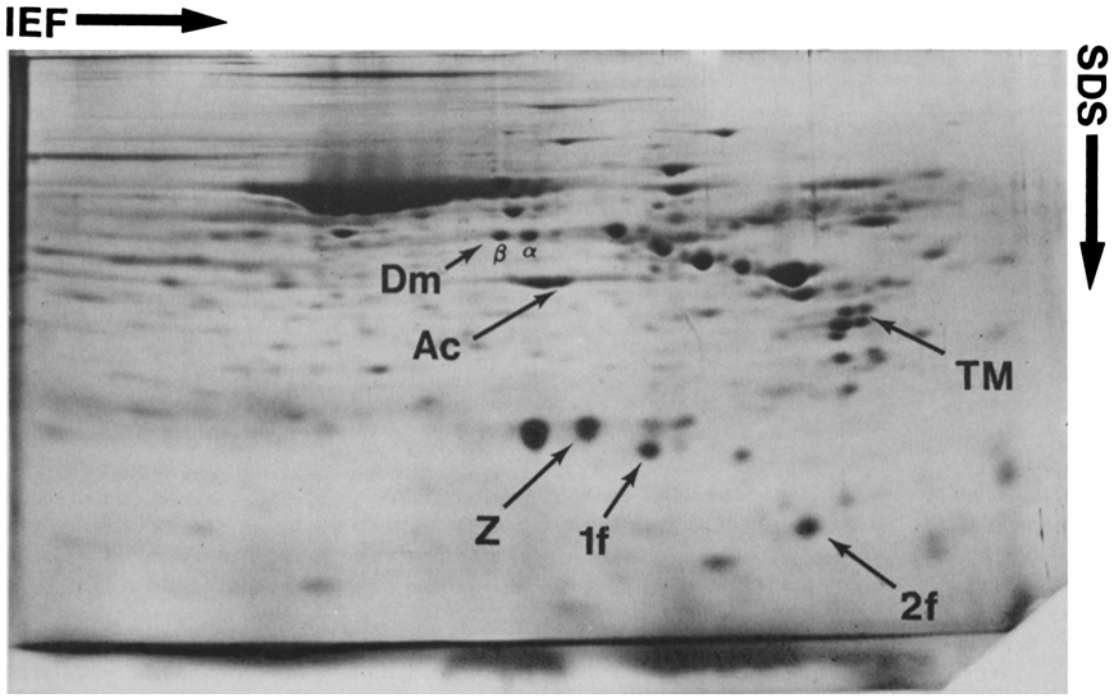

FIGURE 2 Two-dimensional polyacrylamide gel electrophoresis of total lysate protein of cultured muscle cells. Electrophoresis was performed as described in Materials and Methods using a first dimension isoelectric focusing gel containing ampholytes in the pH 5–7 range. Proteins were stained with Coomassie Blue as described in the text. *1f*, myosin light chain 1f; *2f*, myosin light chain 2f; *Ac*, α-actin; *TM*, tropomyosin; *Dm*, α,β-desmin; *Z*, unknown protein Z.

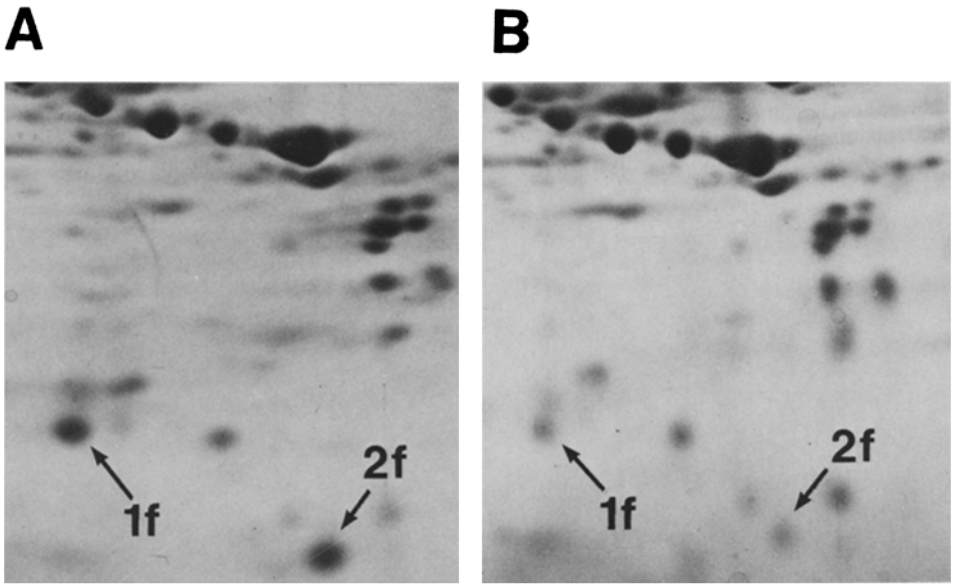

FIGURE 3 Two-dimensional polyacrylamide gel electrophoresis of total lysate protein of control (*A*) and 72 h A23187-treated (*B*) cultures. Electrophoresis was performed as described in the legend for Fig. 2. Only the myosin light chain region of the gel is shown.

[^{35}S]methionine was carried out for up to 96 h (several times the half-lives of the proteins being studied). Such measurements reflect both protein synthesis and degradation. At these longer times (48–96 h), incorporation of [^{35}S]methionine approximates a true accumulation measurement. This is in contrast to estimates of protein synthesis (see below), in which [^{35}S]methionine incorporation is measured after short labeling times (1 h). In control cultures, there was rapid incorporation of [^{35}S]methionine into MHC, LC1f, LC2f, α-actin (Ac), and tropomyosin (TM) until 48 h of incubation (Fig. 4, *A–E*). After this time, there was no further net uptake of label into these proteins or into total protein. This cessation of incorporation occurs because rates of protein synthesis are equal to rates of protein degradation. Consistent with these observations, there was no difference in the amount of protein on the plate between 0 and 96 h, as judged by the Bradford assay (4). In A23187-treated cultures, at 24 h of incubation, rates of isotope incorporation were similar to those of control cultures (Fig. 4, *A–E*). However, after a 24-h exposure to A23187, there was a net reduction of the contractile proteins (MHC, LC1f, LC2f, Ac, TM), which became even more apparent after 48- and 96-h exposures to the ionophore (Fig. 4, *A–E*). These data are consistent with the decreased accumulation of the contractile proteins seen by Coomassie Blue staining (Fig. 3). Interestingly, no phosphorylated form of LC2f could be detected in these cultures, either by Coomassie Blue staining or by autoradiography of [^{35}S]methionine-labeled proteins.

Accumulation of certain other proteins was not affected by the calcium ionophore. For example, after 96 h of incubation, there was no difference in the accumulation of an unidentified protein Z between control and ionophore-treated cultures (Fig. 4*F*). Protein Z is a soluble myotube-specific protein, since primary chick fibroblast cultures do not synthesize this protein (data not shown).

Similar results were obtained using the highly specific calcium ionophore, ionomycin (28). Like A23187, ionomycin (0.38 μM) decreased rates of accumulation of the two myosin light chains, 1f and 2f (Fig. 5). In addition, ionomycin, after 96 h of treatment, had no effect on total protein accumulation, as measured by either the Bradford assay or by incorporation of [^{35}S]methionine into TCA-precipitable material (data not shown). Thus, the two calcium ionophores caused decreased accumulation of many of the myofibrillar proteins in primary myotube cultures.

Calcium-dependent Regulation of Protein Degradation

Accumulation of a protein is determined by the net balance between the rates of protein synthesis and degradation. Therefore, we investigated the extent to which the effects of A23187 on myofibrillar proteins result from changes in synthesis and degradation. Cultures were labeled for 24 h with [^{35}S]methionine and protein degradation measured by pulse-chase analysis. A23187 had marked effects on the half-lives of the two myosin light chains (Fig. 6). The half-life of LC2f decreased from 22.1 h in control cultures to 16.8 h in A23187-treated cells. Similarly, the half-life of LC1f decreased from 28.8 h in controls to 19.2 h in A23187-treated cultures. To survey the effects of A23187 on the degradation of the other myofibrillar proteins, cultures were labeled with [^{35}S]methionine and chased for 24 h in the presence and absence of A23187. The effect of the ionophore is expressed as the percent difference in cpm remaining in each protein resolved on one- and two-dimensional polyacrylamide gels. In addition to increasing the degradation of the two myosin light chains, A23187 also increased degradation of TM. In contrast, A23187 caused decreased degradation of Ac and troponin-C and had no effect on the degradation of MHC, troponin-T, troponin-I, or of the cytoskeletal and Z-line proteins, α,β desmin (Dm) (Table I).

Increasing the amount of A23187 administered to the cells, from 0.38 μM to 2.0 μM, did not substantially affect the A23187-induced increases in protein degradation. However, the increased dose was still subthreshold for contraction in these cells. In contrast, decreasing the amount of A23187 added, to 0.1 μM, resulted in a loss of the A23187 effect (data not shown). Similar to the results on accumulation, A23187 had no effect on total protein degradation, as determined by the cpm remaining in TCA-precipitable material after 96 h of incubation (data not shown). A23187 also had no effect on the degradation of the total myofibrillar, membrane, or soluble protein fractions (data not shown).

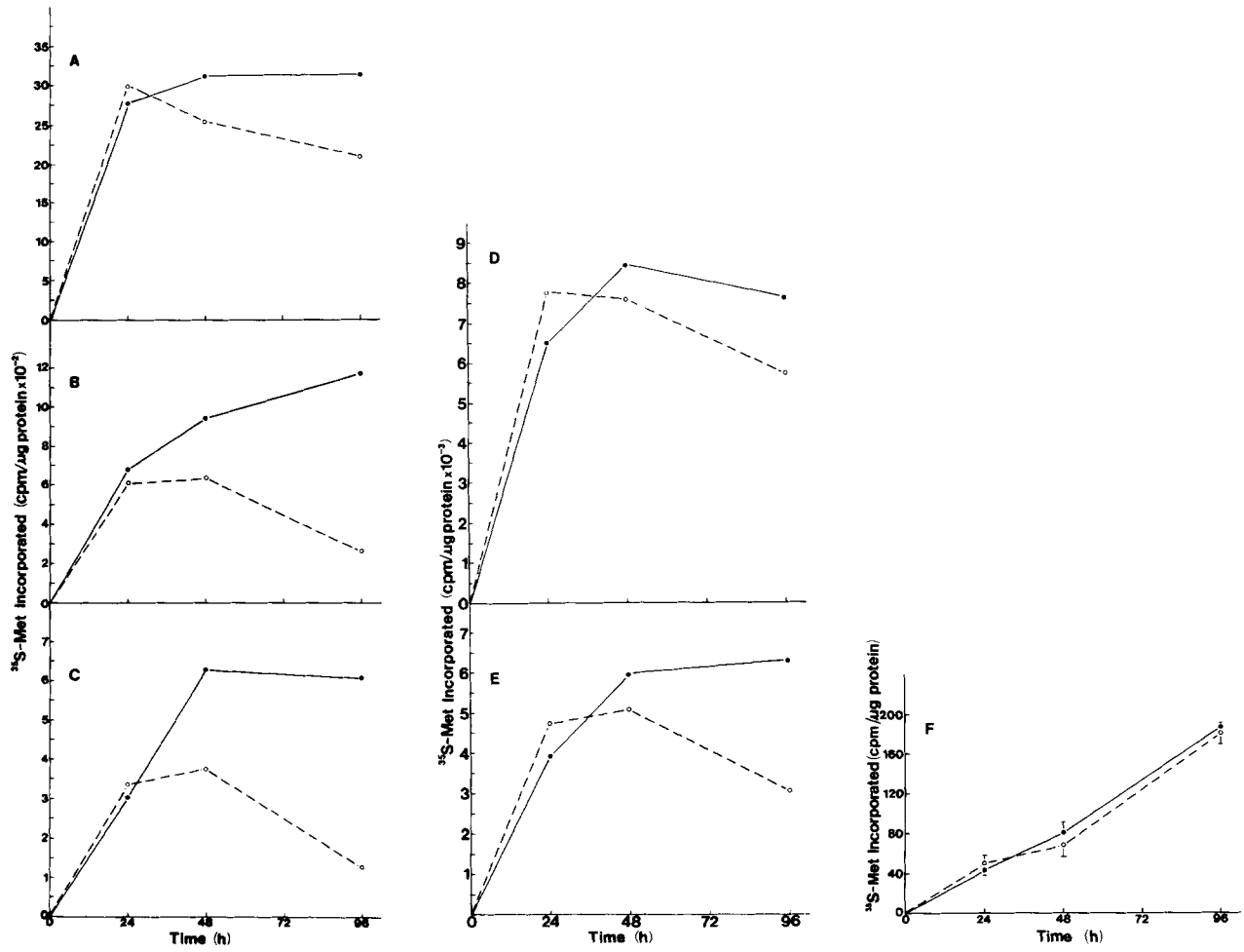

FIGURE 4 Effect of A23187 on the accumulation of myotube proteins: MHC (*A*), myosin LC1f (*B*), myosin LC2f (*C*), Ac (*D*), TM (*E*), protein Z (*F*). 7-d-old myotube cultures were continuously labeled with [^{35}S]methionine (5 μCi/ml) in the presence (○) and absence (●) of A23187 (0.38 μM). After various times, cell protein was separated by one- and two-dimensional gel electrophoresis and analyzed as described in Materials and Methods. Individual proteins were identified by co-electrophoresis with purified marker proteins. Each point represents the average of two determinations, which agreed within 5%. Similar results were obtained in at least three independent experiments.

Calcium-dependent Regulation of Protein Synthesis

The effect of A23187 on protein synthesis in myotube cultures was examined. Cells were incubated in the presence and absence of A23187 for various periods of time, and [^{35}S]methionine was added for 1 h before the solubilization of cell protein. A23187 had little or no effect on the synthesis of total myotube protein (Table II). However, after 48 h of incubation, there was a marked decrease in the synthesis of MHC, LC2f, LC1f, Tm, Ac, and Dm (Table III). For the majority of the myofibrillar proteins (LC2f, TM, Ac, and Dm), this effect was even more apparent after 72 and 96 h of incubation in the presence of A23187. However, for two of these proteins, MHC and LC1f, the maximal effect was obtained after 48 h of incubation with A23187 and was maintained at this level for the remainder of the experiment (Table III). Thus, there appears to be decreased synthesis of many of the myofibrillar proteins in A23187-treated cultures.

Therefore, the large decrease (75%) in the accumulation of LC1f, LC2f, and TM in A23187-treated cultures results from both decreased synthesis and increased degradation of these proteins. The smaller decrease (30%) in accumulation of MHC is consistent with decreased synthesis, accompanied by no change in degradation, of this protein in A23187-treated cultures. Finally, the small decrease (25%) in the accumulation of Ac in the presence of A23187 is accounted for by a larger decrease in the synthesis of the protein, relative to the observed decrease in its degradation.

Characterization of Calcium-dependent Proteolysis

In muscle, lysosomal as well as nonlysosomal proteolytic activities have been implicated in the breakdown of endogenous proteins (14, 17). We therefore used agents with different target specificities to define the system responsible for the increased breakdown of the myofibrillar proteins in A23187-treated cultures. One of the agents used was leupeptin, which is a low molecular weight inhibitor of many thiol proteases, including the nonlysosomal Ca^{++}-dependent proteases, as well as the lysosomal proteases, cathepsins B, H, and L (3, 26, 42). Myotube cultures were incubated in the presence and absence of A23187 and leupeptin (50 μg/ml) for the indicated chase period. The A23187-induced increases in the degradation of

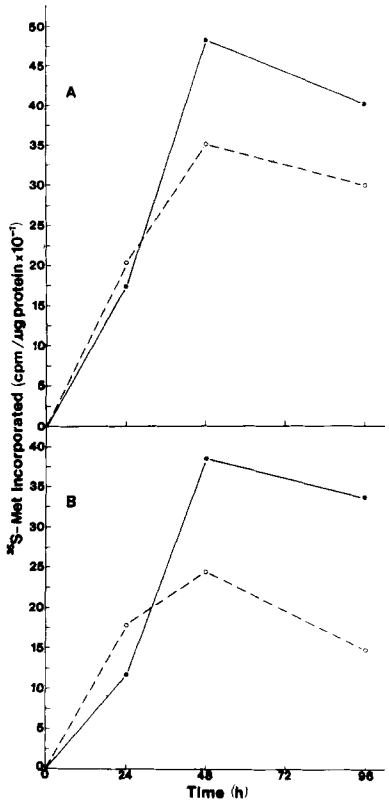

FIGURE 5 Effect of ionomycin on the accumulation of myotube proteins: myosin LC1f (*A*) and myosin LC2f (*B*). 7-d-old myotube cultures were continuously labeled with [^{35}S]methionine (2 μCi/ml) in the presence (○) and absence of (●) of ionomycin (0.38 μM). After various times, cell protein was separated by two-dimensional gel electrophoresis and analyzed as described in Materials and Methods. Each point represents the average of two determinations, which agreed within 5%. Similar results were obtained in at least three independent experiments.

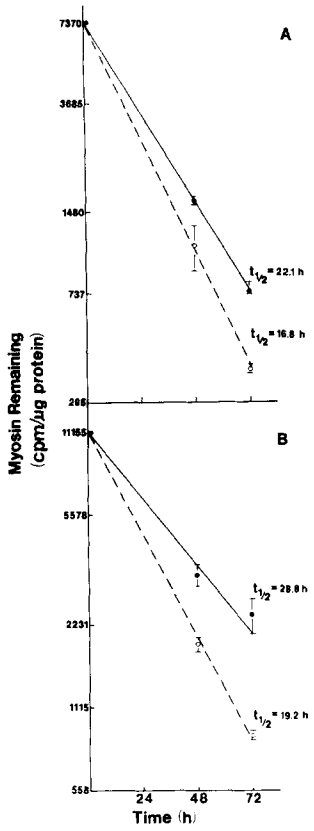

FIGURE 6 Effect of A23187 on the degradation of myosin LC2f (*A*) and LC1f (*B*). 7-d-old myotube cultures were labeled for 24 h with [^{35}S]methionine (20 μCi/ml) and then thoroughly washed in medium containing 10-fold excess cold methionine. The cells were then incubated for various periods of time in the presence (○) and absence (●) of A23187 (0.38 μM), in medium containing 10-fold excess cold methionine. cpm remaining in individual myofibrillar proteins was determined after two-dimensional gel electrophoresis of muscle cell lysates; $n = 3$ for each point. Similar results were obtained in at least three independent experiments.

TABLE I. *Effect of A23187 on Protein Degradation in Myotube Cultures*

	Degradation
	% Stimulation
Myosin LC2f	15.5*
Myosin LC1f	17.1‡
TM	13.9*
Ac	−28.0*
Troponin-C	−36.5*
MHC	3.2 (NS)
Troponin-T	−3.7 (NS)
Troponin-I	−5.9 (NS)
Dm	5.5 (NS)

A23187 (0.38 μM) was added during the 24-h chase period, as described under Materials and Methods. The percent stimulation represents the difference in cpm remaining in individual proteins between control and A23187-treated cultures, relative to control values. The Student's *t*-test (*P* values) indicates the significance of the difference in each protein. $n = 6$ for each point. NS, not significant.
* $P < 0.001$.
‡ $P < 0.025$.

the two myosin light chains were blocked by leupeptin (Table IV).

To distinguish between lysosomal and nonlysosomal proteolysis, we used the lysosomotropic agent, methylamine. Methylamine is thought to act by raising the pH of the lysosome and/or by inhibiting the fusion of the autophagic vesicles with the lysosome (23). Myotube cultures were incubated in the presence and absence of A23187 and methylamine (10 mM) for the indicated chase period. Methylamine blocked the A23187-induced increases in the degradation of LC2f, LC1f, and TM (Table V). Another lysosomotropic agent, chloroquine (10 μM), had the same effect as methylamine in these cells (Table V). Therefore, the increased degradation of the myofibrillar proteins appears to involve a lysosomal pathway. Interestingly, the A23187 effects occur in the presence of serum, a condition in which no lysosomal component of overall protein degradation can be detected (1, 27).

DISCUSSION

We have shown that the calcium ionophores A23187 and ionomycin, at a concentration that was subthreshold for contraction, caused decreased accumulation of the myofibrillar proteins, MHC, LC1f, LC2f, TM, and Ac in primary chick myotube cultures. These agents had no effect on overall protein accumulation, which can be accounted for by several means. Firstly, it is possible that the ionophores can also cause increased accumulation of other proteins in the myotube, thereby opposing the negative effect that the drugs have on myofibrillar protein accumulation. Such an effect has been demonstrated in cultured chicken pectoralis muscle, where A23187 and ionomycin have been shown to increase the synthesis of two membrane-bound proteins of 80,000 and 100,000 D (37, 45). Secondly, unlike adult muscle, the myofibrillar proteins in the embryonic myotube cultures comprise a relatively small proportion of the total cellular protein; <10% of the protein synthesized in mature myotubes consists of myosin, actin, and tropomyosin (data not shown). There-

TABLE II. *Effect of A23187 on Total Protein Synthesis in Myotube Cultures*

Time	Protein synthesis		
	Control	A23187	Difference
h	*cpm/μg protein × 10⁻⁵*		%
24	4.15	4.16	−0.3
48	2.58	2.36	8.5
72	2.08	2.02	2.8
96	2.07	1.92	7.2

7-d-old myotube cultures were incubated in the presence and absence of A23187 (0.38 μM). [^{35}S]Methionine (100 μCi/ml) was added for 1 h before the solubilization of cell protein. Incorporation of label into total cellular protein was determined after TCA precipitation. Values shown are duplicates that vary <5%. Similar results were obtained in at least three independent experiments.

TABLE III. *Effect of A23187 on Myofibrillar Protein Synthesis in Myotube Cultures*

Time	Percent inhibition of synthesis					
	MHC	Myosin LC2f	Myosin LC1f	TM	Ac	Dm
h						
24	11.0	8.5	6.5	3.6	−5.5	—
48	27.5	18.6	57.6	31.7	17.2	45.5
72	29.0	46.1	52.3	27.0	25.7	30.2
96	28.4	60.1	62.9	58.9	41.7	62.1

7-d-old myotube cultures were labeled as described under Materials and Methods. Incorporation of label into individual proteins was determined after two-dimensional gel electrophoresis of muscle cell lysates. Values shown are duplicates that vary <5%. Percent inhibition represents the difference in cpm remaining in individual proteins between control and A23187-treated cultures, relative to control values. Similar results were obtained in at least three independent experiments.

TABLE IV. *Effect of Leupeptin on Myosin Degradation in Myotube Cultures*

	Degradation	
	LC2f	LC1f
	% Stimulation	
Control	—	—
Leupeptin (50 μg/ml)	−26.4*	10.4 (NS)
A23187	10.8*	23.5‡
Leupeptin + A23187	−27.0§	0.5 (NS)

A23187 (0.38 μM) and leupeptin (50 μg/ml) were added during the 24-h chase period as described under Materials and Methods. Percent stimulation represents the difference in cpm remaining in individual proteins between the control and each experimental condition, relative to control values. $n = 6$ for each point. NS, not significant.
* $P < 0.05$.
‡ $P < 0.0025$.
§ $P < 0.025$.

TABLE V. *Effect of Methylamine and Chloroquine on Proteolysis in Myotube Cultures*

	Degradation		
	Myosin LC2f	Myosin LC1f	TM
	% Stimulation		
Control	—	—	—
A23187	20.4*	19.8*	18.2*
Methylamine (10 mM)	−0.6 (NS)	−1.1 (NS)	−28.1‡
Methylamine + A23187	−11.1 (NS)	−6.7 (NS)	−39.5§
Chloroquine (10 μM)	−2.8 (NS)	−5.5 (NS)	−27.8§
Chloroquine + A23187	−6.6 (NS)	−9.1 (NS)	−33.8§

A23187 (0.38 μM), methylamine (10 mM), and/or chloroquine (10 μM) were added during the 24-h chase period, as described under Materials and Methods. Percent stimulation represents the difference in cpm remaining in individual proteins between the control and each experimental condition, relative to control values. $n = 6$ for each point. NS, not significant.
* $P < 0.025$.
‡ $P < 0.05$.
§ $P < 0.01$.

fore, decreased accumulation of the myofibrillar proteins might not be detected in overall protein accumulation in these cells.

It is probable that the effects of the ionophores on protein metabolism are mediated by movement of calcium in the cell, i.e., by increased influx of the ion across the plasma membrane and/or by increased transport of calcium between various intracellular pools. Indeed, the A23187-stimulated efflux of $^{45}Ca^{++}$ from the myotubes (Fig. 1) strongly suggests that an increase in the free intracellular calcium concentration is produced by this drug (38). The magnitude and time course of the efflux experiment resembles that seen in other studies, which have also shown that low concentrations of A23187 can increase intracellular calcium levels in a wide variety of cell types including muscle (37, 38). The efflux reaction is completed quickly because the labeled calcium is rapidly exhausted (Fig. 1); this does not indicate whether or not the calcium elevation persists over a longer time course. Indeed, we have found increased rates of $^{45}Ca^{2+}$ efflux even after 24 h of exposure to the ionophore (data not shown). In addition, A23187 and ionomycin, which are structurally distinct, both had the same effects on protein accumulation in the myotube cultures. Thus, it is unlikely that the effects on protein metabolism result from effects of the drugs that are unrelated to their calcium ionophore properties.

Our studies demonstrate that A23187 had selective effects on the half-lives of some of the myofibrillar proteins. A23187 increased the degradation of LC1f, LC2f, and TM, decreased the degradation of Ac and troponin-C, and had no effect on the degradation of MHC, troponin-T, troponin-I, and Dm. Again, similar to the effects on accumulation, A23187 had no effect on total protein degradation or on the degradation of the total myofibrillar, membrane, or soluble protein fractions. The A23187-induced increases in myofibrillar protein degradation appear to involve a lysosomal pathway, since the effects can be blocked by the protease inhibitor leupeptin and the lysosomotropic agents, methylamine and chloroquine. The way in which calcium affects lysosomal function is unclear, but it could involve either direct or indirect effects of calcium on calcium-dependent proteins, for example, calmodulin, calcimedin, and/or protein kinases. However, synergistic involvement of a calcium-activated neutral protease cannot be discounted, since its activity can also be blocked by leupeptin (42).

Interestingly, the effect of A23187 on myofibrillar protein degradation occurs in the presence of serum, a condition in which no lysosomal component of overall protein degradation can be detected (1, 27). Lysosomal proteolytic activity appears to be largely responsible for the enhanced proteolysis seen during serum deprivation (1). This implies that A23187 may bypass the inhibition of lysosomal proteolysis due to the presence of serum, or that A23187 can activate a lysosomal pathway that is serum-insensitive. Calcium may act at a prelysosomal step such as autophagy (15). It is also interesting to note that both leupeptin and the lysosomotropic agents (Tables IV and V) had effects on the basal protein degradation that varied with respect to the specific myofibrillar protein.

The lysosomotropic agents inhibited proteolysis of TM, whereas degradation of LC1f and LC2f were unaffected; leupeptin inhibited degradation of LC2f, but had no effect on proteolysis of LC1f. This suggests that these proteins do not turn over coordinately, and moreover, that the basal degradation of these proteins may be accomplished via different pathways. In addition, the small inhibition of basal proteolysis compared with the complete inhibition of A23187-induced degradation suggests the possibility that distinct mechanisms may be responsible for basal and calcium-accelerated proteolysis.

Other studies have demonstrated that calcium can cause increased intracellular proteolysis in muscle and in other cell types (15, 21, 25, 36, 40). However, important differences exist between these studies and those reported here. Such studies used adult muscles over a time course of several hours and doses of A23187 that were much larger (5–50×) than those used in the present experiments. Rodemann et al. have concluded that the calcium-induced increases in overall protein degradation were mediated by a lysosomal pathway, based on an inhibition of the effect with leupeptin (36). However, recent studies in our laboratory have led to a reinterpretation of their conclusions. Using several lysosomotropic agents as well as leupeptin, it has been concluded that a nonlysosomal pathway is responsible for the stimulation of overall proteolysis by A23187 in adult rat skeletal muscles (47). Thus, calcium appears to have multiple actions on distinct proteolytic pathways in muscle.

Similar to its effects on degradation, A23187 had little or no effect on total protein synthesis. However, A23187 selectively decreased the synthesis of the myofibrillar proteins MHC, LC1f, LC2f, Ac, TM, and Dm. Calcium has been previously shown to affect protein synthesis at the levels of both transcription and translation (16, 45). The effect of A23187 on contractile protein synthesis and degradation was not affected by contaminating fibroblasts, since primary fibroblast cultures do not synthesize the myotube -specific myofibrillar proteins (data not shown).

Other agents that would be expected to involve alterations in calcium can also influence the rates of synthesis and degradation of myofibrillar proteins in chick myotube cultures. Tetrodotoxin and elevated intracellular potassium (K^+), both of which inhibit spontaneous contractions, selectively increased the degradation of the myofibrillar proteins, while having no effect on the synthesis of these proteins (2, 6). The co-carcinogen 12-O-tetradecanoyl phorbol-13-acetate selectively increased the degradation and decreased the synthesis of the myofibrillar proteins (44). These results differ from those presented here since these agents affected all of the contractile proteins similarly; in contrast, the calcium ionophore A23187 affected individual myofibrillar proteins differently. Thus, the effects of K^+, tetrodotoxin, and 12-O-tetradecanoyl phorbol-13-acetate do not appear to be simply mediated by changes in calcium. Likewise work-induced hypertrophy of muscle, although associated with increased average cytosolic calcium levels, may also involve other factors, and thus it is probably not surprising that the present results do not mimic the increased muscle mass resulting from enhanced activity (13). In addition, the precise compartmentalization or mechanism of calcium elevation may be of importance in determining multiple effects associated with calcium fluxes, i.e., endocrine, or activity-induced effects on muscle composition (5, 13, 39).

The differential effect of A23187 on the degradative rates of the myofibrillar proteins suggests that there might be selective release of individual proteins from the myofilaments, and/or some degree of localized myofibrillar disassembly. A pool of individual contractile protein subunits might be degraded by lysosomal and/or nonlysosomal proteases. The existence of soluble pools of unassembled contractile proteins has been proposed for the myosin light chains and troponin-I, based upon kinetic labeling arguments and the existence of immunoreactive material in soluble subcellular fractions (18, 29, 33, 46). Calcium could possibly act by regulating the equilibrium of protein between assembled and free pools, and/or it could act to affect the degradation of proteins in the dissociated state. In any case, together with the selective inhibition of synthesis of the myofibrillar proteins, the movement of calcium appears to act so as to alter the relative proportion of contractile proteins in the myotube.

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REFERENCES

1. Amenta, J. S., T. J. Hlivko, A. G. McBee, H. Shinozuka, and S. Brocher. 1978. Specific inhibition by NH_4Cl of autophagy-associated proteolysis in cultured fibroblasts. *Exp. Cell Res.* 115:357–366.
2. Bandman, E., and R. C. Strohman. 1982. Increased K^+ inhibits spontaneous contractions and reduces myosin accumulation in cultured chick myotubes. *J. Cell Biol.* 93:698–704.
3. Bird, J. W. C., and J. H. Carter. 1980. Proteolytic enzymes in striated and non-striated muscle. *In* Degradative Processes in Heart and Skeletal Muscle. K. Wildenthal, editor. Elsevier/North-Holland, Amsterdam. 51–86.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72:248–254.
5. Brown, R., K. Berger, and P. Taylor. 1984. α1-Adrenergic receptor activation mobilizes cellular calcium in a muscle cell line. *J. Biol. Chem.* 259:7554–7562.
6. Crisona, N., and R. C. Strohman. 1983. Inhibition of contraction of cultured muscle fibers results in increased turnover of myofibrillar proteins but not of intermediate filament proteins. *J. Cell Biol.* 96:684–692.
7. David, J., W. See, and C. Higginbotham. 1981. Fusion of chick embryo skeletal myoblasts: role of calcium influx preceding membrane union. *Dev. Biol.* 82:297–307.
8. Ebashi, S. 1976. Excitation-contraction coupling. *Annu. Rev. Physiol.* 38:293–313.
9. Etlinger, J. D., T. Kameyama, D. van der Westhuyzen, D. Erlij, and K. Matsumoto. 1981. Roles of calcium and tension in the regulation of protein turnover in muscle. *In* Mechanism of Muscle Adaptation to Functional Requirements. F. Guba, G. Marechal, O. Takacs, editors. Pergamon Press, New York. 241–253.
10. Etlinger, J. D., H. McMullen, R. F. Rieder, A. Ibrahim, R. A. Janeczko, and S. Marmorstein. 1985. Mechanisms and control of ATP-dependent proteolysis. *In* V International Symposium on Intracellular Proteolysis. E. Khairallah, J. S. Bond, and J. W. C. Bird, editors. Alan R. Liss, Inc., New York. 47–60.
11. Etlinger, J. D., S. Speiser, E. Wajnberg, and M. J. Glucksman. 1981. ATP-dependent proteolysis in erythroid and muscle cells. *Acta Biol. Med. Ger.* 40:1285–1291.
12. Fischman, D. A. 1970. The synthesis and assembly of myofibrils in embryonic muscle. *Curr. Top. Dev. Biol.* 5:235–280.
13. Goldberg, A. L., J. D. Etlinger, D. F. Goldspink, and C. Jablecki. 1975. Mechanism of work-induced hypertrophy of skeletal muscle. *Med. Sci. Sports.* 7:248–261.
14. Goldberg, A. L., and A. C. St. John. 1976. Intracellular protein degradation in mammalian and bacterial cells: part II. *Annu. Rev. Biochem.* 45:747–803.
15. Grinde, B. 1983. Role of Ca^{2+} for protein turnover in isolated rat hepatocytes. *Biochem. J.* 216:529–536.
16. Halbreich, A. 1979. Differential effect of Ca^{2+} on the translation of yeast mitochondrial and soma viral RNA's in an *E. coli* cell-free system. *Biochem. Biophys. Res. Commun.* 86:78–87.
17. Hershko, A., and A. Ciechanover. 1982. Mechanisms of intracellular protein breakdown. *Annu. Rev. Biochem.* 51:335–364.
18. Horvath, B., and E. Gaetjens. 1972. Immunochemical studies on the light chains from skeletal muscle myosin. *Biochim. Biophys. Acta.* 263:779–793.
19. Ishiura, S. 1981. Calcium-dependent proteolysis in living cells. *Life Sci.* 29:1079–1087.
20. Janeczko, R. A., and J. D. Etlinger. 1984. Inhibition of intracellular proteolysis in muscle cultures by multiplication-stimulating activity. *J. Biol. Chem.* 259:6292–6297.
21. Kameyama, T., and J. D. Etlinger. 1979. Calcium-dependent regulation of protein synthesis and degradation in muscle. *Nature (Lond.).* 279:344–346.

22. Kay, J., L. M. Siemankowski, R. F. Siemankowski, J. A. Greweling, and D. E. Goll. 1982. Degradation of myofibrillar proteins by trypsin-like serine proteinases. *Biochem. J.* 201:279–285.
23. Kovacs, A. L., A. Reith, and P. O. Seglen. 1982. Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinblastine, leupeptin, or a lysosomotropic amine. *Exp. Cell Res.* 137:191–201.
24. Laemmli, U., and J. King. 1971. Polypeptides of the tail fibers of bacteriophage T4. *J. Mol. Biol.* 62:465–477.
25. Lewis, S. E. M., P. Anderson, and D. F. Goldspink. 1982. The effects of calcium on protein turnover in skeletal muscles of the rat. *Biochem. J.* 204:257–264.
26. Libby, P., and A. L. Goldberg. 1978. Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. *Science (Wash. DC).* 199:534–536.
27. Libby, P., and A. L. Goldberg. 1981. Comparison of the control and pathways for degradation of the acetylcholine receptor and average protein in cultured muscle cells. *J. Cell. Physiol.* 107:181–194.
28. Liu, C., and T. Hermann. 1978. Characterization of ionomycin as a calcium ionophore. *J. Biol. Chem.* 253:5892–5894.
29. Martin, A. 1981. Turnover of cardiac troponin subunits: kinetic evidence for a precursor pool of troponin I. *J. Biol. Chem.* 258:8955–8962.
30. Matsuda, R., T. Obinata, and Y. Shimada. 1981. Types of troponin components during development of chicken skeletal muscle. *Dev. Biol.* 82:11–19.
31. Millward, D. J., P. C. Bates, J. G. Brown, M. Cox, R. Giugliano, M. Jepson, and J. Pell. 1985. Role of thyroid, insulin and corticosteroid hormones in the physiological regulation of proteolysis in muscle. *In* Intracellular Protein Catabolism. E. Khairallah, J. Bond, and J. W. C. Bird, editors. Alan R. Liss, Inc., New York. 531–542.
32. Morkin, E. 1970. Post-natal muscle fiber assembly localization of newly synthesized myofibrillar proteins. *Science (Wash. DC).* 167:1499–1501.
33. Morkin, E., Y. Yazaki, T. Katagiri, and P. J. Laraia. 1973. Comparison of the synthesis of light and heavy chains of adult skeletal myosin. *Biochim. Biophys. Acta.* 324:420–429.
34. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
35. Rasmussen, H., and P. Q. Barrett. 1984. Calcium messenger systems: an integrated view. *Physiol. Rev.* 64:939–984.
36. Rodemann, H. P., L. Waxman, and A. L. Goldberg. 1982. The stimulation of protein degradation in muscle by Ca^{2+} is mediated by prostaglandin E2 and does not require the calcium activated protease. *J. Biol. Chem.* 257:8716–8723.
37. Roufa, D., F. S. Wu, and A. N. Martonosi. 1981. Effect of calcium ionophores upon the synthesis of proteins in cultured skeletal muscle. *Biochim. Biophys. Acta.* 674:225–237.
38. Schudt, C., and D. Pette. 1975. Influence of the ionophore A23187 on myogenic cell fusion. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 59:36–38.
39. Schudt, C., and D. Pette. 1978. Ca^{2+} ions as coupling agents in enzymatic differentiation and carbohydrate metabolism of cultured muscle cells. *Adv. Enzyme Regul.* 16:121–139.
40. Sugden, P. H. 1980. The effects of calcium ions, ionophore A23187, and inhibitors of energy metabolism on protein degradation in the rat diaphragm and epithochlearis muscles *in vitro*. *Biochem. J.* 190:593–603.
41. Tischler, M. E. 1981. Hormonal regulation of protein degradation in skeletal and cardiac muscle. *Life Sci.* 28:2569–2576.
42. Toyo-oka, T., T. Schimizu, and T. Masaki. 1978. Inhibition of proteolytic activity of calcium activated neutral protease by leupeptin and antipain. *Biochem. Biophys. Res. Commun.* 82:484–491.
43. van der Westhuyzen, D. R., Matsumoto, K., and J. D. Etlinger. 1981. Easily releasable myofilaments from skeletal and cardiac muscles maintained *in vitro*. *J. Biol. Chem.* 256:11791–11797.
44. West, C., and H. Holtzer. 1982. Protein synthesis and degradation in cultured muscle is altered by a phorbol diester tumor promoter. *Arch. Biochem. Biophys.* 219:335–350.
45. Wu, F. S., Y. Park, D. Roufa, and A. Martonosi. 1981. Selective stimulation of the synthesis of an 80,000 dalton protein by calcium ionophores. *J. Biol. Chem.* 256:5309–5312.
46. Zak, R., A. F. Martin, G. Prior, and M. Rabinowitz. 1977. Comparison of turnover of several myofibrillar proteins and critical evaluation of double isotope method. *J. Biol. Chem.* 252:3430–3435.
47. Zeman, R. J., T. Kameyama, K. Matsumoto, P. Bernstein, and J. D. Etlinger. 1985. Regulation of protein degradation in muscle by calcium: evidence for enhanced nonlysosomal proteolysis associated with elevated cytosolic calcium. *J. Biol. Chem.* 260:13619–13624.