AN EMERGING ROLE FOR CALPAIN IN SKELETAL MUSCLE ADAPTATION

by

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ABSTRACT

In skeletal muscle, molecular and cellular adaptation occurs in response to changes in functional demand. The changes in phenotype are well described, however the signalling mechanisms which lead to adaptation are poorly understood. Our purpose was to determine whether acute changes in myofibril integrity caused by calpain activation are related to changes in myoblast proliferation and differentiation. We hypothesized that degradation products of muscle, capable of stimulating myogenesis, are produced in response to acute exercise. Wistar rats ran downhill on a motorized treadmill (16% grade, 30 m/min) for 60 min or remained sedentary. Saline extracts prepared from hindlimb muscle following exercise increased L6 myoblast proliferation and differentiation ($p<0.01$). Extracts from sedentary animals had no effect (p=NS). The active factor(s) had an apparent molecular weight of ≥ 10 kDa. A subset of animals in each group received E64c, a cysteine protease (calpain) inhibitor. The inhibitor attenuated the increase in differentiation associated with exercise ($p<0.05$), suggesting that a cysteine protease was involved in the release of soluble factors that affect myogenesis. Many of the proteins that undergo remodelling in response to exercise are calpain substrates, therefore we further hypothesized that calpain-derived myofibril fragments are capable of stimulating myoblast proliferation and differentiation. Purified myofibrils were treated with m-calpain in the presence of calcium. The autolysis of calpain was followed under the same conditions in the absence of myofibrils. Both preparations showed equal ability to stimulate myoblast proliferation and differentiation. Therefore, the primary finding of this study was that autolytic fragments of m-calpain stimulate myogenesis and calpain-derived myofibril fragments do not. Further studies showed that autolytic fragments of μ-calpain also stimulate myoblast proliferation and differentiation. The active fragment was a low molecular weight peptide (<10 kDa) and was an early product of calpain autolysis. The results suggest a novel consequence for calpain activation. Overall, the results support a relationship between the acute changes in myofibril integrity caused by calpain activation and adaptation in muscle.

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CHAPTER 1

STATEMENT OF THE PROBLEM

Skeletal muscle can alter its functional, morphological and metabolic characteristics in response to changes in the quantity and/or pattern of neuromuscular activity. Persistent changes in neuromuscular activity result in quantitative and qualitative changes in gene expression and protein metabolism (reviewed in 1). These changes alter the size and/or metabolic properties of muscle and produce an altered phenotype that is better suited to the imposed demand. The changes in phenotype have been well described (Table 1); however, the signalling mechanisms which lead to adaptation are still poorly understood. It is unclear what the initial signals are or whether they are the same for all adaptational stimuli. In this study, we investigate the relationship between the acute loss in myofibril integrity caused by protease activation and adaptation in skeletal muscle. The results of these experiments will be useful in understanding basic muscle function and will also be of clinical significance.

BACKGROUND

Muscle Adaptation

Persistent changes in neuromuscular activity induce a series of regulated changes in gene expression, effecting fast-slow protein isoform transitions in muscle phenotype (reviewed in 2). The extensive remodelling involves contractile (myosin heavy chain; MHC) and regulatory proteins (tropomyosin, troponin), soluble and membrane-bound calcium-binding proteins (parvalbumin, SR-ATPase) and metabolic enzymes (creatine kinase, hexokinase). Transition of protein isoforms involved in calcium-regulation occurs in parallel with the fast-slow transition of the MHC isoforms, suggesting a correlation between molecular and functional changes (3).

Persistent changes in neuromuscular activity can also induce regulated changes in protein metabolism. All intracellular proteins undergo a continuous process of degradation and resynthesis and the rate of flux through these two opposing pathways determines the change in protein mass observed in vivo during normal growth, hypertrophy and atrophy of skeletal muscle (4). In addition to controlling total protein mass, regulated changes in protein synthesis and/or degradation rapidly and selectively alter the content of individual structural, catalytic and regulatory proteins. Nevertheless, protease involvement in adaptation has not been well studied.

Early in the adaptation process, extensive remodelling occurs within the muscle (5-9). Various structural abnormalities, including sarcomere Z-line alterations, swollen mitochondria, fragmented or swollen sarcoplasmic reticulum elements, dilated t-tubules and lesions in the plasma membrane have been described. In most cases, cellular ion regulation is also affected. It is not clear whether acute morphological changes in the myofibril (the loss of structural integrity) are linked mechanistically to adaptation. The relationship may involve the release of soluble factors from muscle and the subsequent activation of satellite cells. **The overall purpose of this study was to investigate whether products of muscle catabolism participate in adaptation and repair.**

Post-natal muscle contains a population of muscle precursor cells (or satellite cells), located between the sarcolemma and the basal lamina (10). Satellite cells constitute between 2-10% of the total fiber-associated myonuclei in young and adult vertebrates (11) and have been identified in skeletal muscle from a variety of species (reviewed in 12). Satellite cells are characterized by a heterochromatic nucleus, relatively little cytoplasm and few organelles (13). Using conventional light microscopy, they are indistinguishable from peripherally located myonuclei (true myonuclei).

Satellite cells fuse with existing myofibers and contribute additional nuclei during post-natal growth (14). Muscle maintains a relatively constant DNA to protein ratio thus changes in myonuclear number are associated with, and appear to be necessary for, changes in myofiber size (reviewed in 15). Most studies agree that satellite cell involvement is also essential for adaptation (16-19). Satellite cells are mitotically quiescent in normal muscle (20) but are capable of migration, proliferation and differentiation in response to exercise (21,22), stretch (23), hypertrophy (24), suspension (25) and denervation (26). Satellite cell proliferation has also been reported following mild mechanical compression (27). Collectively, the data from these studies suggest that satellite cell activity is regulated by locally produced factors in muscle.

A variety of purified growth factors, each with pleiotropic effects, can influence the growth and differentiation of satellite cells in vitro (reviewed in 28). Allen and Boxhorn (29) showed that by altering the combination and concentration of purified growth factors satellite cells can be induced to proliferate, differentiate or remain quiescent. There is evidence that some of these factors are present in muscle (30,31) however, the interaction among tissues is complex and it is unclear how growth factors interact to govern the behaviour of satellite cells in vivo. Indeed, Bischoff (32) showed that satellite cells in contact with the surface of myofibers are less responsive to growth factor levels than cells in contact with the basal lamina.

The relationship between acute damage to the myofibril and adaptation is unclear but it may involve the release of soluble growth-promoting factors. Numerous investigators have shown that saline extracts from gently crushed muscle are mitogenic in a variety of myoblast culture systems (31,33-39). The composition of the saline extract has been partially characterized and most studies suggest that the extract contains a combination of known growth factors (31,36-38,40). Extracts from heterologous tissue (liver, lung, heart and kidney) and uncrushed muscles do not have the same mitogenic properties (33,36).

A similar approach has been used to determine whether anabolic factors are released from muscle in response to chronic changes in functional demand. In the avian wing stretch model, extracts from rapidly growing (chicken) patagialis muscle contained soluble mitogens that increased nearly 2-fold during stretch-induced hypertrophy (41). Muscle-derived extracts from rats trained by grid-climbing also contain soluble mitogens (42). These models do not distinguish between the acute changes in muscle and other compensatory processes. In models of chronic adaptation, mitogens or growth factors could arise separately or in combination from the vascular system (43,44), the myofiber (30,31), connective tissue or the basal lamina (45), or as a result of nerve contact and motor end plate formation (46). The extent to which mitogenic activity increases in muscle in response to an acute physiological stimulus has not been described.

HYPOTHESIS 1. Soluble factors capable of stimulating myoblast proliferation and differentiation are produced in response to periods of increased contractile activity.

We subjected rats to a single bout of downhill running on a motorized treadmill (16% grade, 60 min) and prepared saline extracts from hindlimb muscle immediately following exercise. We assayed the ability of the extracts from sedentary and exercised animals to promote growth and differentiation in a myoblast culture system. Since muscle oxidative capacity may influence susceptibility to damage (47), individual extracts were prepared from soleus (mostly Type I; oxidative) and plantaris (mostly Type II; glycolytic) muscles.

The run protocol employed in this study has been shown to result in the presence of clotted fibers and produce significant morphological changes in the organization of the sarcomere including focal

disruptions in the A-band and localized dissolution of Z-lines (48). Ultrastructural evidence suggests that there may also be lesions in the plasma membrane (49,50). A loss of membrane integrity is believed to cause the loss of cellular ion homeostasis, resulting in the activation of calcium-dependent degradative processes. We have proposed that the loss of myofibril integrity occurs secondary to protease activation (51). The mechanism responsible for promoting the release of soluble mitogens has not been described. However, it likely also occurs secondary to protease activation (52). It is not clear whether these processes share a common mechanism.

Myogenesis can be observed in both primary cultures (derived from embryonic, neonatal or adult muscle) and in immortalized myogenic cell lines. The process is outwardly similar in either case. The advantage of primary culture, which may more closely model the in vivo process, is offset by the inability to maintain a characterized or transduced cell-line over a long period of time and the heterogeneity of cell types present. For these reasons, a myogenic cell line was used the present study. L6 myoblasts were isolated originally by Yaffe (53) from primary cultures of rat hindlimb muscle maintained for the first two passages in the presence of methyl cholanthrene.

Calpain Biochemistry

Calpain (EC 3.4.22.17) is an intracellular non-lysosomal cysteine protease. Two isozymes of calpain, differing in calcium requirement, exist ubiquitously in mammalian tissues (54). The low (μM) and high (mM) calcium-requiring types are designated μ-calpain and m-calpain respectively. Both isozymes are heterodimers and consist of a catalytic (80 kDa) and a regulatory (30 kDa) subunit. The primary structure of both subunits has been deduced from cDNA cloning studies (55,56). The catalytic (80 kDa) subunit can be divided into four domains (I-IV), whereas the regulatory subunit (30 kDa) consists of two domains (IV' and V; Fig 1-1A).

Domain I shows little sequence homology with other known proteins. The NH_2-terminus inhibits assembly of the active site and regulates calcium sensitivity through association with the regulatory subunit (57). It is also removed during autocatalytic activation (58). Domain II contains the catalytic site (59). It shows sequence homology to other cysteine proteases, such as papain and cathepsins B, H and L (60). Site directed mutagenesis studies reveal that cys^{105}, his^{262} and asn^{286} residues are essential for proteolytic activity (61). Domain III is structurally related to C2 domains, calcium-regulated lipid binding modules in many enzymes (62). It has also been proposed that domain III of calpain interacts with its endogenous inhibitor calpastatin. Domains IV and IV' are well-characterized calcium-binding domains, each containing five EF-hand motifs. These domains are responsible for dimerization of the large and small subunits through a unique interaction between their fifth EF-hand motifs (59).

The small (30 kDa) subunit is involved in the regulation of calcium sensitivity. It contains five potential calcium-binding regions in the C-terminus (domain IV'). Domain V contains a cluster of hydrophobic residues in the NH_2-terminus, which is largely unresolved in the crystal structure (59). It possibly interacts with membrane phospholipids to localize or stabilize the enzyme complex (63,64).

Recent x-ray crystallography studies of rat (57) and human (65) m-calpain reveal some of the conformational changes that occur during calpain activation. In the absence of calcium, the active (catalytic) site is not assembled. The binding of calcium causes a conformational change that allows domains I and II to associate, thereby generating a competent active site (reviewed in 59). Initially, this involves subtle conformational changes in domains IV and IV', abrogating the interaction between the NH_2-terminus of domain I and the second EF motif of domain IV'. The second step in the process involves the realignment of the active site cleft caused by the cooperative binding of calcium to domain II.

Calpastatin is an endogenous inhibitor of calpain. It prevents both enzyme activation and the expression of catalytic activity (reviewed in 66). The calpastatin from mammalian sources can be classified into two types, based on molecular weight (67). The erythrocyte inhibitor (70 kDa) is smaller than the inhibitor from other tissues (110 kDa). Diversity among calpastatin species may reflect alternative splicing (68,69) and/or post-translational modifications, including proteolytic cleavage and phosphorylation (70).

The amino acid sequence of calpastatin has also been determined by cDNA cloning studies (71). Calpastatin has a repetitive domain structure (Fig 1-1B) and contains as many as four identical inhibitory sequences, each with independent activity (72). Highly conserved residues among the four repetitive domains (1-4) are clustered in three regions designated A, B and C. Region B is the inhibitory locus and regions A and C potentiate its activity by interacting with the calmodulin-like domains of the large and small subunit, respectively (66). The N-terminal region of the protein (L-domain) lacks inhibitory activity; its function has not been defined.

In the presence of calcium and possibly upon phosphorylation, calpastatin binds to calpain with high affinity, inhibiting the protease at calcium concentrations lower than those required for autolysis or proteolytic activity (73). The reactive site of calpastatin shows no apparent homology to the reactive sites of other known protease inhibitors (72) and probably does not interact with the active site cysteine of calpain, since the latter can be masked by covalent inhibitors without impairing the binding of calpastatin (74). Inhibition of calpastatin is removed either by phosphorylation of calpastatin, proteolysis of calpastatin by calpain or by competition with substrates at the active site. The interaction is reversible thus its amount relative to calpain may be important.

Calpain causes the limited degradation of various membrane and cytoskeletal proteins, enzymes and transcription factors (reviewed in 75). Cleavage occurs at specific sites, producing large polypeptide fragments with altered functional properties. For example, the limited proteolysis of protein kinase C produces an active fragment whose activity is independent of the cofactors normally required for activation of the intact molecule (76). The physiological consequences of calpain activation are uncertain however, many calpain substrates are involved in signal transduction (77), cell cycle regulation (78), differentiation and apoptosis (79). Calpain has also been implicated in several pathological conditions including cerebral and cardiac ischemia, cataract formation and Alzheimer's disease (reviewed in 80).

CALPAIN'S EFFECT ON SKELETAL MUSCLE

There is evidence for the involvement of calpain in the early events of myogenesis (81-92). Myoblast fusion is a calcium-dependent process which involves reorganization of the cytoskeleton and the redistribution of membrane components. The involvement of calcium at the ultrastructural level coincides with the formation of gap junctions which are involved in the synchronization and metabolic coupling of myoblasts prior to fusion (93). Most studies agree that m-calpain is involved in membrane reorganization prior to fusion (81,82,84,88,92) and that μ-calpain is involved in other calcium-dependent signalling cascades (83,87,92).

In normal postnatal muscle, calpain is closely associated with the I- and Z-band regions (94-98). Denervation and fasting increase the concentration of enzyme but do not change its distribution (95). In dystrophic muscle, calpain is distributed more uniformly than in normal muscle (96,99) however it is unclear whether less calpain is bound to myofibril structures. Calpain's role in muscle has been linked to the proteolysis of muscle proteins, resulting in the initiation of myofibrillar turnover and organelle disassembly. The transient and specific removal of Z-lines suggests that calpain initiates the turnover of myofibrillar proteins by uncoupling the three-dimensional structure of the myofibril.

Prolonged running exercise promotes the early activation and redistribution of calpain activity in muscle (100) and increases the susceptibility of myofibrillar proteins to degradation by calpain (101). Belcastro and co-workers (102) described the specific disruption and/or loss of Z-line structure in 22% of myofibrils isolated from exercised skeletal muscle in the rat. It has been proposed that acute changes in muscle structure and function following periods of increased contractile activity are related to calpain activation (51).

We propose that a mechanism exists which links calpain activation and the acute loss of myofibril integrity with subsequent adaptation in muscle. This relationship may involve the release of soluble anabolic factors from muscle. It appears that soluble mitogens are released from muscle concurrent with transient increases in the rate of protein degradation. To our knowledge, this relationship has never been tested experimentally.

HYPOTHESIS 2. The release of anabolic factors is mediated by a cysteine protease.

To assess whether the release of anabolic factors from muscle following exercise is mediated by the activity of a protease, a subset of animals in each group (sedentary, exercise) was treated with a cysteine protease inhibitor. (2s,3s)-Trans-epoxysuccinyl-l-leucyclamido-3-methylbutane (E64c) is a peptide inhibitor which is capable of forming irreversible sulfide linkages with the active site thiol (cysteine) of calpain (103). The application of this inhibitor to rats one hour prior to exercise effectively attenuates the reported increase in calpain activity (104). We prepared saline extracts from the soleus and plantaris and assayed their ability to promote L6 myoblast differentiation.

A variety of muscle proteins have been identified as potential substrates for calpain under in vitro conditions (reviewed in 105). In many cases, the resulting protein fragments have been identified (reviewed in 106). However, it is possible that the conformation of substrate proteins is altered by their assembly into the myofibril and that these alterations change their susceptibility to degradation by calpain. Little is known about calpain's effect on complexed proteins in organelles.

Purified myofibrils are fully competent fragments of the contractile apparatus capable of calcium-dependent shortening in the presence of ATP. The incubation of these myofibrils with purified calpain (in the presence of calcium) results in a loss of periodicity along the thin filaments without producing visible changes in the thick or thin filaments themselves (94,107-109). There is a rapid loss of N-line structure (titin and nebulin) which is followed by a gradual loss in density of the Z-line and the release of undegraded alpha-actinin (110). Calpain also rapidly degrades costameres (attachments of the myofibril to the sarcolemma) which contain desmin, vimentin, vinculin, dystrophin, spectrin and other cytoskeleton-associated proteins (111). The degradation of costameres results in a separation of the sarcolemma from the myofibril without degradation of extracellular matrix proteins. Calpain causes no bulk degradation of the sarcoplasmic protein fraction (94), thus its action is directed exclusively at myofibrillar and cytoskeletal proteins.

In other cell systems, calpain alters, by limited proteolysis, the activity or function of structural proteins (112). For example, the activation of intermediate filament proteins, such as desmin, can be achieved by the limited truncation of the NH_2-termini by calpain (113,114). The fragments retain the ability to activate nucleosomes during transcription initiation and elongation however, they have a diminished capacity to form and maintain filaments (115). It is unclear whether calpain-derived myofibril fragments can function as regulatory factors in the extracellular environment.

The calpain-mediated release of myofibrillar proteins from the sarcomere appears to be an early component of the adaptation response in muscle and many of the proteins that undergo remodelling in

response to altered loading are calpain substrates (116). Most studies suggest that calpain-derived myofibril fragments are ubiquitinated and further degraded by the 26S proteasome (117,118). We proposed that the fragments have a more important biological role.

HYPOTHESIS 3. Fragments derived from the calpain-mediated degradation of myofibrillar proteins are anabolic to myoblasts.

The arrangement of sarcomeric proteins within the myofibril complex is retained by the isolation and purification procedure (119) used in these experiments. Purified myofibrils were incubated with m-calpain in the presence of calcium and the ability of the resulting fragments to promote myoblast proliferation and differentiation was assessed. To control for the direct effects of calpain alone, the autolysis of m-calpain was followed under identical assay conditions in the absence of myofibrils.

CONSEQUENCES OF CALPAIN ACTIVATION

Both isozymes of calpain undergo rapid autolysis in presence of calcium (58). It is plausible that autolysis arises as a consequence of the conformational changes induced by calcium binding (120). It is also plausible that autolysis removes the NH_2-terminal sequence which normally suppresses protease activity or that it causes a conformational change in the molecule, leading to enzyme activation (121). Autolysis lowers the calcium requirement for substrate proteolysis (122), however the physiological significance is unclear.

Kunimatsu and co-workers (123) showed that the autolytic fragments of human erythrocyte (μ-) calpain are chemoattractive to neutrophils. They (124) further showed that synthetic peptides corresponding to the NH_2-terminal sequence of the large and small subunits of μ-calpain have chemotactic activity toward neutrophils, T and B cells and monocytes. The amino acid sequences of the regulatory subunits of μ- and m-calpain are identical and well-conserved among species (125,126), therefore both calpain isozymes could be the source of the same chemotactic factor.

A role for autolytic calpain fragments in muscle has not previously been defined, however it is plausible that autolytic fragments might also show bioactivity toward myoblasts. An interesting possibility might be that myoblasts share common receptors for N-acetyl peptides of calpain. Indeed, widespread binding capacity among cell types has been observed for other chemoattractants (reviewed in 124). For example, interleukin 8 (IL-8) is secreted from various cell types including macrophages, fibroblasts and endothelial cells and it is chemoattractive to T lymphocytes, basophils and neutrophils.

HYPOTHESIS 4. Autolytic fragments of calpain are anabolic to myoblasts.

Autolysis of μ-calpain was allowed to proceed in presence of calcium. Aliquots of the reaction mixture were removed at 10, 30 and 60 minutes and the reaction was terminated using EDTA. The ability of the autolytic fragments to stimulate myoblast proliferation and differentiation was considered.

In summary, these studies showed that a soluble factor capable of stimulating myoblast proliferation and differentiation is released from skeletal muscle in response to exercise. Furthermore, these studies suggest a novel consequence of calpain activation and support a relationship between the acute loss in myofibril integrity caused by calpain activation and adaptation in muscle.

LIST OF HYPOTHESES

1. Soluble factors capable of stimulating myoblast proliferation and differentiation are produced in response to periods of increased contractile activity.

2. The release of anabolic factors is mediated by a cysteine protease.

3. Fragments derived from the calpain-mediated degradation of myofibrillar proteins are anabolic to myoblasts.

4. Autolytic fragments of calpain are anabolic to myoblasts.

TABLE 1-1. EFFECTS OF ALTERED LOADING ON THE GROSS MORPHOLOGY, FUNCTIONAL PROPERTIES AND PROTEIN EXPRESSION IN SKELETAL MUSCLE

PROPERTY:	FUNCTIONAL OVERLOAD	WEIGHTLESSNESS
Fiber cross-sectional area	Increase*	Decrease
Maximal force (P_o)	Increase	Decrease
Normalized force	Decrease	Decrease
Maximal power output	Increase	Decrease
Shortening V_{max}	Decrease	Increase
Relaxation velocity	No change	Increase
Slow myosin phenotype	Increase	Decrease
Fast myosin phenotype	Decrease	Increase
Calcium-release proteins	No change	Increase
Calcium-sequestering proteins	No change	Increase

*change relative to control. Adapted from (1).

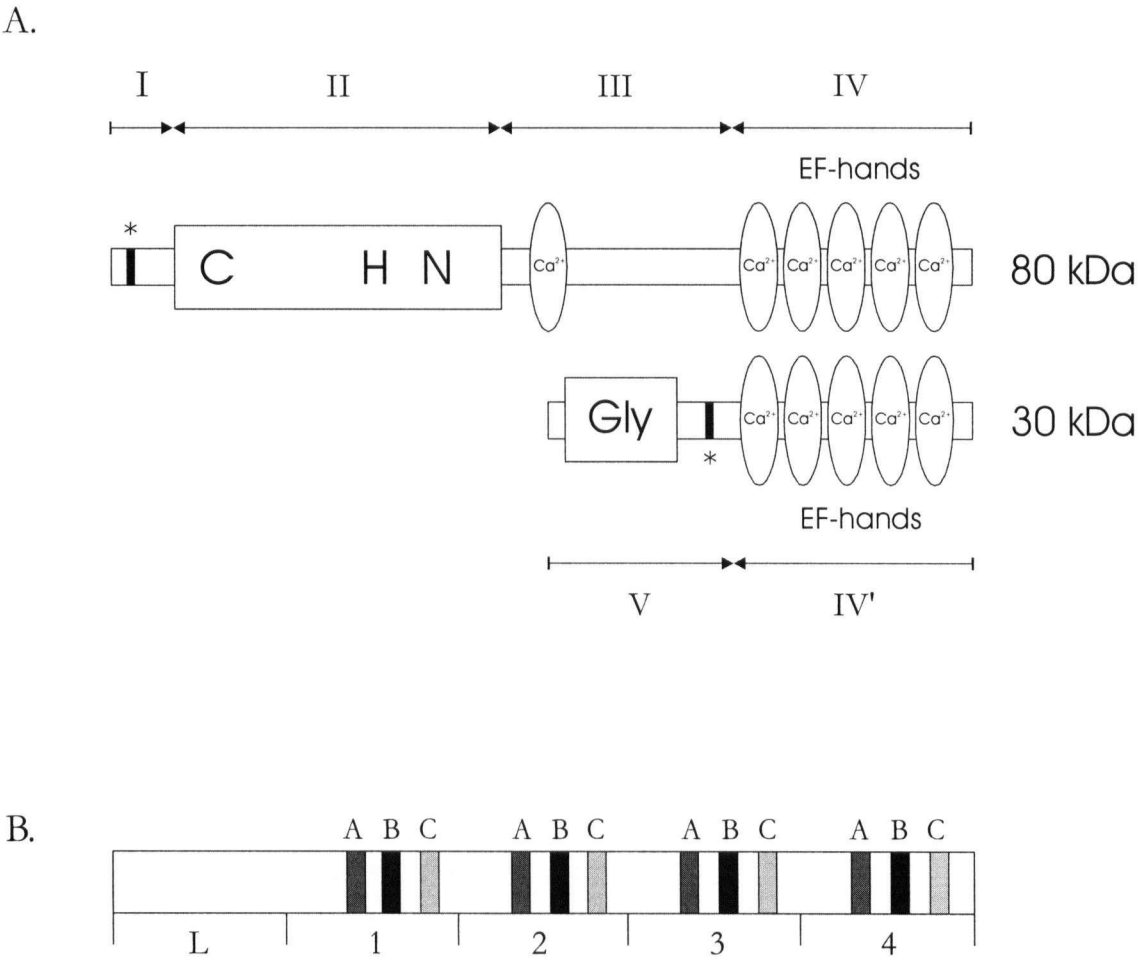

FIG 1-1. SCHEMATIC STRUCTURE OF CALPAIN AND CALPASTATIN. (A) Calpain is composed of a catalytic (80 kDa) and a regulatory subunit (30 kDa). Domain II contains the catalytic site with cysteine (C), histidine (H) and asparagine (N) residues. Domains IV and IV' are calcium-binding domains, each containing 5 EF-hand structures. * denotes sites of autolysis in the NH_2-terminus of each subunit. (B) Calpastatin has a repetitive domain structure and contains as many as four identical inhibitory sequences. Highly conserved residues among the four repetitive domains (1-4) are clustered in three regions designated A, B and C. Region B is the inhibitory locus and regions A and C potentiate its activity by interacting with the large and small subunits of calpain. The function of the L domain has not been defined. Adapted from (66).

CHAPTER 2

SOLUBLE EXTRACTS DERIVED FROM HINDLIMB MUSCLE FOLLOWING EXERCISE STIMULATE MYOBLAST PROLIFERATION AND DIFFERENTIATION

In skeletal muscle, molecular and cellular adaptation occurs in response to changes in functional demand. The changes in phenotype have been well described (reviewed in 1), however the signalling mechanisms which lead to adaptation are still poorly understood. In particular, the role of protein degradation and its specificity in this process has not been well studied.

When changes in demand are imposed, extensive remodelling occurs within the muscle. There are significant morphological changes in the organization of the sarcomere including focal disruptions in the A-band and localized dissolution of Z-lines (48). Ultrastructural evidence suggests that there may also be lesions in the plasma membrane (49,50). During the repair process, adaptation occurs which renders the muscle less susceptible to further injury (127). It is not clear whether the acute loss of myofibril integrity is linked mechanistically to this functional outcome.

Post-natal muscle contains a population of muscle precursor cells (or satellite cells), located between the sarcolemma and the basal lamina (10). These cells are mitotically quiescent in intact muscle (20) but are capable of migration, proliferation and differentiation under a variety of conditions including exercise (21,22), stretch (23), compression (27), hypertrophy (24) and suspension (25). Satellite cells fuse with existing fibers following injury (128,129) and most studies agree that their involvement is essential for adaptation (16-19).

Satellite cell proliferation has been measured in enzymatically isolated fiber segments following a single bout of downhill running exercise (21). In the soleus, ^{3}H-thymidine incorporation reaches a maximum within 24 hours. This observation is consistent with histological evidence of injury to the muscle. The underlying mechanism was not described, however satellite cell activity was probably regulated by factors produced locally in muscle.

Saline extracts from crushed muscle have been used in a variety of myoblast culture systems to identify which factors are released from muscle following injury (31,33-39). In this model, intact muscle is gently crushed along its length with blunt forceps and soluble factors are collected in the supernatant following a 60-90 min incubation in Tris- or phosphate buffered saline. A similar muscle extract approach has been used to determine whether the abundance and/or availability of growth factors change in response to a chronic increase in loading. Summers and co-workers (41) showed that extracts from rapidly growing chicken patagialis muscles contain soluble growth factors that increase nearly 2-fold during stretch induced hypertrophy. The extracts from stretched muscle produced a dose-dependent increase in embryonic chick myoblast proliferation. Muscle-derived extracts from rats trained by grid climbing also contain soluble mitogens (42). Chronic training models do not distinguish between acute changes in myofibril integrity and other compensatory processes related to exercise. The extent to which mitogenic activity increases in response to an acute exercise stimulus has not been well described.

The mechanism responsible for promoting the release of soluble mitogens in response to injury has also not been well described. Prolonged running exercise promotes the early activation and redistribution of calpain activity in muscle (100) and increases the susceptibility of myofibrillar proteins to degradation by calpain (101). It has been proposed that the loss of Z-line integrity that occurs following exercise is related to calpain activation (51). It is reasonable to predict that growth factor release from muscle also occurs secondary to calpain activation.

We prepared saline extracts from rat hindlimb muscle following an acute bout of downhill-running exercise and assayed their ability to promote myogenesis in an established cell line. We hypothesized that soluble factors, capable of stimulating myoblast proliferation and differentiation, are released from muscle immediately following downhill running exercise. The oxidative capacity of muscle may influence its susceptibility to damage (47), therefore we considered fiber-type differences using individual extracts from soleus (mostly Type I; oxidative) and plantaris (mostly Type II; glycolytic) muscles. To assess whether the release of anabolic factors is mediated by protease activity, a subset of animals in each group was treated with a cysteine protease inhibitor. (2s,3s)-Trans-epoxysuccinyl-l-leucyclamido-3-methylbutane (E64c) is a peptide which is capable of forming irreversible sulfide linkages with the active site thiol (cysteine) of calpain.

METHODS

ANIMALS AND GROUPS

All experimental procedures were carried out in strict accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of British Columbia Ethics Committee. Male Wistar rats (225-250 g; Charles River Laboratories, Inc.) were housed in a temperature-controlled facility, maintained on a 12 h light/dark cycle and fed commercial pellets and water ad libitum. The rats were randomly assigned to two groups, control (CONTROL) and exercise (RUN). A subset of animals in each group received a subcutaneous injection of (2s,3s)-trans-epoxysuccinyl-L-leucylamide-3-methylbutane (E64c; 1 mg/kg; Sigma-Aldrich Corp.), a cysteine protease inhibitor, 1 h prior to exercise or rest. Each experimental group contained at least 4 animals and all assays were performed on each rat individually, unless noted otherwise.

EXPERIMENTAL PROTOCOL

Animals were familiarized (15 m/min, 5 min) on a motorized treadmill 2 d prior to the run protocol. Rats ran downhill (30 m/min; 16% grade) for 60 min (RUN) or remained in their cages (CONTROL). The animals were encouraged to run using mild noise and air stimulation directed at the tail region. The treadmill was equipped with 10 x 50 cm plexiglass-enclosed lanes and a photoelectric beam which, when broken, delivered compressed air at a right angle to the animals' haunches. The animals were under continuous supervision and food and water was readily accessible at all times except while running. The animals were not forced to run and were not run to exhaustion.

At the completion of exercise or rest, rats were anaesthetized with pentobarbital sodium (65 mg/kg ip; MTC Pharamaceuticals) and the hindlimb muscle was exposed via an incision through the skin and fascia. The soleus and plantaris muscles from both hindlimbs were excised under aseptic conditions and incubated in cold phosphate-buffered saline (PBS; 0.9% NaCl, 0.20 g/L KCl, 1.15 g/L Na_2HPO_4, 0.20 g/L KH_2PO_4, pH 7.4) with gentle shaking for 60 min. The soluble factors were collected in the supernatant after centrifugation at 1000 g. The extracts were sterile filtered using a 0.22 μm Millex-GS filter (Millipore) and the protein concentration was determined by the method of Bradford using BSA Fraction V as a standard. The supernatant was used in cell culture studies at concentrations of 10-100 μg total protein/mL to determine its effect on myoblast proliferation and differentiation.

CELL CULTURE

Rat L6 myoblasts were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DME; Sigma-Aldrich Corp.) containing 4.0 mM L-glutamine, 1.0 mM sodium pyruvate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate and supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, Inc.). L6 myoblasts fuse to form multinucleated fibers,

however the extent of fusion declines with continued subcultivation. Early passages were frozen in 20% FBS/DME containing 10% dimethylsulfoxide (DMSO; Sigma-Aldrich Corp.) and stored at -135°C. A new ampule was used for each experiment.

CELL PROLIFERATION ASSAY

For experimental analysis of total DNA, myoblasts were plated at sufficiently low density (2.5 x 10^4/cm^2) to allow for subconfluent growth in positive control wells treated with 10% FBS. Cells were maintained in a 5% CO_2 atmosphere at 37°C. Twelve h later, cells were rinsed 2X in serum-free medium and cultured for an additional 24-48 h in medium containing 0.5% FBS/DME and 10 μL of extract in a total volume of 100 μL (0-100 μg/mL total extract protein). Cultures were processed at the time of medium switch (t=0) and at 24, 36 and 48 h thereafter. Cell viability was assayed at each time point by trypan blue exclusion and was always >98%.

DNA content was assessed using Hoechst 33258 dye (bisbenzimide; Sigma-Aldrich Corp.) as previously described (130). Briefly, the cells were rinsed 1X with serum-free medium, fixed with cold 70% ethanol/formalin/acetic acid (20:2:1), then rinsed again. An aqueous working solution of Hoechst 33258 dye (1 μg/mL) was prepared in 150 mM sodium citrate, 0.9% NaCl (1X SSC; pH 7.0) immediately prior to use. 1 mL was added to each well. After 30 min incubation at room temperature, sample fluorescence was measured (355 nm excitation, 460 nm emission) using a microplate reader (Wallac, Inc.). To ensure that sample readings remained within the detection range of the microplate reader, the instrument's gain was set so that the sample containing the highest DNA concentration yielded a fluorescence intensity near the fluorometer's maximum. To minimize photobleaching effects, the time for fluorescence measurement was held constant for all samples. The Hoechst 33258-based assay is selective for dsDNA, does not show significant fluorescence enhancement in the presence of proteins and allows the detection and quantitation of DNA concentrations as low as 10 ng/mL DNA (131). Results were expressed relative to the amount of DNA in myoblast cultures grown in medium containing 0.5% FBS/DME and 10 μL phosphate buffered saline.

ASSAYS OF TERMINAL MUSCLE DIFFERENTIATION

For experimental analysis of total myosin heavy chain (MHC), cells were plated at high density (1 x 10^5/cm^2). When cells are plated at this density (confluence), there is no significant increase in total DNA per well (data not shown). Twelve h later, cells were washed twice in serum-free media and cultured for an additional 48-96 h in media containing 0.5% FBS/DME and 10 μL of extract in a total volume of 100 μL (0-100 μg/mL total extract protein). This media was chosen to maximize the effects of differences in IGF-I responsiveness (data not shown). Media was replaced at 48 h intervals. Cultures were processed at the time of medium switch (t=0) and 48, 72 and 96 h thereafter. Cell viability was assayed at each time point by trypan blue exclusion and was always >95%.

Differentiation was assessed by Western blot analysis of MHC expression as previously described (132). Briefly, cultures were rinsed 3X in PBS and solubilized in Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 0.01% bromophenol blue). Samples were heated to 95°C for 2 min and stored frozen. Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose (0.45 μm; Bio-Rad Laboratories, Inc.) using a Mini Trans-Blot® apparatus (Bio-Rad Laboratories, Inc.) and the buffer system described by Towbin (133). Prestained molecular weight standards (Bio-Rad Laboratories, Inc.) appeared as colored bands on the membrane and provided a good qualitative assessment of transfer efficiency.

The membranes were immersed in Tris-buffered saline (TBS; 0.9% NaCl, 50 mM Tris pH 7.5) and 10% H_2O_2 for 30 min to reduce endogenous peroxidase activity and then blocked for 2 h at Room

temperature in TBS-Tween 20 (TBS-Tw) + 5% nonfat dry milk to reduce non-specific binding. The immobilized proteins were probed with MF-20 (1:20; Developmental Studies Hybridoma Bank, University of Iowa; contributed by Dr D Fischman), a monoclonal antibody directed against sarcomeric myosin heavy chain (134). After 2 h, the membranes were rinsed 3X using a generous volume of TBS-Tw and incubated with affinity-purified peroxidase-conjugated goat anti-mouse IgG (1:5000; Life Technologies, Inc.) for an additional hour. The blots were rinsed as before, and the bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Inc.) as described in the instructions provided by the manufacturer. Band intensities were quantified by densitometry (UNSCAN-IT; Silk Scientific, Inc.). Results were expressed relative to MHC expression in myoblast cultures grown in medium containing 0.5% FBS/DME and 10 μL phosphate buffered saline.

Terminal differentiation was also assessed by immunocytochemical staining as described previously (135). Briefly, cultures were rinsed 3X with serum-free media, fixed with cold 70% ethanol/formalin/glacial acetic acid (20:2:1) and rinsed again. The fixed cells were treated with TBS containing 1% normal goat serum (TBS-NGS; Life Technologies, Inc.) at 4°C overnight to reduce non-specific binding. Cultures were exposed to MF-20 (1:5 v/v in TBS-NGS) for 1 hour at room temperature and rinsed 4X with TBS containing 0.05% Tween-20 (TBS-Tw). Cells were then exposed to affinity-purified fluorescein isothyocyanate (FITC)-labelled goat anti-mouse IgG (Cappel) at 1:500 in TBS-NGS for 1 h at room temperature. During the last 10 min of incubation, cells were counterstained with ethidium bromide (0.01%). Finally, cells were rinsed 4X with TBS-Tw and mounted under a coverslip in glycerol:TBS (9:1). Cells were viewed with epifluorescence optics using a Nikon Optiphot 2 microscope (Tokyo, Japan). The total number of nuclei per field and the number of nuclei within MHC-positive cells were counted in five randomly selected fields (per dish).

RESULTS

All animals in RUN completed 60 min of downhill running (16% decline) at 30 m/min. To determine whether an extract derived from rat hindlimb muscle following exercise can stimulate myoblast proliferation, cells were cultured at low density in medium containing 0-100 μg/mL total extract protein. DNA content was assessed using Hoechst dye 36 h following the switch to low-serum medium. At equivalent protein concentrations (50 μg/mL), extracts from RUN caused a 30% increase ($p<0.01$) in total DNA (Fig 2-1A) compared to vehicle (saline only). Extracts from CONTROL showed no effect. The increase in DNA induced by extracts from RUN was significantly different from CONTROL only at 36 h (Fig 2-1B). The effect of RUN on total DNA was greater ($p<0.01$) than CONTROL at all concentrations tested, however there was a dose-dependent response using extracts from RUN only (Fig 2-1C). Extracts from CONTROL were not significantly different from vehicle at any of the concentrations tested. To determine whether the effect of exercise on myoblast proliferation was fiber-type specific, extracts from soleus and plantaris were considered independently (Fig 2-1D). Higher total protein concentrations were recovered in extracts from plantaris (100 μg/mL vs 50 μg/mL; $p<0.05$). At equivalent concentrations (50 μg/mL extract protein), extracts from RUN SOLEUS and RUN PLANTARIS caused equivalent increases in total DNA (p=NS). The data suggest that extracts from RUN can stimulate myoblast proliferation and that the effect is probably not fiber-type specific.

Terminal differentiation involves the induction of muscle-specific genes required for specialized function of the mature fiber (contractile proteins, metabolic enzymes, cell surface synaptic molecules). MHC was chosen as a marker of differentiation because it is produced only in differentiated myoblasts and myotubes. To determine whether an extract derived from muscle following exercise can stimulate differentiation, myoblasts were cultured at high density in media containing 0-100 μg/mL total extract protein. MHC expression was assessed 48, 72 and 96 h later using Western blot analysis. Extracts from

CONTROL had no effect on total MHC (relative to vehicle) whereas extracts from RUN caused a 255% increase ($p<0.01$) after 72 h (Fig 2-2A). Differences ($p<0.01$) between CONTROL and RUN were observed across all time points measured (Fig 2-2B). The biggest difference was observed at 48 h. The effect of RUN on MHC expression was greater than CONTROL at all concentrations tested ($p<0.01$) and there was a dose-dependent response using extracts from RUN only ($p<0.01$; Fig 2-2C). When considered independently, extracts from RUN SOLEUS and RUN PLANTARIS caused equivalent (p=NS) increases in total MHC (239 and 276%, respectively; Fig 2-2D). The data suggest that extracts from RUN can stimulate myoblast differentiation and that the effect is probably not fiber-type specific.

To distinguish between increased differentiation and hypertrophy as a mechanism underlying the increase in total MHC, myoblasts were plated at confluent density (6×10^5) in 35 mm dishes. Pooled extracts from 5 animals were added to the medium (100 μg/mL total extract protein) and MHC expression was evaluated 72 h later using Western blot analysis (Fig 2-3A) and immunocytochemical staining (Figs 2-3B and 2-3C). Western blot analysis revealed an increase in total MHC associated with extracts from CONTROL and RUN. In the soleus, this increase was approximately equal. In the plantaris, RUN extracts resulted in significantly more MHC (compared to extracts from CONTROL; $p<0.05$). Ethidium bromide staining revealed an increase in the total number of nuclei (Fig 2-3B) using extracts from CONTROL and RUN (compared to vehicle). The effect was not fiber-type specific as the increases were approximately equal for soleus and plantaris. Overall, there were a greater number of nuclei within MHC+ cells for CONTROL and RUN (compared to vehicle). The increase was greater for extracts from RUN (in both muscle groups). There were significantly more nuclei within MHC+ cells for RUN PLANTARIS (compared to RUN SOLEUS). Photomicrographs illustrating typical results are shown in Fig 2-4. The data show that hypertrophy was not the mechanism responsible for the increase in MHC expression. Rather, extracts from RUN increased the number of differentiated nuclei in the myoblast cultures.

To characterize the source of bioactivity based on molecular weight, size exclusion chromatography was employed on pooled extracts from 5 animals. Unfractionated extracts (60 μg/mL) from CONTROL and RUN caused equivalent increases in total DNA (compared to vehicle; $p<0.05$). There was no effect associated with the <10 kDa fraction in either group. The ≥10 kDa fraction caused equivalent increases in both CONTROL and RUN. The lack of difference between pooled extracts from CONTROL and RUN could not be explained by this data set. Unfractionated extracts from CONTROL had no effect on total MHC, whereas those from RUN resulted in a modest increase (60%; $p<0.05$). There was no effect on myoblast differentiation associated with the <10 kDa fraction in either group. The ≥10 kDa fraction caused equivalent increases in total MHC in both CONTROL and RUN ($p<0.05$). The results fail to demonstrate that soluble factors of low molecular weight (<10 kDa) increase in abundance or availability following exercise. Rather, the active factor(s) had an apparent molecular weight of ≥10 kDa

To consider a role for calpain in the release of soluble factors following exercise, a subset of animals was treated with E64c one hour prior to exercise or rest. The administration of the inhibitor caused an increase in MHC expression associated with control extracts from both muscle types (compared to no inhibitor). The inhibitor attenuated the increase in MHC expression associated with exercise in extracts from plantaris only ($p<0.05$).

DISCUSSION

The overall purpose of this study was to determine whether anabolic factors are released from muscle following periods of increased contractile activity. The results showed that saline extracts derived from rat hindlimb muscle after 60 minutes of downhill running cause an increase in myoblast proliferation and

differentiation. Extracts from soleus (mostly type I; oxidative) and plantaris (mostly type II; glycolytic) muscle caused equivalent increases per mg protein. Immunocytochemical analysis suggested that the increase in MHC expression was probably not related to hypertrophy. Size exclusion chromatography was used to characterize the source of activity. There was no activity associated with the less than 10 kDa fraction. The administration of (the protease inhibitor) E64c to animals one hour prior to exercise attenuated the increase in myoblast differentiation, suggesting that cysteine protease activity was involved. These data indicate that a soluble factor, capable of stimulating myoblast proliferation and differentiation, is released from rat hindlimb muscle immediately following a single bout of downhill running exercise.

The histological appearance of the soleus and plantaris was not directly assessed in the current study, however other studies have shown that downhill running can induce focal injury in the soleus and gastrocnemius while the plantaris remains largely unaffected (127,136,137). Previous reports from our laboratory (Ball and Belcastro, unpublished data) showed that plasma levels of the muscle specific isoform of creatine kinase (CK) increase two-fold immediately following this run protocol. This report and others (49,136) suggest that lesions in the plasma membrane occur and provide a plausible mechanism whereby soluble factors might be released from the muscle. Our data support the release of a soluble factor.

Fiber oxidative capacity may be important in determining the extent of fiber damage that occurs immediately following eccentric activity (47). Higher total protein concentrations were derived in extracts from the plantaris however, at equivalent protein concentrations, the magnitude of effect on proliferation and differentiation was the same as for soleus. This suggests that the same factor or combination of factors was present in the extracts from both fiber types, albeit in differing amounts. The idea that the regulation of proliferation and fusion is controlled locally is consistent with our findings. The idea that regulation might differ for muscle fibers of different functional roles and different fiber-types was not supported by our data (138). Further, the histological differences between soleus and plantaris reported by others (127,136,137) do not appear to correlate with release of mitogenic or differentiation-stimulating activity following exercise in the current study.

The soluble factor(s) described in this study effectively stimulated myoblast proliferation and differentiation. The activity fractionated with an apparent molecular weight of greater than 10 kDa. Most of the known purified growth factors are peptides with molecular weights of less than 30 kDa. The best understood purified growth factors that modulate myoblast activity are fibroblast growth factor (FGF), the insulin-like growth factors (IGF-I and -II) and transforming growth factor beta TGF-beta; (reviewed in 28). FGF and IGF have mitogenic properties whereas TGF-beta has no effect or suppresses myoblast proliferation. FGF and TGF-beta are potent inhibitors of differentiation whereas IGF can effectively stimulate both differentiation and hypertrophy.

Based on the ability of the run extract to stimulate both proliferation and differentiation, it is likely that IGF was present. IGF is low molecular weight (7.5 kDa) polypeptide that is structurally related to insulin (139). There is a progressive stimulation of myoblast differentiation at low concentrations of IGF, whereas higher concentrations lead to a decrease in differentiation and a stimulation of proliferation (140). Chen and co-workers (36,37) and Haugk and co-workers (38) provide evidence that IGF-I is a component of muscle extracts derived following a crush injury. The size exclusion data argues against IGF as the source of bioactivity since no effect on proliferation or differentiation was observed in the <10 kDa fraction. However, the availability of IGF is regulated by a family of high molecular weight binding proteins (IGFBP; reviewed in 141) which could easily account for the IGF-like activity in the greater than 10 kDa fraction.

Our data could not determine whether the saline extract contained one or more additional factors, each with independent activity. Studies performed with combinations of growth factors reveal potent and specific interactions (reviewed in 28,142). These studies suggest that growth factors bind to different receptors and generate intracellular signals which act synergistically to initiate an integrated response in myoblasts.

We used E64c to assess whether cysteine protease activity was involved in the release of anabolic factors. Since E64c inhibits other cysteine proteases such as cathepsin B and L (103), it is difficult to assess whether the in vivo effects described for this inhibitor were a result of calpain inhibition alone. Therefore, the data cannot exclude the possibility that calpain was not responsible for the activity observed in the extracts. Notwithstanding, Raj and co-workers (104) and others (143) have shown that the increase in calpain activity associated with exercise can be completely attenuated with E64c. Other studies have shown that acute morphological changes in muscle associated with calpain activation can be prevented by the in vivo administration of E64c (144).

The observation that E64c pretreatment produced different results in the soleus and plantaris following exercise was expected. Running exercise results in a greater increase in calpain activity in plantaris than in soleus (143). Since soleus calpain activity is not greatly influenced by run exercise, the lack of response to E64c pretreatment in our study was not surprising. The acute morphological changes in soleus associated with exercise might be related to an increased susceptibility of proteins to degradation rather than a net increase in protease activity (101). In the plantaris, cysteine protease activity is much higher following run exercise thus the result of E64c pretreatment was more evident in our myoblast system.

The observation that extracts from control animals treated with E64c caused an increase in MHC expression was somewhat unexpected. Previous studies have shown that this dose of inhibitor is non-toxic (144) and does not affect resting levels of calpain activity (145). However, in vitro studies show that E64c binds less effectively to calpain (146) in the absence of calcium and possibly changes the interaction between the enzyme and its endogenous inhibitor calpastatin. The activation of calpain by calpastatin repression is a possible explanation for our findings, but the extent to which this interaction changed in the current study cannot readily be determined.

Preliminary reports demonstrating a relationship between increased calpain activity and indices of muscle damage following run exercise (143) lend additional support to the suggestion that calpain activation may be responsible for release of the bioactivity described in this study. In addition, prolonged running exercise promotes the activation and redistribution of calpain activity in muscle (100). Exercise also increases the susceptibility of myofibrillar proteins to degradation by calpain (101).

Overall, the results suggest that an acute increase in contractile activity can promote the release of factors from muscle which stimulate myoblast proliferation and differentiation. Although no direct assessment of long-term adaptation was planned for in these experiments, the observation lends support to the hypothesis that acute changes in muscle are linked to long-term adaptation. The extent to which repeated bouts of exercise would continue to promote the release of soluble factors is unknown and warrants further investigation. Stupka and co-workers (147) report that a single bout of eccentric exercise can induce adaptation in muscle that results in a reduced force deficit and attenuated CK release. The role of calpain in the adaptation process warrants further consideration.

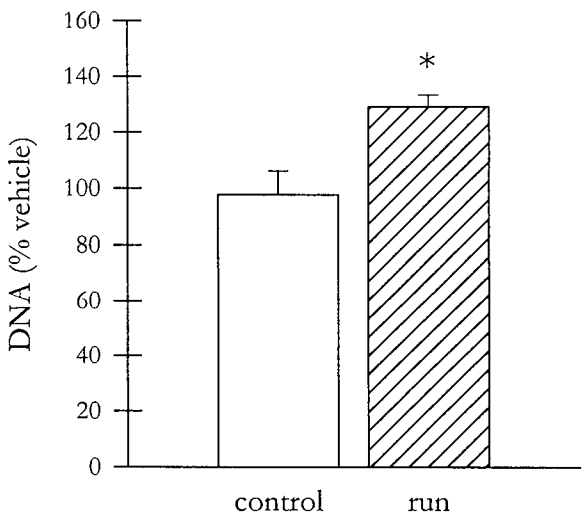

FIG 2-1A. TOTAL DNA IN MYOBLASTS PLATED AT LOW DENSITY IN MEDIUM CONTAINING EXTRACTS FROM CONTROL OR RUN. Medium was 0.5% FBS/DME + 50 μg/mL extract. DNA was assessed at 36 h using Hoechst dye and expressed relative to vehicle (saline only). Values represent mean ± SD (n=10/group). *denotes $p<0.01$ using a t-test.

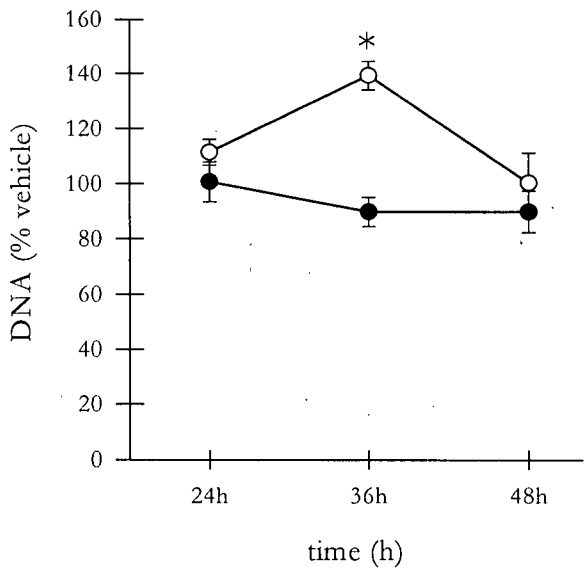

FIG 2-1B. TOTAL DNA IN MYOBLAST CULTURES 24-48 H FOLLOWING SWITCH TO MEDIUM CONTAINING EXTRACTS FROM CONTROL (●) OR RUN (○). Medium was 0.5% FBS/DME + 50 μg/mL extract. DNA was assessed using Hoechst dye and expressed relative to vehicle (saline only). Values represent mean ± SD (n=10/group). Muscle groups combined. Overall effect by two-way ANOVA. *denotes difference from control, $p<0.01$.

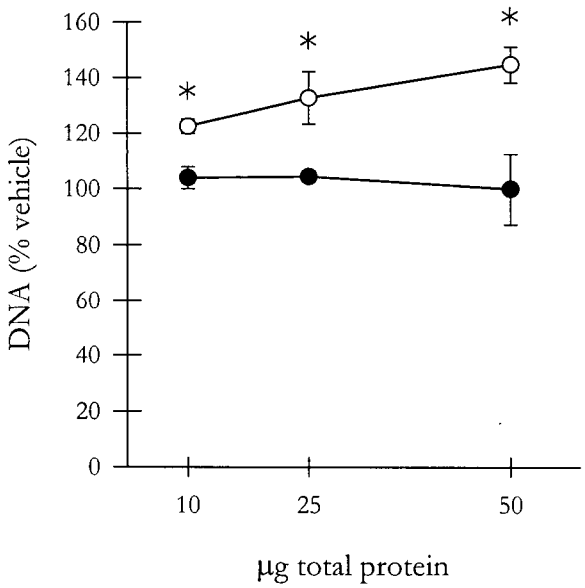

FIG 2-1C. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING EXTRACTS (10-100 µg/mL) FROM CONTROL (●) OR RUN (○). DNA was assessed after 36 h using Hoechst dye and expressed relative to vehicle (saline only). Values represent mean ± SD (n=10/group). Muscle groups combined. Overall effect by two-way ANOVA. *denotes difference from control, $p<0.01$.

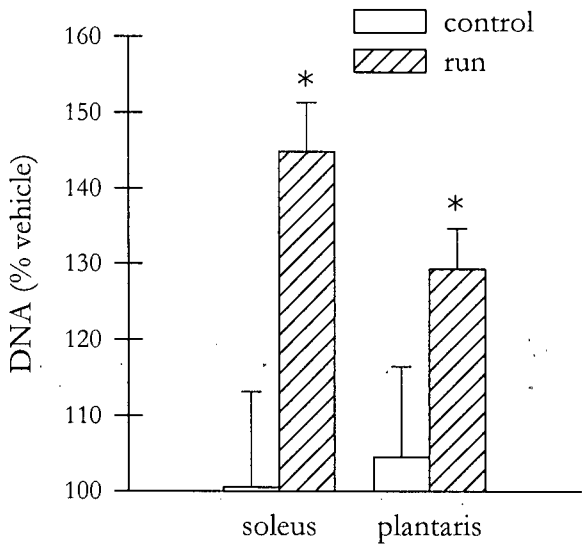

FIG 2-1D. FIBER-TYPE SPECIFIC EFFECTS ON TOTAL DNA IN MYOBLASTS PLATED AT LOW DENSITY IN MEDIUM CONTAINING EXTRACTS FROM CONTROL OR RUN. Medium was 0.5% FBS/DME + 50 µg/mL extract. DNA was assessed at 36 h using Hoechst dye and expressed relative to vehicle (saline only). Values represent mean ± SD (n=5/group). Overall effect by two-way ANOVA. *denotes difference from control, $p<0.01$

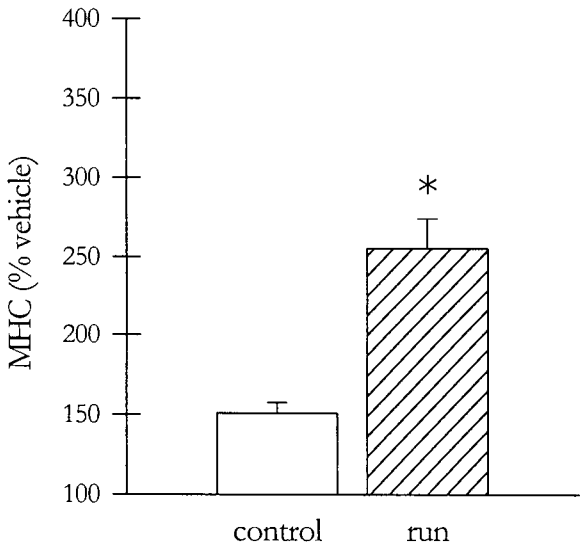

FIG 2-2A. MHC EXPRESSION IN MYOBLASTS PLATED AT HIGH DENSITY IN MEDIUM CONTAINING EXTRACTS (100 μg/mL) FROM CONTROL OR RUN. MHC was assessed by Western blot analysis using MF-20; bands were quantified by densitometry and expressed relative to vehicle (saline only). Values represent mean ± SD (n=8/group). Muscle groups combined. *denotes $p<0.01$ using t-test.

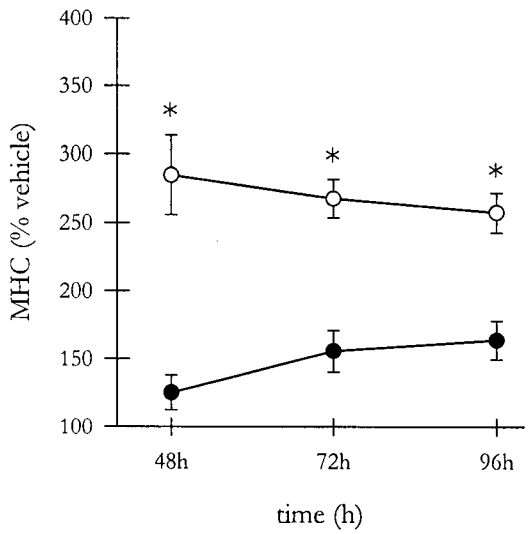

FIG 2-2B. MHC EXPRESSION IN MYOBLAST CULTURES 48-96 H FOLLOWING SWITCH TO MEDIUM CONTAINING EXTRACTS FROM CONTROL (●) OR RUN (○). MHC was assessed by Western blot analysis; bands were quantified by densitometry and expressed relative to vehicle (saline only). Values represent mean ± SD (n=8/group). Muscle groups combined. Overall effect by two-way ANOVA. *denotes difference from control, $p<0.01$.

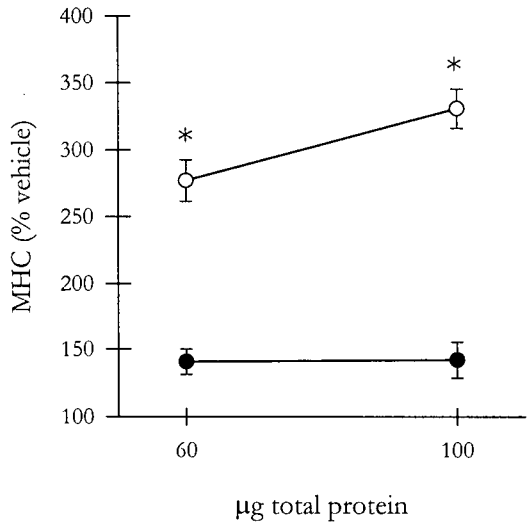

FIG 2-2C. MHC EXPRESSION IN MYOBLASTS PLATED AT HIGH DENSITY IN MEDIUM CONTAINING EXTRACTS (60-100 μg/mL) FROM CONTROL (●) OR RUN (○). MHC was assessed by Western blot analysis; bands were quantified by densitometry and expressed relative to vehicle (saline only). Values represent mean ± SD (n=4/group) using extracts from plantaris only. Overall effect by two-way ANOVA. *denotes difference from control, $p<0.01$.

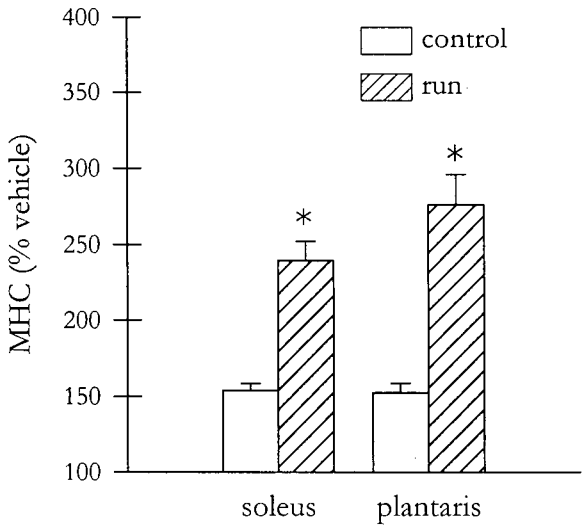

FIG 2-2D. FIBER-TYPE SPECIFIC EFFECTS ON MHC EXPRESSION IN MYOBLASTS PLATED AT HIGH DENSITY IN MEDIUM CONTAINING EXTRACTS FROM CONTROL OR RUN. MHC was assessed by Western blot analysis; bands were quantified by densitometry and expressed relative to vehicle (saline only). Values represent mean ± SD (n=4/group). Overall effect by two-way ANOVA. *denotes difference from control, $p<0.01$.

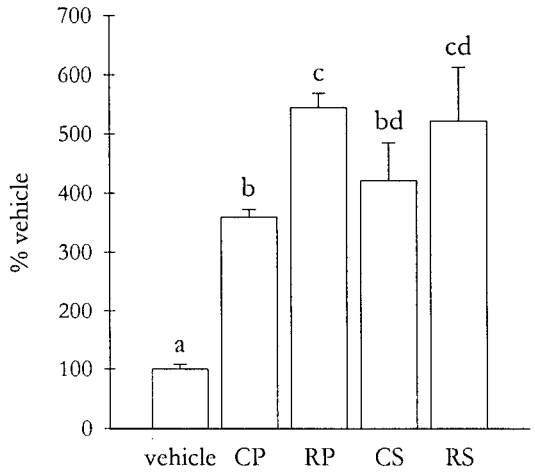

FIG 2-3A. WESTERN BLOT ANALYSIS OF MHC EXPRESSION IN MYOBLASTS 72 H FOLLOWING SWITCH TO MEDIUM CONTAINING EXTRACT FROM CONTROL OR RUN. Pooled extracts from 5 animals. Medium was 0.5% FBS/DME + 100 μg/mL extract. Values represent mean ± SD (n=2/group). Significant effects by two-way ANOVA. Values without a common letter are significantly different, $p<0.05$. CP=control plantaris; RP=run plantaris; CS=control soleus; RS=run soleus.

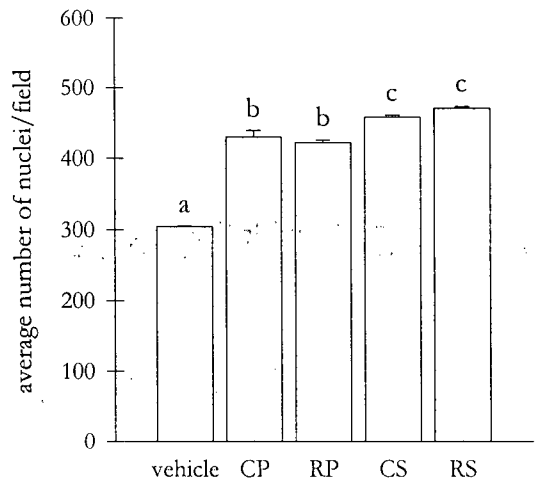

FIG 2-3B. TOTAL NUMBER OF NUCLEI PER FIELD IN MYOBLASTS PLATED AT CONFLUENCE AND MAINTAINED FOR 72 H IN MEDIUM CONTAINING EXTRACT FROM CONTROL OR RUN. Nuclei were stained with ethidium bromide (0.1%). Individual data points represent the mean of five fields. Values are mean ± SD (n=2/group). Significant effects by two-way ANOVA. Values without a common letter are significantly different, $p<0.05$.

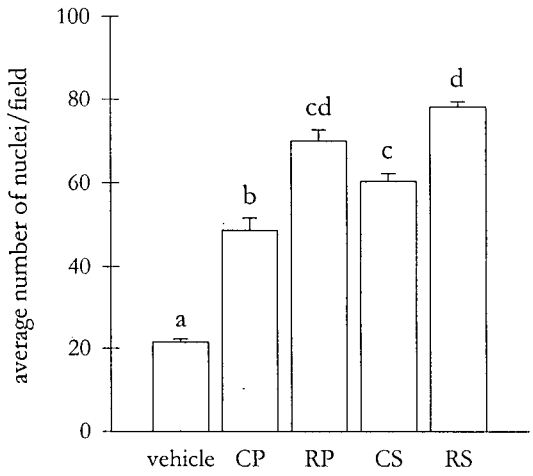

FIG 2-3C. NUMBER OF NUCLEI WITHIN MHC+ CELLS PER FIELD IN MYOBLASTS PLATED AT CONFLUENCE AND MAINTAINED FOR 72 H IN MEDIUM CONTAINING EXTRACT FROM CONTROL OR RUN. Nuclei were stained with ethidium bromide (0.1%). MHC+ cells were identified using MF-20 and fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Individual data points represent mean of five fields. Values are mean ± SD (n=2/group). Significant effects by two-way ANOVA. Values without a common letter are significantly different, $p<0.05$.

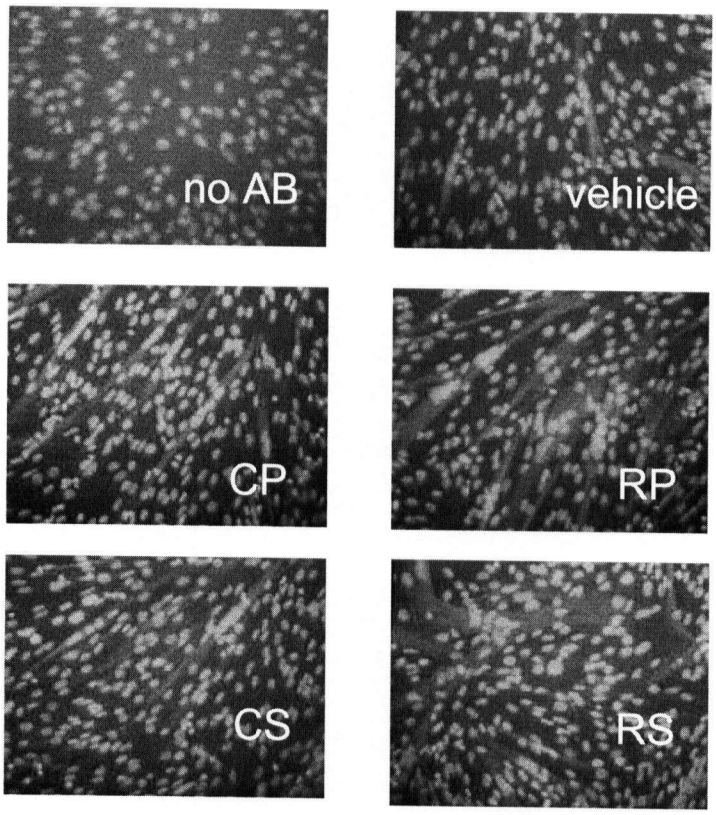

FIG 4. IMMUNOCYTOCHEMICAL STAINING OF DIFFERENTIATION IN MYOBLASTS USING ANTI-SARCOMERIC MHC. Nuclei were stained with ethidium bromide. Cells were photographed using epifluorescence optics. Results are typical of experiments performed twice. CP=control plantaris; RP=run plantaris; CS=control soleus; RS=run soleus.

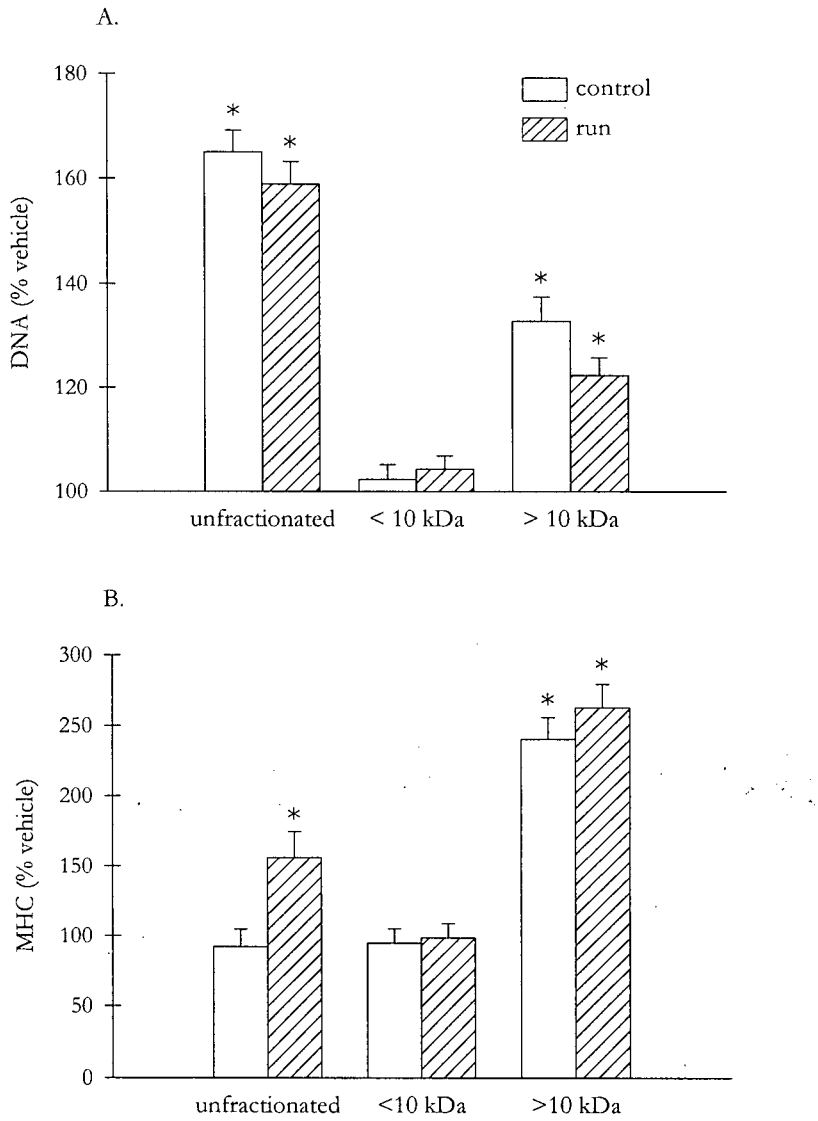

FIG 2-5. ESTIMATION OF MOLECULAR WEIGHT USING SIZE EXCLUSION CHROMATOGRAHPY. Pooled extracts from 5 animals (CONTROL and RUN) were fractionated using 10 kDa molecular weight cut-off filters (MWCF) and applied to myoblast cultures as previously described. (A) DNA was assessed using Hoechst dye 36 h following switch to low-serum medium containing 60 μg/mL extract from control or run. (B) MHC content was assessed by Western blot analysis 72 h following switch to low-serum medium containing 60 μg/mL extract from CONTROL OR RUN. *denotes $p<0.01$ (compared to vehicle).

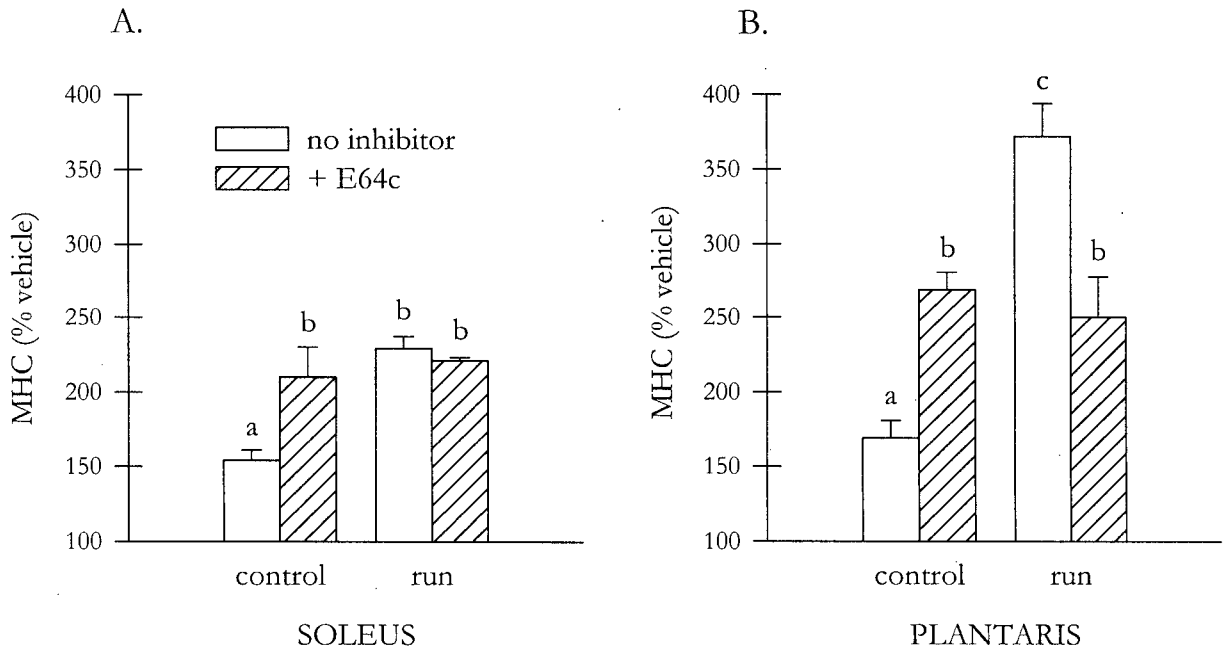

FIG 2-6. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN LOW SERUM MEDIUM CONTAINING EXTRACTS FROM ANIMALS TREATED WITH E64c. (A) 60 μg/mL extract protein (SOLEUS) and (B) 100 μg/mL extract protein (PLANTARIS). MHC content was assessed 72 h after switch to low-serum medium containing extracts from C and R. E64c, a cysteine protease inhibitor, was administered to a subset of animals in each group 1 h prior to exercise or rest.. The extract from each animal was assayed individually. Data is expressed relative to vehicle (saline only). Values represent mean ± SD (n=4/group). Overall effect by two-way ANOVA. Values without a common letter are significantly different, $p<0.05$.

CHAPTER 3

FRAGMENTS PRODUCED BY THE CALPAIN-MEDIATED DEGRADATION OF PURIFIED MYOFIBRILS ARE NOT ANABOLIC TO MYOBLASTS

Calpain (EC 3.4.22.17) is a calcium-dependent cysteine protease. It is believed to function in various biological processes, including integrin-mediated signalling, cell differentiation and apoptosis. Under pathological conditions, calpain activation has been implicated in muscular dystrophy, cardiac and cerebral ischemia, platelet aggregation, rheumatoid arthritis, cataract formation and Alzheimer's disease (reviewed in 80). Calpain causes the limited degradation of membrane and cytoskeletal proteins, enzymes and transcription factors (reviewed in 75). Calpain also plays a role in initiating the turnover of myofibrillar proteins (94).

A variety of muscle proteins have been identified as substrates for calpain under in vitro conditions (desmin, filamin, C-protein, tropomyosin, troponin I, troponin T, titin, nebulin, vimentin, gelsolin, and vinculin; reviewed in 105). Calpain does not degrade actin, alpha-actinin or myosin heavy chain (110,148). In many cases, the protein fragments generated by calpain have been identified for isolated proteins (reviewed in 106). However, it is possible that the conformation of substrate proteins is altered by their assembly into the myofibril and that these alterations change their susceptibility to degradation by calpain. Little is known about how calpain activation affects muscle function in vivo.

Two isozymes of calpain, differing in calcium requirement, exist ubiquitously in mammalian tissues (54). Both isozymes are heterodimers and consist of a catalytic (80 kDa) and regulatory (30 kDa) subunit. Both isozymes of calpain are typically localized throughout the muscle cell (95,97,98). Within the sarcomere, calpain is twice as abundant at the Z-disk than in the I-band region (95). The incubation of myofibrils with purified calpain (in the presence of calcium) results in discrete changes in morphology. A loss of periodicity along the thin filaments occurs in the absence of structurally detectable changes in the thick or thin filaments themselves (94,107-109). The rapid loss of N-line structures is followed by a gradual loss in density of the Z-disk (110). SDS-PAGE analysis suggests that calpain degrades desmin, nebulin, titin and troponin-T in the myofibril and releases undegraded alpha-actinin (149).

Exercise promotes the redistribution of active calpain from the cytosol to protein/membrane structures in cardiac and skeletal muscle (100). Purified myofibrils from exercised muscle demonstrate a pattern of protein loss similar to that of purified myofibrils treated with calpain (102). Belcastro and co-workers (102) described the specific loss and/or disruption of Z-line structure in 22% of myofibrils isolated from exercised skeletal muscle in the rat. There was an extensive loss of two proteins (58 kDa and 95 kDa), thought to be desmin and alpha-actinin. Whether the loss and/or modification of specific myofibrillar proteins are linked mechanistically to adaptation is not well understood. It is possible that regulated changes in protein degradation could rapidly and selectively alter the properties of structural, catalytic and regulatory proteins in muscle and produce fragments with altered function.

In other cell systems, fragments of structural or regulatory proteins have the potential to initiate a biological response. For example, a 20-kDa cleavage product of collagen interacts with integrins on the surface of epithelial cells to inhibit angiogenesis (150). In vitro studies identified cathepsin L as an enzyme capable of generating endostatin, whereas metalloproteases produce larger fragments of collagen in a parallel pathway (151).

Calpain activation occurs in response to exercise (100,101). Many of the proteins that undergo remodelling in response to exercise are calpain substrates (116). Indeed, the calpain-mediated release of myofibrillar proteins from the sarcomere appears to be an early component of the adaptation response in

muscle. Previous observations (Chapter 2) showed that the release of anabolic factors from muscle following exercise is mediated by cysteine protease activity. We hypothesized that peptide fragments produced by the calpain-mediated degradation of myofibrillar proteins are anabolic to myoblasts. In this experiment, purified myofibrils were incubated with m-calpain in the presence of calcium. The autolysis of m-calpain was followed under the same conditions in the absence of myofibrils. The ability of both preparations to stimulate myoblast proliferation and differentiation was considered.

METHODS

All experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of British Columbia Ethics Committee. Male Wistar rats (225-250 g; Charles River Laboratories, Inc.) were housed in a temperature-controlled facility, maintained on a 12 h light/dark cycle and fed commercial pellets and water ad libitum. Rats were anaesthetized with pentobarbital sodium (65 mg/kg ip; MTC Pharmaceuticals) and the hindlimb muscle was exposed via an incision through the skin and fascia. The plantaris muscle was removed under aseptic conditions, trimmed of visible fat and connective tissue and placed in liquid N_2 with precooled tongs. Samples were stored at -70°C prior to analysis.

MYOFIBRIL ISOLATION PROCEDURE

Purified myofibrils were prepared from control plantaris muscle as previously described (152,153). Briefly, samples were suspended in 10 vol of buffer (containing 39 mM sodium borate, 25 mM KCl, 200 μM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich Corp.) and 5 mM ethylene glycol-bis(beta-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA), pH 7.0) using an Ultra-Turrax tissue homogenizer (model T25; Ika Laboratories) at half maximal setting. The suspension was centrifuged at 1000 g for 12 min and the supernatant was discarded. The pellet was washed with 1.0% Triton X-100 to remove membrane-bound proteins and resuspended in 100 mM KCl, 50 mM Tris (pH 7.0) and 1.0 mM dithiothreitol (DTT). The pellet was washed 2X in buffer containing 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris (pH 7.0) and 1.0 mM DTT. All procedures were performed at 4°C. Protein concentration was determined by the method of Lowry (154) using bovine serum albumin (Fraction V) as a standard. The arrangement of sarcomeric proteins within the myofibril complex is retained by this isolation and purification procedure (119).

CALPAIN ACTIVITY DETERMINATION

m-Calpain (purity ≥98% by SDS-PAGE; Calbiochem) was supplied in 100 mM NaCl, 50 mM Tris-HCl (pH 7.8), 5 mM EDTA, 5 mM 2-mercaptoethanol and 40% glycerol (m-calpain storage buffer). Calpain activity was determined colorimetrically by measuring the release of trichloroacetic-acid (TCA)-soluble peptides from Hammerstein casein (155). Briefly, the reaction mixture (1 mL) contained 4% casein in 0.5 M imidazole-HCl pH 7.5 (100 μL), 1 M imidazole-HCl pH 7.5 (100 μL), 50 mM cysteine (100 μL), 50 mM $CaCl_2$ (100 μL) and m-calpain (0.2-0.5 units). After 30 min, the reaction was terminated using an equal volume of ice cold 5% TCA. The mixture was centrifuged at 10 000 g for 10 min and the absorbance of the supernatant was measured at 750 nm. One unit (U) of activity is defined as the amount of enzyme that will increase absorbance at 750 nm by 1.0 in 30 min at 30°C. Estimates of calpain activity were performed in triplicate.

MYOFIBRIL DIGEST PREPARATIONS

Purified myofibrils (80 μg) were incubated with m-calpain (3 U) in a reaction mixture containing 10 mM 2-mercaptoethanol, 5 mM $CaCl_2$ and 20 mM Tris, pH 7.6 (myofibril digest). The digest was allowed to proceed at 30°C for 60 min. At selected intervals (10, 30, 60 min), an aliquot was removed and the

reaction was terminated using 20 mM EDTA. Myofibrils were also incubated without calpain (undigest) and the autolysis of m-calpain was followed in the absence of myofibrils (mCANP+calcium). The composition of each digest preparation is described in Table 2.

To visualize individual protein bands, samples were mixed with an equal volume of Laemmli sample buffer and heated to 95°C for 2 min. Proteins were resolved by electrophoresis under denaturing and reducing conditions using one-dimensional polyacrylamide gels (12%) and a discontinuous buffer system (156). The gels were run at constant voltage, according to the manufacturer's protocols. Proteins were stained with Coomassie Brilliant Blue R-250 (0.02%; Bio-Rad Laboratories, Inc.) using standard protocols. Gels were destained, dried between 2 sheets of porous cellophane and mounted in a plexiglass frame. The molecular weight of individual protein bands was estimated from the relative mobility of recombinant molecular weight standards (Bio-Rad Laboratories, Inc.). It was assumed that the distance travelled by the band was inversely proportional to the log molecular weight of that band.

Where indicated, digest preparations were desalted using a 3 kDa molecular weight cut-off filter (MWCF; Centricon). This was accomplished by repeated concentration and reconstitution of the concentrated sample using 20 mM Tris, pH 7.6. Greater than 98% of the interfering substances were removed following three spins (according to the manufacturer's specifications). Following the final spin, the concentrated samples were reconstituted to the original volume using 20 mM Tris, pH 7.6 (vehicle).

CELL CULTURE

L6 myoblasts, derived from rat skeletal muscle, were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DME; Sigma-Aldrich Corp.) containing 4 mM L-glutamine, 1.0 mM sodium pyruvate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate and supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, Inc.).

MYOBLAST PROLIFERATION ASSAY

For experimental analysis of total DNA, myoblasts were plated at low density ($2.5 \times 10^4/cm^2$) and maintained in a 5% CO_2 atmosphere at 37°C. After 12 h, the medium was replaced with low-serum medium containing 0.5% FBS and an aliquot of one of three digest preparations described earlier (undigest, myofibril digest, mCANP+calcium). Cultures were processed at the time of medium switch (t=0) and 36 and 48 h thereafter.

DNA content was assessed using Hoechst 33258 dye (bisbenzimide; Sigma-Aldrich Corp.) as previously described (157). Briefly, the cells were rinsed 1X with serum-free medium, fixed with cold 70% ethanol/formalin/acetic acid (20:2:1), then rinsed again. An aqueous working solution of Hoechst 33258 dye (1 μg/mL) was prepared in 150 mM sodium citrate, 0.9% NaCl (1X SSC; pH 7.0) immediately prior to use. 1 mL was added to each well. After a 30 min incubation at Room temperature, sample fluorescence was measured (355 nm excitation, 460 nm emission) using a microplate reader (Wallac, Inc.). The results represent the mean of 4 wells at each dilution of factor in a typical experiment.

TERMINAL MUSCLE DIFFERENTIATION ASSAY

For experimental analysis of total myosin heavy chain (MHC), myoblasts were plated at high density ($1 \times 10^5/cm^2$) and maintained in a 5% CO_2 atmosphere at 37°C. After 12 h, the medium was replaced with low-serum medium containing 0.5% FBS and an aliquot of one of three digest preparations described earlier (undigest, myofibril digest, mCANP+calcium). Cultures were processed at the time of medium switch (t=0) and 48 and 72 h thereafter. Medium was replaced after 48 h.

Differentiation was assessed by Western blot analysis of MHC expression as previously described (132).

Briefly, cultures were rinsed 3X in phosphate-buffered saline (PBS; 0.9% NaCl, 0.20 g/L KCl, 1.15 g/L Na_2HPO_4, 0.20 g/L KH_2PO_4, pH 7.4) and solubilized in Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 0.01% bromophenol blue). Samples were heated to 95°C for 2 min and stored frozen. Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (0.45 μm; Bio-Rad Laboratories, Inc.) using a Mini Trans-Blot® apparatus (Bio-Rad Laboratories, Inc.) and the buffer system described by Towbin (133). Prestained molecular weight standards (Bio-Rad Laboratories, Inc.) appeared as colored bands on the membrane and provided a good qualitative assessment of transfer efficiency.

The membranes were immersed in Tris-buffered saline (TBS; 0.9% NaCl, 50 mM Tris pH 7.5) and 10% H_2O_2 for 30 min to reduce endogenous peroxidase activity and then blocked for 2 h at Room temperature in TBS-Tween 20 (TBS-Tw) + 5% nonfat dry milk to reduce non-specific binding. The immobilized proteins were probed with MF-20 (Developmental Studies Hybridoma Bank, University of Iowa; contributed by Dr D Fischman), a monoclonal antibody directed against sarcomeric myosin heavy chain (134). After 2 h at Room temperature, the membranes were rinsed 3X using a generous volume of TBS-Tw and incubated with affinity-purified peroxidase-conjugated goat anti-mouse IgG (1:5000; Life Technologies, Inc.) for an additional hour. The blots were rinsed as before, and the bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Inc.) as described in the instructions provided by the manufacturer. Band intensities were quantified by densitometry (UNSCAN-IT; Silk Scientific, Inc.) and results expressed as % of vehicle (20 mM Tris, pH 7.6).

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to identify differences between groups. Individual mean differences were determined using Tukey post-hoc analysis, $p<0.05$.

RESULTS

Changes in myofibril composition were documented using SDS-PAGE. In control preparations (t=0 min), the major proteins had apparent molecular weights (MW) of 200 kDa (myosin heavy chain), 95 kDa (alpha-actinin), 58 kDa (desmin) and 42 kDa (actin). The 80 kDa and 18 kDa bands represent the catalytic and regulatory subunits of exogenous m-calpain, respectively. Purified myofibrils treated with m-calpain showed a distinct pattern of degradation or removal, characterized by the loss of Coomassie stained bands with molecular weights of 37, 55, 98 and 140 kDa, presumably troponin-T, desmin, alpha-actinin and C-protein (Fig 3-1A). Additional bands appeared below the level of C-protein (140 kDa) which are degradation products from other high MW proteins. Neither MHC nor actin was degraded by calpain. The pattern of loss can be directly attributed to calpain since digests performed in the absence of calcium showed no evidence of protein degradation (data not shown).

The pattern of m-calpain autolysis is shown in Fig 3-1B. In 5 mM $CaCl_2$, autolysis of m-calpain was evident following 10 min incubation. The 78 kDa polypeptide produced in the first step of autolysis could easily be distinguished from the 80 kDa subunit of native, unautolyzed m-calpain. Conversion of the 80 kDa subunit to a 78 kDa polypeptide was nearly complete within 30 min. Additional bands (42 kDa, 20 kDa) appeared which represent degradation products from the catalytic (80 kDa) subunit. Density of the 18 kDa (regulatory) subunit did not change.

The NH_2-terminus of the catalytic (80 kDa) subunit is rapidly cleaved in the presence of calcium. Loss of the NH_2-terminus was followed using anti-m-calpain IgG. The reaction proceeded quickly following the addition of calcium (t=0 min) and the rate was approximately equal in the presence (myofibril digest; Fig 3-1C) and absence (mCANP+calcium; Fig 3-1D) of myofibrils. At 10 min, less than two-thirds of

the native, unautolyzed protease remained in both preparations. Conversion of the 80 kDa to 78 kDa subunit was complete within 30 min.

To determine whether digest preparations (myofibril digest, mCANP+calcium) could stimulate myoblast proliferation, cells were plated at low density in medium containing an aliquot (1/500) of each digest sampled at 10, 30 and 60 min. DNA content was assessed after 36 and 48 h using Hoechst dye. After 36 h, neither myofibril digest nor mCANP+calcium caused any measurable increase in total myoblast DNA (data not shown). After 48 h, both preparations sampled at 10 min caused a modest increase ($p<0.01$) in myoblast proliferation (Fig 3-2A). The magnitude of effect was the same for myofibril digest and mCANP+calcium. No differences in myoblast DNA were observed using 30 or 60 min samples of either preparation (data not shown).

To determine whether digest preparations (myofibril digest, mCANP+calcium) could stimulate myoblast differentiation, cells were plated at high density in medium containing an aliquot (1/500) of each digest sampled at 10, 30 and 60 min. Terminal differentiation was assessed after 48 and 72 h by Western blot analysis using a monoclonal antibody directed against sarcomeric MHC. There was no effect of either preparation on myoblast differentiation after 48 (data not shown) or 72 h (Fig 3-2B).

When digest preparations were added to myoblast cultures at 1/100 dilution, cells showed changes in morphology (rounding, lack of adherence to the plate bottom) and a loss of viability. An independent series of experiments was performed to determine whether components of the digest preparation (described in Table 1) had deleterious effects on cell proliferation and differentiation. To determine their effect, 2-mercaptoethanol and EDTA were tested over a range of concentrations. 2-mercaptoethanol caused a dose-dependent reduction in DNA (Fig 3-2C) and MHC (Fig 3-2D) at concentrations greater than 10 μM. EDTA caused a similar reduction in DNA at concentrations >10 μM (Fig 3-2E). A decrease in MHC expression was observed at concentrations of EDTA >50 μM (Fig 3-2F). To eliminate the interfering effects of these agents, 2-mercaptoethanol and EDTA were removed from the digest preparations using 3 kDa molecular weight cut-off filters (Centricon YM-3; Millipore). This was accomplished by repeated concentration and reconstitution of the samples using 20 mM Tris (pH 7.6). Following the final spin, the concentrate was reconstituted to the original volume using 20 mM Tris (vehicle).

Digest samples reconstituted in 20 mM Tris (pH 7.6) were used in all subsequent experiments to determine effects on myoblast proliferation and differentiation. Total myoblast DNA was measured 36 h following the switch to low-serum medium containing an aliquot (1:100 v/v) of the digest preparations (undigest, myofibril digest, mCANP+calcium) and the data are presented in Fig 3-3A. Digest preparations sampled at 10 min caused an increase ($p<0.01$) in total myoblast DNA. The magnitude of the increase was equal for myofibril digest and mCANP+calcium. Undigested myofibrils (undigest) had no effect. MHC expression was measured 72 h following the switch to low-serum medium containing an aliquot (1:100 v/v) of the digest preparations (undigest, myofibril digest, mCANP+calcium). Following 10 min of digestion, myofibril digest and mCANP+calcium induced a three-fold increase ($p<0.01$) in myoblast differentiation compared with vehicle (Fig 3-3B). Undigested myofibrils (undigest) had no effect. The results show that both myofibril digest and mCANP+calcium had the ability to stimulate myoblast proliferation and differentiation.

To determine whether time of digest influenced either myoblast proliferation or differentiation, an aliquot of each digest preparation (undigest, myofibril digest, mCANP+calcium) was removed at intervals along the continuum of the digest (10, 30 and 60 min) and the reaction was terminated using 20 mM EDTA. The ability of each aliquot to stimulate myoblast proliferation is shown in Fig 3-4A. There was no effect of sampling time for undigest or mCANP+calcium. Samples of undigest had no effect on

myoblast proliferation. mCANP+calcium caused equivalent increases ($p<0.01$) in myoblast DNA when sampled at 10, 30 and 60 min (compared to vehicle). For myofibril digest, the 10 min sample caused the largest increase in total myoblast DNA. The 30 and 60 min samples caused smaller increases in myoblast proliferation, however the effect was still different from vehicle. Fig 3-4B/C/D represents dose-response curves for digest preparations sampled at 10 min. There was an inverse relationship between the amount of undigest added to the culture medium and total myoblast DNA (Fig 3-4B). There was a positive relationship between the amount of myofibril digest (Fig 3-4C) added to the medium and total myoblast DNA. The same positive relationship was observed for mCANP+calcium (Fig 3-4D). The results show that myofibril digest and mCANP+calcium stimulate myoblast proliferation in a dose-dependent manner.

DISCUSSION

Previous observations showed that calpain activation occurs following exercise (100,101) and that the release of anabolic factors from muscle following exercise can be attenuated using a cysteine protease inhibitor (Chapter 2). Since many of the proteins that undergo remodelling in response to exercise are calpain substrates (116), the purpose of the present study was to assess whether peptide fragments produced by the calpain-mediated degradation of myofibrillar proteins are anabolic factors. Purified myofibrils were treated with m-calpain in the presence of calcium. The autolysis of m-calpain was followed under the same conditions in the absence of myofibrils. Both preparations showed the same ability to stimulate myoblast proliferation and differentiation. The primary finding of this study was that autolytic fragments of m-calpain were responsible for the observed increase in myoblast proliferation and differentiation. These data suggest that the active peptide is an early product of calpain autolysis that persists without further modification even as the catalytic (80 kDa) subunit undergoes additional processing. Further, the release of the active peptide does not depend on the presence of calpain substrates. The results support calpain's involvement in the release of anabolic factors following exercise and lend support for a mechanism linking calpain activation and acute changes in myofibril integrity to long-term adaptation in muscle.

The in vitro model used in the current study is relevant to physiological processes in vivo based on evidence that the activation and redistribution of active calpain from the cytosol to membrane/protein structures occur following exercise (100) and that there is a characteristic and consistent pattern of calpain's action on substrate proteins in muscle (94,107,108). Indeed, the increase in calpain activity observed immediately following exercise is accompanied by a heightened calcium sensitivity of the enzyme and an increased susceptibility of its substrate proteins to degradation (101). Large changes in cellular metabolism occur following prolonged running exercise and it is possible that dynamic changes such as phosphorylation (158), oxidation status (159) and/or sulfhydryl group reactivity (119) alter protein susceptibility to degradation by calpain. In this study, myofibrils were isolated from the plantaris muscle of sedentary (control) animals so that the calpain-mediated effects on muscle could be studied independently from other variables that change during exercise.

In this study, purified myofibrils from control plantaris muscle treated with m-calpain showed a pattern of protein degradation and/or loss consistent with other reports using SDS-PAGE (106,109). This pattern can be directly attributed to calpain's action since digests performed in the absence of calcium showed no evidence of protein degradation. Reports from other laboratories show that the incubation of myofibrils with purified μ- or m-calpain in the presence of calcium results in the dissolution of Z-lines and the release of alpha-actinin (110). The Z-line anchors the thick and thin filaments together to maintain the three-dimensional architecture of the myofibril (160), thus the specificity in degrading the Z-line suggests that calpain is responsible for the initial steps in myofibril turnover.

Purified myofibrils from exercised muscle show a pattern of protein degradation consistent with the in vitro actions of calpain reported here (102,110). The histological appearance of injured fibers (Z-line dissolution) correlates with the loss of desmin and alpha-actinin. Since force is transmitted longitudinally within the myofibril and laterally to adjacent myofibrils via the intermediate filament system, the loss of Z-line integrity could also account for the loss in force-generating capacity associated with exercise. We have proposed that calpain activation is responsible for the changes in muscle structure and function that occur immediately after exercise (51).

Calpain alters the activity or function of substrate proteins by limited proteolysis (112). This suggests that calpain-mediated degradation of structural proteins in muscle may serve more than a digestive function. For example, the activation of intermediate filament proteins, such as desmin, can be achieved by the limited truncation of the NH_2-termini by calpain (113,114). The fragments have a diminished capacity to form and maintain filaments, however they retain the ability to activate nucleosomes during transcription initiation and elongation (115). In other cell types, calpain has been implicated in the calcium-dependent processing of receptors that become associated with the nucleus (161) and in the cytoskeletal-directed condensation of chromosomes (162). Although such functions have been identified for intracellular processes, these studies did not address whether calpain-derived fragments can act as regulatory molecules outside the cell. Raj and co-workers (104) reported a positive correlation between calpain activity and neutrophil accumulation in skeletal muscle following exercise, however the chemotactic factor was not characterized. The results of our study suggest that calpain-derived myofibril fragments do not stimulate myoblast proliferation and differentiation.

The observation that autolytic fragments of m-calpain could stimulate myoblast proliferation and differentiation was a novel finding and lends support to a mechanism linking acute changes in myofibril integrity to long-term adaptation in muscle. In vitro, the NH_2-terminus of both calpain subunits is rapidly cleaved in the presence of calcium (58). Our results showed that conversion of the 80 kDa to 78 kDa subunit was complete within 30 min (in the presence and absence of myofibrils). This observation is consistent with other reports which suggest that at calcium concentrations greater than 1 mM, neither enzyme concentration nor the presence of substrate affects the rate of autolysis of m-calpain (163). Gabrijelcic-Geiger and co-workers (164) described a complex pattern of transient (80 kDa, 78 kDa, 76 kDa, 55 kDa, 30 kDa, 21 kDa) and stable (40 kDa, 28 kDa, 24 kDa, 18 kDa) autolysis products from purified human erythrocyte μ-calpain. The results of the current study are consistent with this observation, suggesting that the active peptide is an early (by 10 min) product of autolysis that remains in a form capable of stimulating myoblast proliferation and differentiation even as the catalytic subunit undergoes further processing at 30 and 60 min.

It is interesting to note that autolytic fragments of calpain show bioactivity toward other cell types. Kunimatsu and co-workers (123) showed that autolytic fragments of μ-calpain have chemotactic activity toward neutrophils. The active peptide is an acetylated nonapeptide with the sequence N-acetyl Ser-Glu-Glu-Ile-Ile-Thr-Pro-Val-Tyr. The peptide is identical to the NH_2-terminal sequence of the catalytic (80 kDa) subunit of μ-calpain deduced from the cDNA sequence (165) except that it is lacking a methionine residue and is acetylated at the NH_2-terminus. Kunimatsu and co-workers (124) further showed that synthetic peptides corresponding to the NH_2-terminal sequence of the regulatory (30 kDa) subunit of μ-calpain have chemotactic activity toward neutrophils, T and B cells and monocytes. Since the amino acid sequences of the regulatory subunit of μ- and m-calpain are identical (126), both isozymes could mediate chemotaxis. Our data are the first to suggest a role for autolytic calpain fragments in myogenesis. The active peptide was not characterized in the current study and warrants further investigation.

In summary, the results of this study suggest that autolytic fragments of m-calpain can stimulate myoblast proliferation and differentiation. Truncation of the NH_2-terminus of the catalytic subunit

increases calcium sensitivity of the enzyme and lowers the calcium requirement for proteolytic activity (58,122,166). The results of this study suggest that calpain autolysis may have additional physiological significance. The calpain-mediated release of myofibrillar proteins from the sarcomere seems to be an early component in the adaptation response to changes in functional demand. Since the effect of the autolytic calpain fragments on myoblast proliferation and differentiation was the same in the absence and presence of myofibrils, the results suggest a possible role for calpain in adaptation in addition to that of initiating myofibrillar turnover.

Table 3-1. DIGEST COMPOSITION

Composition	Working concentration	digest vehicle	undigest	myofibril digest	mCANP+ calcium
myofibrils	80 μg		*	*	
Low Salt Buffer		*			*
2-mercaptoethanol	10 mM	*	*	*	*
$CaCl_2$	5 mM	*	*	*	*
Tris (pH 7.6)	20 mM	*	*	*	*
m-calpain	3 U			*	*
calpain storage buffer	see methods	*	*		
EDTA	20 mM	*	*	*	*

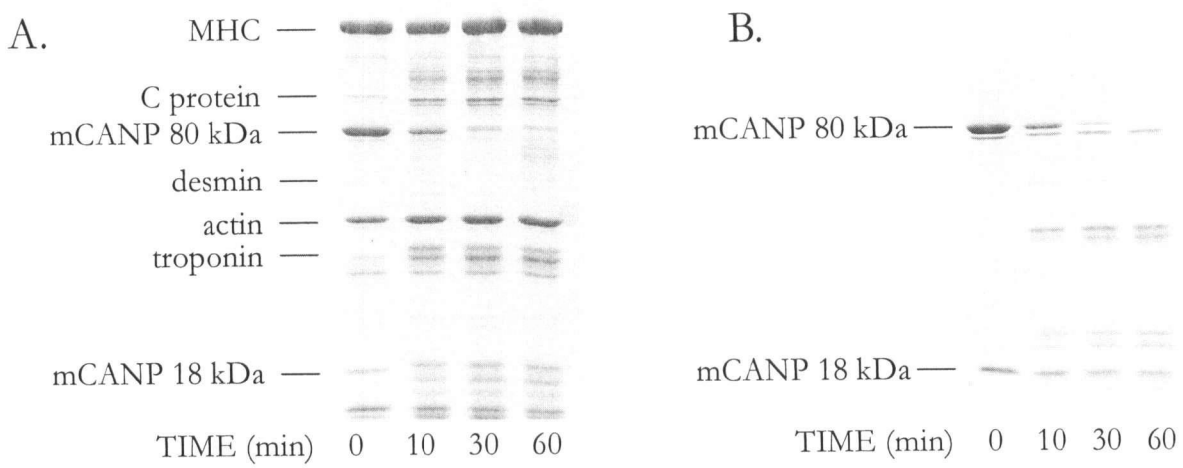

FIG 3-1A. SDS-POLYACRYLAMIDE GEL OF PURIFIED MYOFIBRILS FROM CONTROL PLANTARIS DIGESTED WITH m-CALPAIN. The reaction was terminated at t=0, 10, 30 and 60 min using 20 mM EDTA. Bands lost during the digest correspond to proteins with apparent MW of 37, 55, 98 and 140 kDa. MW are as indicated. These data show the result of a typical digest.

FIG 3-1B. SDS-POLYACRYLAMIDE GEL SHOWING THE TIME COURSE OF m-CALPAIN AUTOLYSIS IN THE PRESENCE OF 5 mM $CaCl_2$. The reaction was terminated at t=0, 10, 30 and 60 min using 20 mM EDTA. Bands lost during autolysis correspond to proteins with apparent MW of 80 kDa (catalytic subunit).

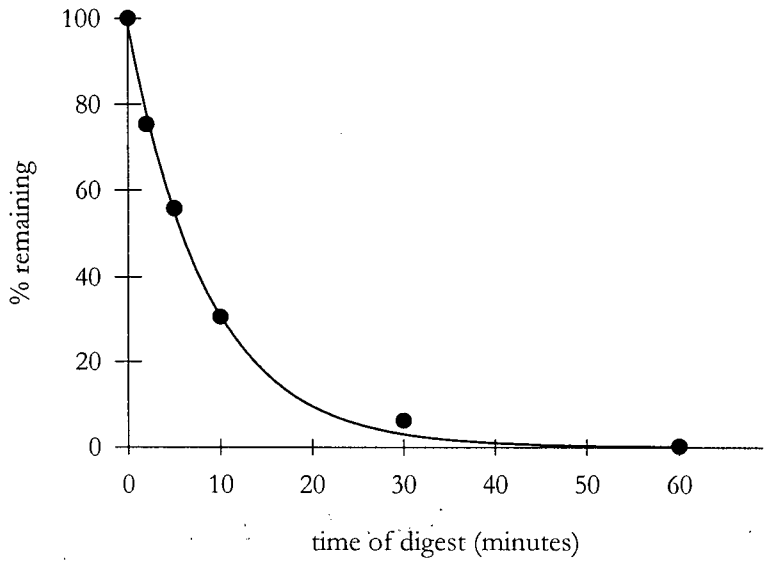

FIG 3-1C. WESTERN BLOT ANALYSIS DOCUMENTING LOSS OF THE NH_2 TERMINUS OF THE CATALYTIC SUBUNIT OF m_s-CALPAIN IN MYOFIBRIL DIGEST. The reaction was terminated at t=0, 2, 5, 10, 30 and 60 min using 20 mM EDTA. Bands were quantified by densitometry and expressed relative to the band intensity of control preparations (t=0 min).

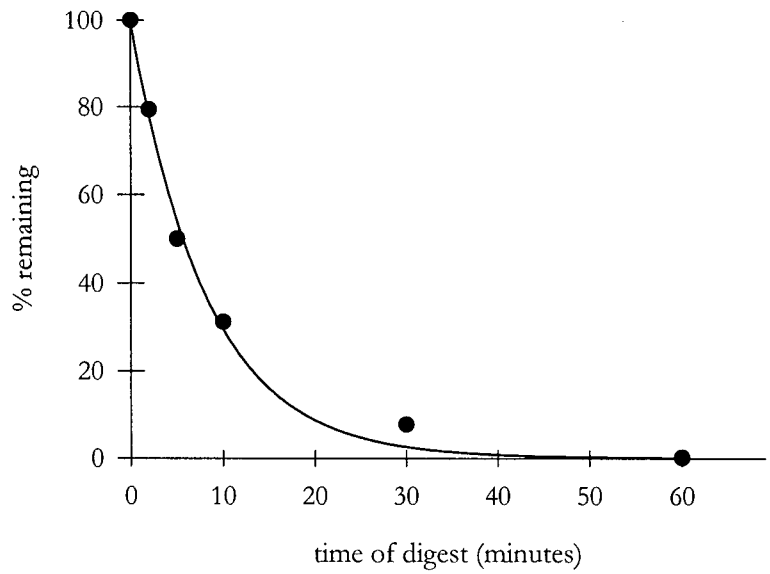

FIG 3-1D. WESTERN BLOT ANALYSIS DOCUMENTING LOSS OF THE NH_2 TERMINUS OF THE CATALYTIC SUBUNIT OF m-CALPAIN IN THE PRESENCE OF 5 mM $CaCl_2$. The reaction was terminated at t=0, 2, 5, 10, 30 and 60 min using 20 mM EDTA. Bands were quantified by densitometry and expressed relative to the band intensity of control preparations (t=0 min).

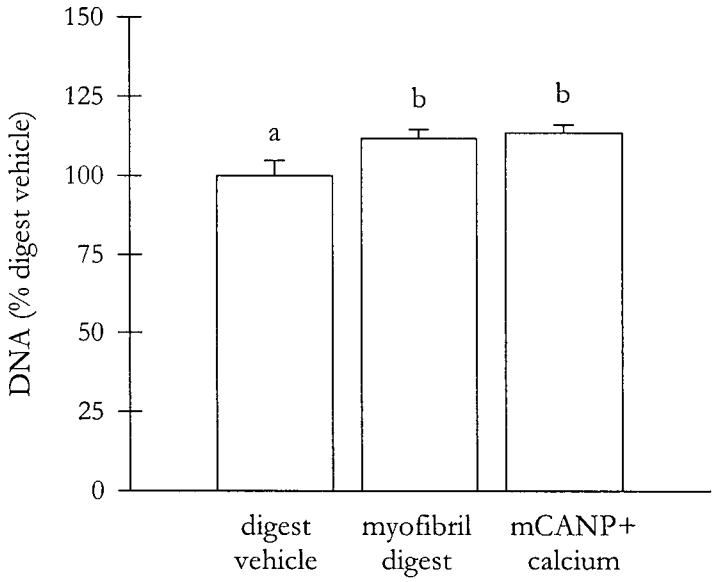

FIG 3-2A. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING AN ALIQUOT OF THE CRUDE DIGEST PREPARATIONS. DNA was assessed at 48 h using Hoechst dye and expressed relative to digest vehicle (described in Table 1). Values represent mean ± SD (n=4/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different.

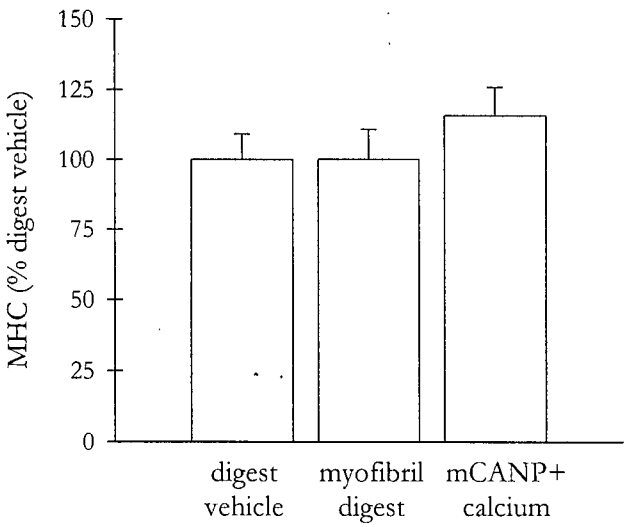

FIG 3-2B. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN MEDIUM CONTAINING AN ALIQUOT OF THE CRUDE DIGEST PREPARATIONS. MHC was assessed at 72 h using Western blot analysis. Bands were quantified by densitometry and expressed relative to digest vehicle. Values represent mean ± SD (n=6/group). NS by one-way ANOVA.

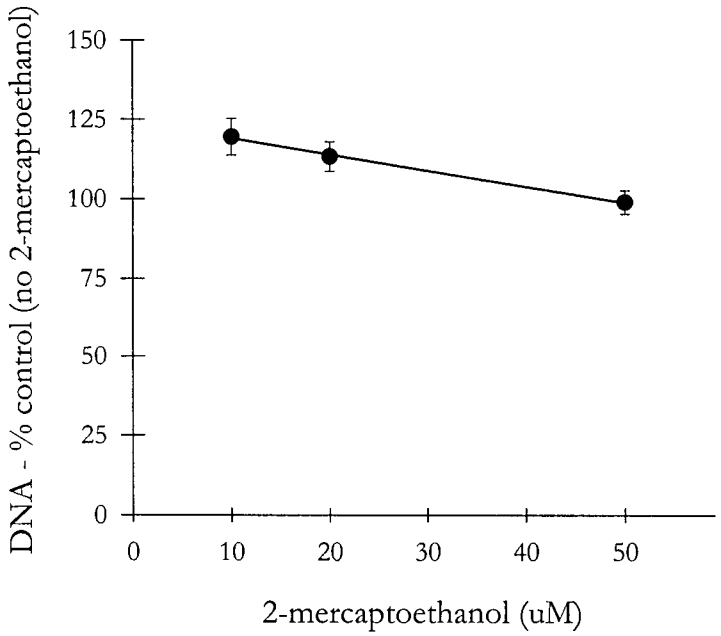

FIG 3-2C. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF 2-MERCAPTOETHANOL. DNA was assessed at 36 h using Hoechst dye and expressed relative to control (no 2-mercaptoethanol). Values represent mean ± SD (n=4/group).

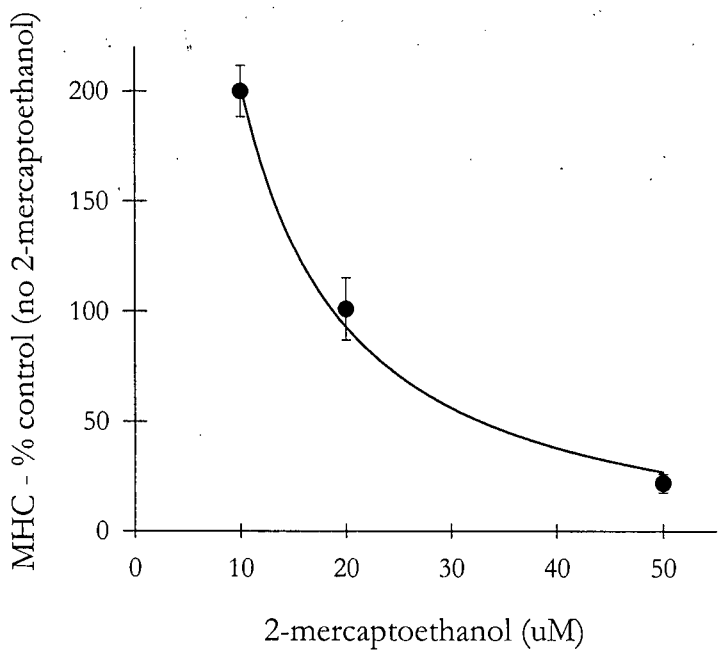

FIG 3-2D. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF 2-MERCAPTOETHANOL. MHC was assessed at 72 h using Western blot analysis. Bands were quantified by densitometry and expressed relative to control (no 2-mercaptoethanol). Values represent mean ± SD (n=2/group)..

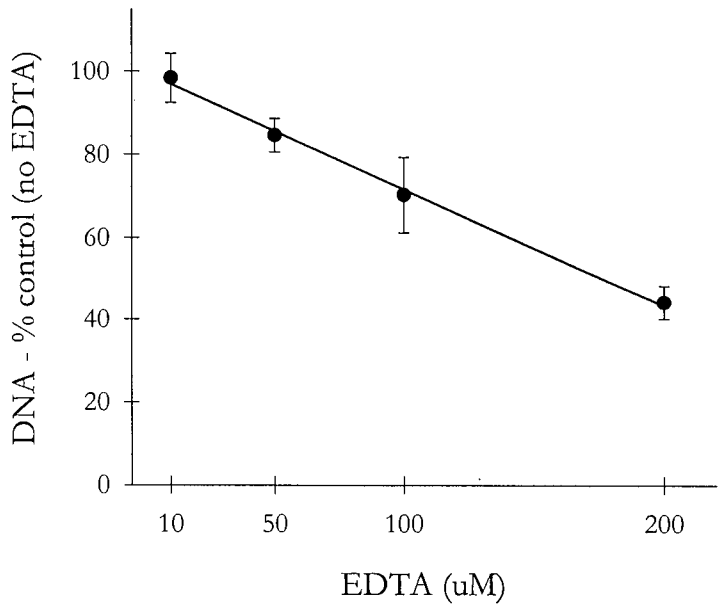

FIG 3-2E. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF EDTA. DNA was assessed at 36 h using Hoechst dye and expressed relative to control (no EDTA). Values represent mean ± SD (n=4/group).

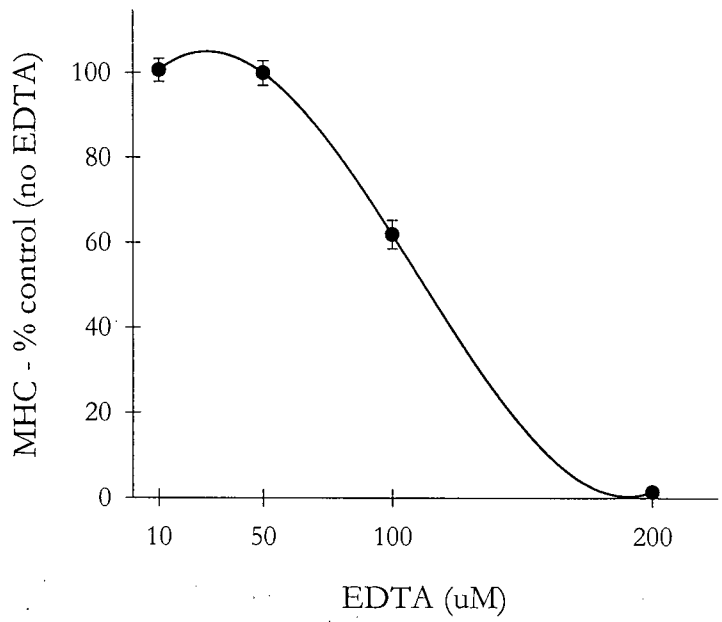

FIG 3-2F. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF EDTA. MHC was assessed at 72 h using Western blot analysis. Bands were quantified by densitometry and expressed relative to control (no EDTA). Values represent mean ± SD (n=2/group).

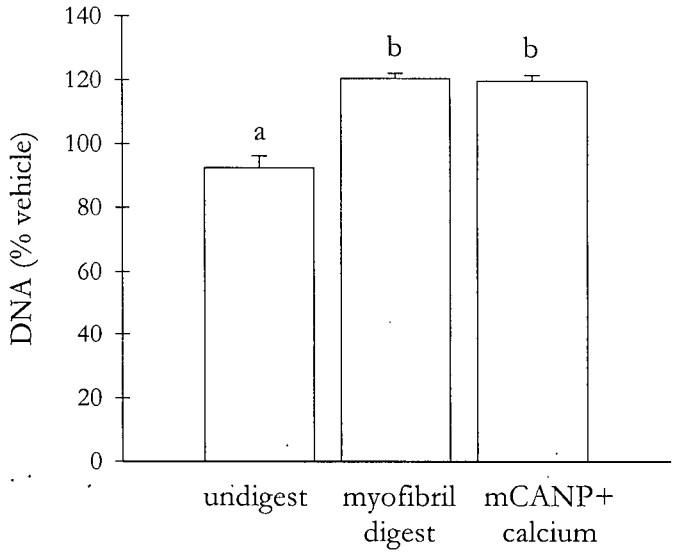

FIG 3-3A. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING AN ALIQUOT (1:100 v/v) OF EACH DIGEST PREPARATION SAMPLED AT 10 MIN. DNA was assessed after 36 h using Hoechst dye and expressed relative to vehicle (20 mM Tris pH 7.6). Values represent mean ± SD (n=4/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different.

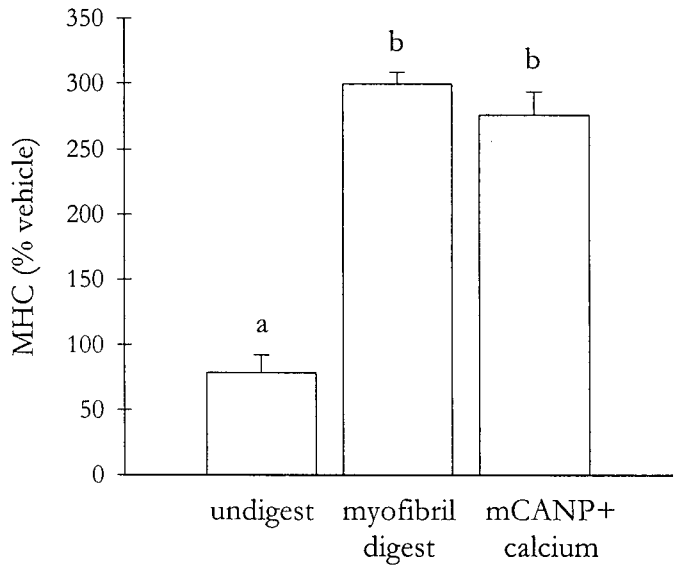

FIG 3-3B. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN MEDIUM CONTAINING AN ALIQUOT (1:100 v/v) OF EACH DIGEST PREPARATION SAMPLED AT 10 MIN. MHC was assessed after 72 h using Western blot analysis. Bands were quantified by densitometry and expressed relative to vehicle (20 mM Tris pH 7.6). Values represent mean ± SD (n=2/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different

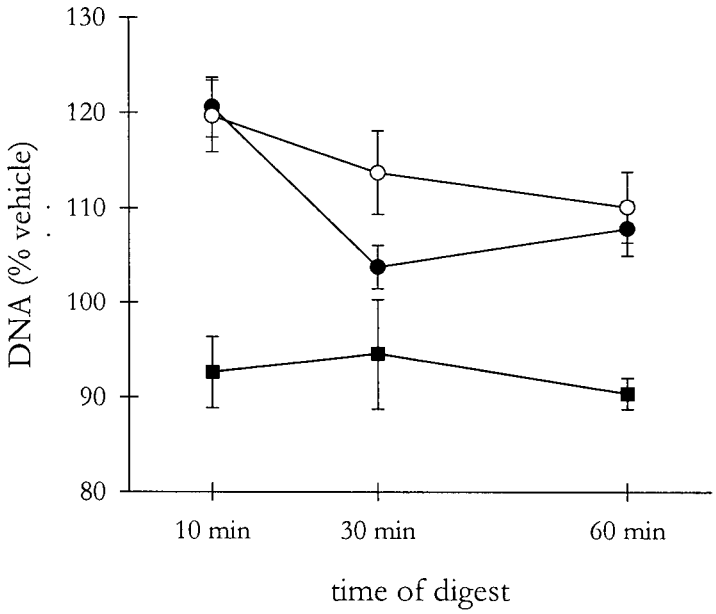

FIG 3-4A. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING AN ALIQUOT (1:100 v/v) OF EACH DIGEST PREPARATION SAMPLED AT 10, 30 AND 60 MIN. DNA was assessed after 36 h using Hoechst dye and expressed relative to vehicle (20 mM Tris pH 7.6). Values represent mean ± SD (n=4/group). ■ undigest ● myofibril digest ○ mCANP+calcium

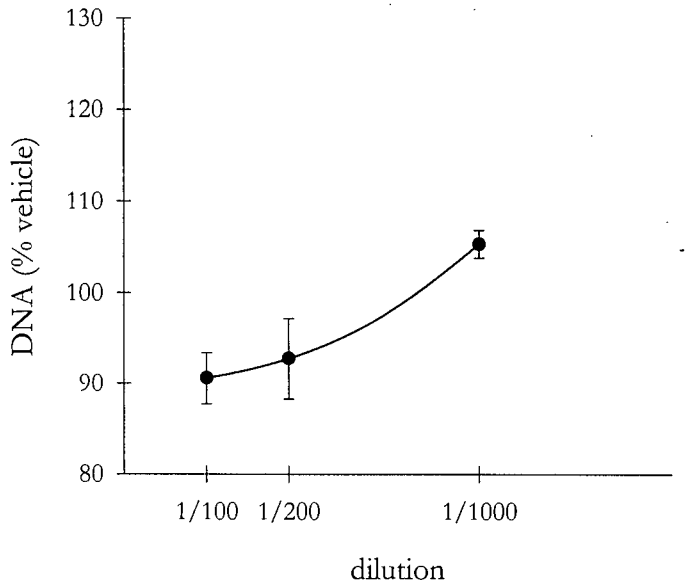

FIG 3-4B. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING VARIOUS DILUTIONS OF UNDIGESTED MYOFIBRILS (UNDIGEST) SAMPLED AT 10 MIN. DNA was assessed after 36 h using Hoechst dye and expressed relative to vehicle. Values represent mean ± SD (n=4/group).

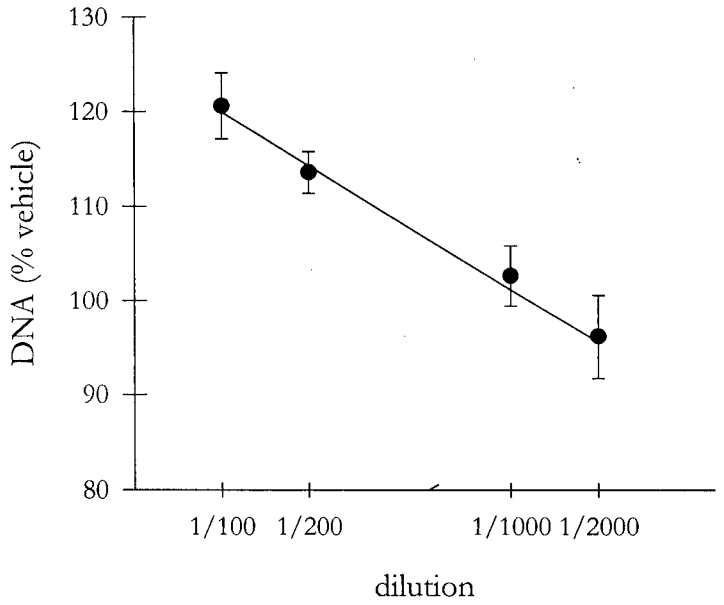

FIG 3-4C. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING VARIOUS DILUTIONS OF MYOFIBRIL DIGEST SAMPLED AT 10 MIN. DNA was assessed after 36 h using Hoechst dye and expressed relative to vehicle. Values represent mean ± SD (n=4/group).

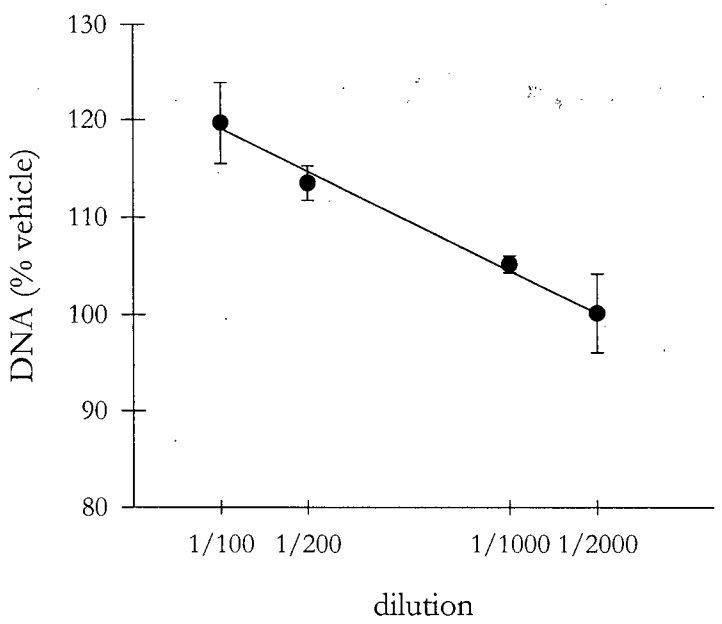

FIG 3-4D. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING VARIOUS DILUTIONS OF mCANP+CALCIUM SAMPLED AT 10 MIN. DNA was assessed after 36 h using Hoechst dye and expressed relative to vehicle. Values represent mean ± SD (n=4/group).

CHAPTER 4

AUTOLYTIC FRAGMENTS OF μ-CALPAIN PROMOTE MYOBLAST PROLIFERATION AND DIFFERENTIATION

Two isozymes of calpain (EC 3.4.22.17) exist ubiquitously in mammalian tissues (54). These low (μM) and high (mM) calcium-requiring types are designated μ-calpain and m-calpain, respectively. Both isozymes are heterodimers and consist of a unique catalytic (80 kDa) subunit and an identical regulatory (30 kDa) subunit. The primary structure of both subunits has been deduced from cDNA cloning studies (55,56). The catalytic (80 kDa) subunit can be divided into four domains (I-IV), whereas the regulatory subunit (30 kDa) consists of two domains (IV' and V). The NH_2-terminus of both calpain subunits is truncated in the presence of calcium (58). Most studies agree that removal of the NH_2-terminus of domain I increases calcium sensitivity of the enzyme and lowers the calcium requirement for proteolytic activity (58,122,166). The kinetics of the process suggest that autolysis of the catalytic subunit precedes that of the regulatory subunit and is followed by proteolysis of the substrate (120).

Recent x-ray crystallography studies of rat (57) and human (65) m-calpain provide insight into some of the conformational changes that occur during calpain activation in vitro. Although mechanistically clear, some aspects of the activation process are still enigmatic. In vitro, the activation of calpain requires calcium concentrations that are seldom attained under normal physiological conditions. It is possible that the actual calcium requirement in vivo is much lower than predicted, due to autolysis (122) and/or the presence of other endogenous activating factors (62,64,167-170).

Autolysis is widely observed among calpain species (171,172), however it is not required for enzyme activation. Recent studies suggest that both calpain isozymes are active in unautolyzed form (173). Notwithstanding, autolyzed species exhibit activity at much lower calcium concentrations in vitro (174). It is possible that autolysis of calpain has some other physiological relevance, which remains unclear.

The autolytic fragments of human erythrocyte (μ-) calpain are chemotactic to neutrophils (123). The active peptide is an acetylated nonapeptide with the sequence N-acetyl Ser-Glu-Glu-Ile-Ile-Thr-Pro-Val-Tyr (123). It is identical to the N-terminal sequence of the catalytic subunit of μ-calpain deduced from the cDNA sequence (165) except that the peptide is lacking a methionine residue and is acetylated at the NH_2-terminus.

The expression of μ- and m-calpain is approximately equal in muscle (175). No differences in substrate specificity have been observed between the two isozymes (94) and the activity of both isozymes is closely regulated by calpastatin (176). It is reasonable to predict that the function of the two isozymes is also closely related. Our earlier data showed that autolytic fragments of m-calpain stimulate the proliferation and differentiation of cultured myoblasts. In the present study, we used purified human erythrocyte calpain to characterize the effects of autolytic μ-calpain fragments on myogenesis. Autolysis was allowed to proceed in presence of 200 μM calcium for 60 min and the ability of the reaction mixture to promote myoblast proliferation and differentiation was assessed.

METHODS

CALPAIN AUTOLYSIS

Human erythrocyte (μ-) calpain (purity ≥99% by SDS-PAGE; Calbiochem) was supplied in 20 mM imidazole-HCl, pH 6.8, 1 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol and 30% glycerol

(calpain storage buffer). The enzyme stock (1.07 mg/mL) was diluted in 9 vol of activation buffer (55 mM Tris, pH 7.5, 5 mM 2-mercaptoethanol). The autolysis reaction was initiated (t=0 min) upon addition of $CaCl_2$ (200 μM final concentration) and allowed to proceed for 60 min at 22°C. At selected intervals (10, 30, 60 min), the reaction was terminated using 1 mM EDTA. Vehicle was a mock incubation performed using 1 vol calpain storage buffer (no enzyme) and 9 vol activation buffer.

SIZE EXCLUSION CHROMATOGRAPHY

An aliquot of the 30 min autolysis reaction mixture was separated on the basis of molecular weight using 10 and 30 kDa molecular weight cut-off filters (Centricon YM-10, YM-30) according to the manufacturer's specifications (Millipore).

CELL CULTURE

L6 myoblasts, derived from rat skeletal muscle, were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DME; Sigma-Aldrich Corp.) containing 4.0 mM L-glutamine, 1.0 mM sodium pyruvate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate and supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, Inc.).

CELL PROLIFERATION ASSAY

For experimental analysis of total DNA, myoblasts were plated at low density ($2.5 \times 10^4/cm^2$) and maintained in a 5% CO_2 atmosphere at 37°C. After 12 h, the medium was replaced with low-serum medium containing 0.5% FBS (0.5% FBS/DME) and an aliquot (1:100-1:500 v/v) of the autolysis reaction mixture. Cultures were processed at the time of medium switch (t=0) and 36 and 48 h thereafter.

DNA content was assessed using Hoechst 33258 dye (bisbenzimide; Sigma-Aldrich Corp.) as previously described (157). Briefly, the cells were rinsed 1X with serum-free media, fixed with cold 70% ethanol/formalin/acetic acid (20:2:1), then rinsed again. An aqueous working solution of Hoechst 33258 dye (1 μg/mL) was prepared in 150 mM sodium citrate, 0.9% NaCl (1X SSC; pH 7.0) immediately prior to use. 1 mL was added to each well. After 30 min incubation at Room temperature, sample fluorescence was measured (355 nm excitation, 460 nm emission) using a microplate reader (Wallac, Inc.). The results were expressed as the mean value of 4 wells at each dilution of factor in a typical experiment.

TERMINAL MUSCLE DIFFERENTIATION ASSAY

For experimental analysis of total myosin heavy chain (MHC), myoblasts were plated at high density ($1 \times 10^5/cm^2$) and maintained in a 5% CO_2 atmosphere at 37°C. After 12 h, the medium was replaced with low-serum medium containing 0.5% FBS (0.5% FBS/DME) and an aliquot (1:100-1:500 v/v) of the autolysis reaction mixture. Medium was replaced after 48 h. Cultures were processed at the time of medium switch (t=0) and 72 h thereafter.

Differentiation was assessed by Western blot analysis of MHC expression as previously described (132). Briefly, cultures were rinsed 3X in phosphate-buffered saline (PBS; 0.9% NaCl, 0.20 g/L KCl, 1.15 g/L Na_2HPO_4, 0.20 g/L KH_2PO_4, pH 7.4) and solubilized in Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol and 0.01% bromophenol blue). Samples were heated to 95°C for 2 min and stored frozen.

Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose (0.45 μm; Bio-Rad Laboratories, Inc.) using a Mini Trans-Blot® apparatus (Bio-Rad Laboratories, Inc.) and the buffer system described by Towbin (133). Prestained molecular weight standards (Bio-Rad Laboratories, Inc.)

appeared as colored bands on the membrane and provided a good qualitative assessment of transfer efficiency.

The membranes were immersed in Tris-buffered saline (TBS; 0.9% NaCl, 50 mM Tris pH 7.5) and 10% H_2O_2 for 30 min to reduce endogenous peroxidase activity and then blocked for 2 h at Room temperature in TBS-Tween 20 (TBS-Tw) + 5% nonfat dry milk to reduce non-specific binding. The immobilized proteins were probed with MF-20 (Developmental Studies Hybridoma Bank, University of Iowa; contributed by Dr D Fischman), a monoclonal antibody directed against sarcomeric MHC (134). After 2 h, the membranes were rinsed 3X using a generous volume of TBS-Tw and incubated with affinity-purified peroxidase-conjugated goat anti-mouse IgG (1:5000; Life Technologies, Inc) for an additional hour. The blots were rinsed as before, and the bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences Inc.) as described in the instructions provided by the manufacturer. Band intensities were quantified by densitometry (UNSCAN-IT; Silk Scientific Inc.) and results expressed in arbitrary units.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to identify differences between groups. Individual mean differences were determined using Tukey post-hoc analysis, $p<0.05$.

RESULTS

Autolysis was allowed to proceed in presence of 200 μM $CaCl_2$ at 22°C for 60 min. Aliquots of the reaction mixture were removed at 10, 30 and 60 min and the reaction was terminated using a 5-molar excess of EDTA. The ability of the reaction mixture to promote myoblast proliferation and differentiation was assessed. There was an increase ($p<0.01$) in total DNA following 10, 30 and 60 min of autolysis (compared to vehicle; Fig 4-1A). There was no difference between the response observed at 10 and 30 min, however both were significantly greater ($p<0.05$) than the response at 60 min. Evidence of myoblast differentiation was barely detectable following 72 h of culture in low-serum media containing vehicle (Fig 4-1B). There was an increase ($p<0.01$) in MHC expression following 10, 30 and 60 min of autolysis (compared to vehicle). There was no difference between the response observed at 10 and 30 min, however both were significantly greater ($p<0.05$) than the response at 60 min. Overall, the data show that autolytic fragments of μ-calpain stimulate myoblast proliferation and differentiation.

Since the maximum response was consistently observed following 30 min of autolysis, an aliquot of the reaction mixture (for this time point only) was fractionated using 10 and 30 kDa MWCF and compared with the unfractionated sample. The low molecular weight fractions (<10 kDa and <30 kDa) showed the same increase in total DNA as the unfractionated (30 min) sample (Fig 4-2A). The ≥30 kDa fraction had a smaller effect ($p<0.01$) on total DNA. All samples (unfractionated, <10 kDa, <30 kDa and ≥30 kDa) were significantly greater than vehicle ($p<0.01$). The low molecular weight fractions (<10 kDa and <30 kDa) showed the same increase in MHC expression as the unfractionated sample ($p<0.01$; compared to vehicle; Fig 4-2B). The ≥30 kDa fraction had a smaller effect ($p<0.01$) on total myosin, however it was still significantly greater than vehicle ($p<0.01$). Overall, the data suggest that a low molecular weight peptide (<10 kDa) can stimulate myoblast proliferation and differentiation.

DISCUSSION

The purpose of the current study was to characterize the effects of autolytic μ-calpain fragments on myogenesis. Autolysis of human erythrocyte (μ-) calpain was allowed to proceed in presence of calcium

for 60 min. Aliquots of the reaction mixture were removed at 10, 30 and 60 min and the reaction was terminated using EDTA. The ability of the reaction mixture to promote myoblast proliferation and differentiation was assessed. At 10 and 30 min, autolytic calpain fragments caused an large increase in total DNA and MHC expression. The size of the active fragment was estimated using 10 and 30 kDa molecular weight cut-off filters (MWCF). The anabolic effect of the <10 kDa fraction was equivalent to the unfractionated sample, suggesting the involvement of a low molecular weight peptide. Overall, the results suggest that autolytic fragments of μ-calpain participate in myogenesis.

The NH_2-terminus of both calpain subunits is rapidly cleaved in the presence of calcium (58). Autolysis decreases the mass of the 80 kDa subunit to 76 kDa and the mass of 28 kDa subunit to 18 kDa (177). Gabrijelcic-Geiger et al (164) recently described a complex pattern of transient (80 kDa, 78 kDa, 76 kDa, 55 kDa, 30 kDa, 21 kDa) and stable (40 kDa, 28 kDa, 24 kDa, 18 kDa) autolysis products from purified human erythrocyte (μ-) calpain. The results of the current study are consistent with this observation, suggesting that active fragments present following 10 and 30 min of autolysis were further processed, resulting in a moderate decline of bioactivity by 60 min. Calpain activity is lost in proportion to the degree of autolysis. Since neither the extent of autolysis nor enzyme activity was measured directly in this study, the data cannot exclude the possibility that active calpain was responsible for the observed changes in myoblast proliferation and differentiation.

Our results suggest that a low molecular-weight (<10 kDa) peptide was responsible for the observed stimulation of myoblast proliferation and differentiation. This is consistent with observations in other systems indicating calpain autolysis results in the release of bioactive peptides with apparent molecular weights of 1-3 kDa (123). Kunimatsu and co-workers (124) showed that synthetic peptides (6-13 aa) corresponding to the NH_2-terminal sequence of the large and small subunits of μ-calpain have chemotactic activity toward neutrophils, T and B cells and monocytes. We observed lower levels bioactivity in samples which fractionated at apparent molecular weights greater than 30 kDa.

Our previous observations showed that autolytic fragments of m-calpain can promote the proliferation and differentiation of myoblasts. Thus, the effect does not appear to be isozyme specific. The catalytic subunits of μ- and m-calpain share 60% sequence homology (55). Since the amino acid sequences of the regulatory subunits of μ- and m-calpain are identical and well-conserved among species (125,126) both calpain species could be the source of a single peptide anabolic factor for myoblasts. Indeed, autolysis of both isozymes may proceed by a common mechanism except for the concentration of calcium required to initiate the reaction (58).

Both calpain isozymes are localized throughout the muscle cell (95,97,98). In vitro, μ- and m-calpain have identical effects on the structure of purified myofibrils (94). In the presence of calcium, both isozymes cause the rapid loss of Z-lines and a loss of periodicity along the length of the thin filaments (94,107,108). This redundancy suggests that the two isozymes cleave identical substrates and are capable of performing the same biological function. Furthermore, this redundancy would enable muscle to initiate myofibrillar turnover in response to different cellular signals. Sultan and co-workers (178) reported a redistribution of μ-calpain from the cytosol to membrane structures following chronic low frequency stimulation. The increase in calpain activity coincides with early changes in MHC isoform composition, suggesting a role for μ-calpain in fiber-type transformation. m-Calpain plays a more prominent role in muscle regeneration following Marcaine-treatment (178) and crush injury (179).

There is also emerging evidence to suggest that μ- and m-calpain have discrete functions in early myogenesis. Poussard and co-workers (89) analysed μ- and m-calpain mRNA transcripts in differentiating rat myoblasts using a competitive polymerase chain reaction. The appearance of the transcripts was not simultaneous, suggesting that the two isozymes had different biological functions.

Balcerzak and co-workers (81) showed that an antisense oligonucleotide to m-calpain mRNA can inhibit fusion in primary myoblast cultures. The specificity of intervention was confirmed using antisense oligonucleotides to μ-calpain and p94 mRNAs, respectively. m-Calpain expression and activity also increase markedly during the fusion of embryonic myoblasts (83,87). Western blot analysis shows that myoblasts contain no detectable μ-calpain before fusion (92), in agreement with other studies that found no μ-calpain activity in primary myoblast cultures (83,87). Collectively, the data suggest that m-calpain is involved in the membrane reorganization that precedes myoblast fusion and that μ-calpain is involved in other calcium-dependent signalling cascades. The precise mechanism underlying these differences is unclear.

In summary, the results of our study suggest that autolytic fragments of μ-calpain can stimulate myoblast proliferation and differentiation. Most studies agree that removal of the NH_2-terminus region of domain I increases calcium sensitivity of the enzyme and lowers the calcium requirement for proteolytic activity (122). The results of this study suggest that calpain autolysis may have additional physiological significance. Whether the autolytic fragments from both calpain isozymes can initiate the same biological response in vivo remains to be tested.

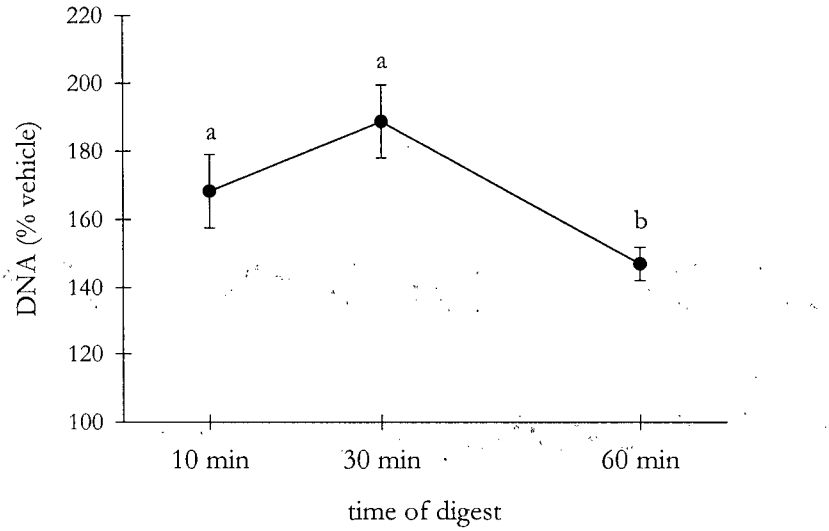

FIG 4-1A. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING AUTOLYTIC FRAGMENTS OF μ-CALPAIN. Autolysis was allowed to proceed in the presence of 200 μM $CaCl_2$ at 22°C for 60 min. Aliquots of the reaction mixture were removed at 10, 30 and 60 min. DNA was assessed after 48 h using Hoechst dye and expressed relative to vehicle. Values are mean ± SD (n=4/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different. Data represent a typical experiment performed twice.

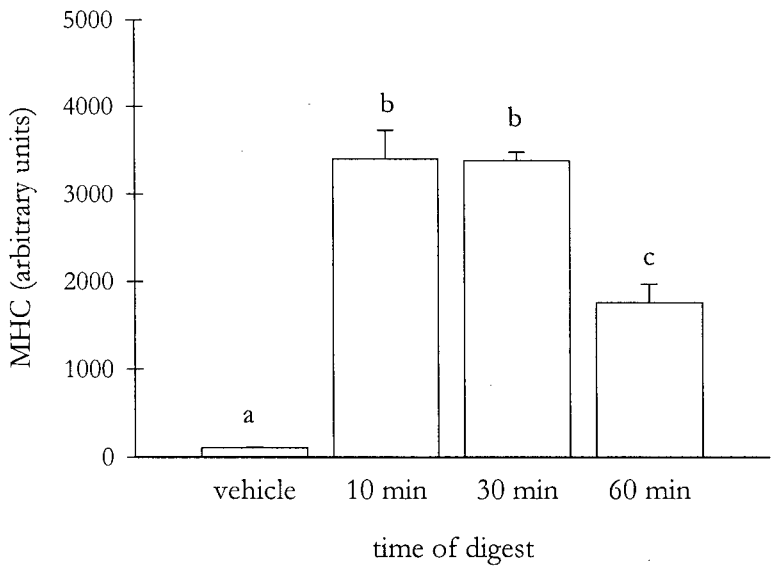

FIG 4-1B. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN MEDIUM CONTAINING AUTOLYTIC FRAGMENTS OF μ-CALPAIN. MHC was assessed after 72 h by Western blot analysis. Bands were quantified by densitometry and expressed in arbitrary units. Only a trace amount of MHC was detectable in cells plated in medium containing vehicle. Values are mean ± SD (n=2/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different.

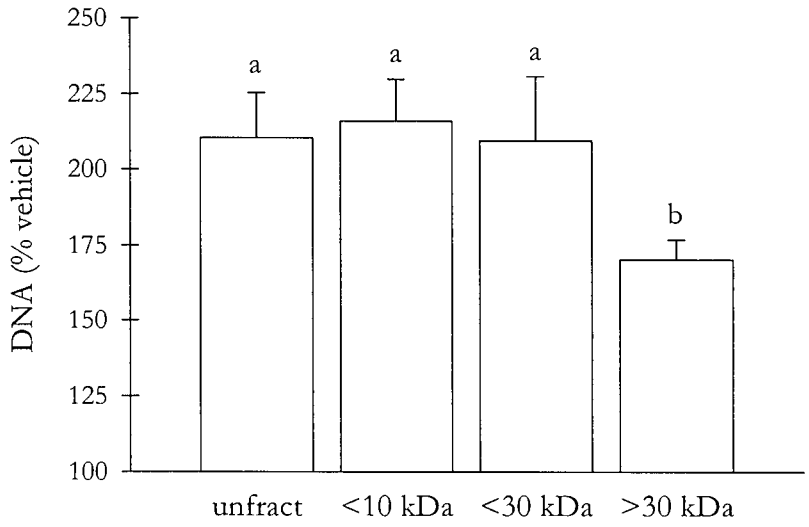

FIG 4-2A. TOTAL DNA IN MYOBLAST CULTURES PLATED AT LOW DENSITY IN MEDIUM CONTAINING AUTOLYTIC FRAGMENTS OF μ-CALPAIN SEPARATED USING SIZE EXCLUSION CHROMATOGRAPHY. Since the maximum response was consistently observed following 30 min of autolysis, an aliquot of the reaction mixture (at this time point only) was fractionated using 10 and 30 kDa molecular weight cut-off filters. DNA was assessed after 48 h using Hoechst dye and expressed relative to vehicle. Values are mean ± SD (n=4/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different. Data represent a typical experiment performed twice.

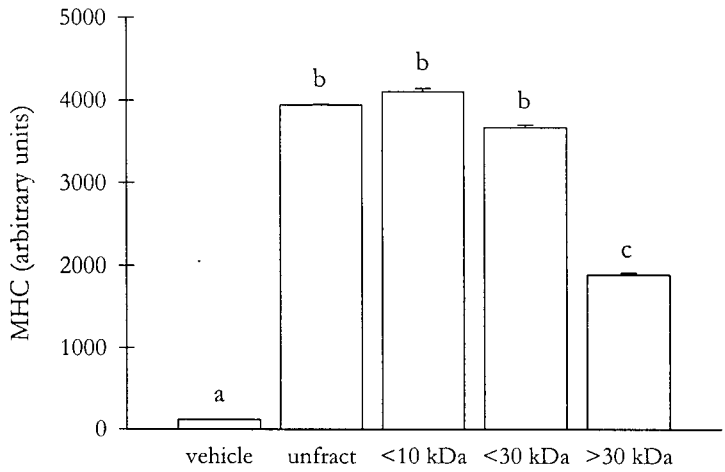

FIG 4-2B. MHC EXPRESSION IN MYOBLAST CULTURES PLATED AT HIGH DENSITY IN MEDIUM CONTAINING AUTOLYTIC FRAGMENTS OF μ-CALPAIN SEPARATED USING SIZE EXCLUSION CHROMATOGRAPHY. MHC was assessed after 72 h by Western blot analysis. Bands were quantified by densitometry and expressed in arbitrary units. Values are mean ± SD (n=2/group). Overall effect by one-way ANOVA ($p<0.01$). Values without a common letter are significantly different.

CHAPTER 5

HYPOTHESES AND CONCLUSIONS

RE-STATEMENT OF THE PROBLEM

Skeletal muscle can alter its functional, morphological and metabolic properties in response to changes in the quantity and/or pattern of neuromuscular activity (reviewed in 1,2). The spatial and temporal changes in phenotype have been well described, however the precise mechanism(s) leading to adaptation are poorly understood. When altered contractile loading is imposed, extensive remodelling occurs within the muscle. In particular, the transient and specific loss of Z-line integrity is an early feature of the remodelling process and has been observed following exercise (48,102), stretch (180), denervation (181) and hindlimb unweighting (182). The initial changes are proposed to occur secondary to protease activation (101). Many of the proteins that undergo remodelling are calpain substrates (116). Based on calpain's ability to disassemble the myofibril complex in a precise and deliberate manner, it has been proposed that the acute changes in muscle structure and function that occur following periods of increased contractile activity are related to calpain activation (51). The experiments described in these studies were designed to facilitate an understanding of whether **products of muscle catabolism participate in adaptation and repair.**

Overall, the results of these studies support a relationship between the acute changes in myofibril integrity caused by calpain activation and adaptation in muscle. The specific hypotheses and conclusions were:

1. SOLUBLE FACTORS CAPABLE OF STIMULATING MYOBLAST PROLIFERATION AND DIFFERENTIATION ARE PRODUCED IN RESPONSE TO PERIODS OF INCREASED CONTRACTILE ACTIVITY

   Saline extracts derived from rat hindlimb muscle after 60 minutes of downhill running caused an increase in myoblast proliferation and differentiation. Extracts derived from the hindlimb muscle of sedentary animals did not stimulate either process.

2. THE RELEASE OF ANABOLIC FACTORS IS MEDIATED BY A CYSTEINE PROTEASE

   The administration of a cysteine protease inhibitor (E64c) to animals one-hour prior to exercise attenuated the increase in myoblast differentiation in extracts from plantaris, suggesting that cysteine protease activity was involved in the release of anabolic factors.

3. FRAGMENTS DERIVED FROM THE CALPAIN-MEDIATED DEGRADATION OF MYOFIBRILLAR PROTEINS ARE ANABOLIC TO MYOBLASTS

   Purified myofibrils from control plantaris treated with m-calpain (in the presence of calcium) showed a pattern of protein degradation and/or loss consistent with other reports. The ability of digested myofibrils to stimulate myoblast proliferation and differentiation was not different from that of autolyzed m-calpain, therefore it was concluded that autolytic fragments of m-calpain are anabolic to myoblasts but calpain-derived myofibril fragments are not.

4. AUTOLYTIC FRAGMENTS OF CALPAIN ARE ANABOLIC TO MYOBLASTS

   Autolytic fragments of m-calpain caused a dose-dependent increase in myoblast proliferation and

differentiation (in the presence and absence of myofibrils). The effect was not isozyme-specific since autolytic fragments of μ-calpain produced the same effect.

OVERALL RESULTS AND DISCUSSION

ACUTE INJURY: A STIMULUS FOR ADAPTATION

Rats ran downhill on a motorized treadmill for 60 min or remained sedentary. Muscle fiber integrity was not directly assessed in the current study, however there is evidence to suggest that downhill running produces significant morphological changes in the organization of the sarcomere including A-band disruptions and Z-line dissolution (48). Additional evidence suggests that there may also be lesions in the plasma membrane (49,50). In this study, saline extracts derived from rat hindlimb muscle immediately following exercise caused a dose-dependent increase myoblast proliferation and differentiation. Extracts derived from the hindlimb of sedentary animals did not stimulate either process. The data suggest that some event associated with exercise increased the abundance or availability of anabolic factors in muscle. Using size exclusion chromatography, we showed that the active factor(s) had an apparent molecular weight of greater than 10 kDa. Based on its size and its ability to stimulate both myoblast processes, the activity of the factor(s) is consistent with an IGF-IGFBP complex. However, this possibility was not directly assessed. To relate the events of the acute exercise stimulus to possible long-term adaptation, it was essential to a) identify a mechanism or process underlying the release of anabolic factors and b) separate acute damage to the myofibril from other compensatory processes related to exercise.

A MECHANISM UNDERLYING THE RELEASE OF ANABOLIC FACTORS: PROTEASE INVOLVEMENT

The in vivo administration of a cysteine protease inhibitor (E64c) attenuated the increase in myoblast MHC expression associated with exercise. These data suggested that the release of anabolic factors associated with exercise was mediated by a cysteine protease. E64c binds irreversibly to the active site thiol (cysteine) of calpain. E64c also inhibits other cysteine proteases such as cathepsin B and L (103), therefore it is difficult to assess whether the in vivo effects described for this inhibitor were a result of calpain inhibition alone. However, changes in cathepsin activity generally occur too late in time to account for the loss of myofibrillar protein found immediately post-exercise (183). Previous reports from our laboratory show that E64c abrogates the increase in calpain activity associated with exercise (104).

SEPARATION OF ACUTE DAMAGE TO THE MYOFIBRIL FROM OTHER COMPENSATORY PROCESSES: CALPAIN-DERIVED MYOFIBRIL FRAGMENTS

The calpain-mediated loss of myofibrillar proteins from the sarcomere seems to be an early component of adaptation, therefore it was of interest to determine whether calpain-derived myofibril fragments could stimulate myogenesis. Myofibrils were isolated from control (sedentary) muscle using a protocol that maintains the arrangement of sarcomeric proteins within the myofibril complex (119) and treated with a purified source of m-calpain. Control tissue was used to eliminate the possibility that dynamic changes such as phosphorylation, oxidation status or sulfhydryl group reactivity that may occur during exercise might influence protein susceptibility to degradation by calpain. Using SDS-PAGE, we demonstrated a pattern of degradation and/or loss consistent with other reports (106,109). There was an extensive loss of proteins with apparent molecular weights of 37, 55 and 140 kDa which could be directly attributed to calpain's action since digests performed in the absence of calcium showed no evidence of protein degradation. Based on calpain's ability to alter the function of substrate proteins in other cell

systems, it seemed possible that calpain-derived fragments of myofibrillar proteins could stimulate myoblast proliferation and differentiation. Since calpain rapidly autolyzes in the presence of calcium (58), the role of autolyzed m-calpain fragments in myogenesis was also considered. The results showed that the ability of autolytic calpain fragments to stimulate myoblast proliferation and differentiation was approximately equal to that of digested myofibrils at all of the time points and dilutions considered. Therefore, it was concluded that autolytic fragments of m-calpain are anabolic to myoblasts but calpain-derived myofibril fragments are not. Both conventional calpain species (μ- and m-calpain) are present in skeletal muscle, however these results did not determine whether both isozymes could exhibit the same effect. Identifying the function of both isozymes is important in understanding the physiological role of the calpain in muscle.

AUTOLYTIC CALPAIN FRAGMENTS: ISOZYME SPECIFICITY

Both isozymes of calpain are equally distributed in muscle (175), have similar substrate specificity (94) and produce the same effects on purified myofibrils (94). Since both isozymes autolyze rapidly in the presence of calcium (58), it was necessary to further characterized the role of autolytic calpain fragments in myogenesis by determining whether the effect on myoblast proliferation and differentiation was isozyme specific. Furthermore, to ensure that no tissue specific variations exist, autolysis of purified human erythrocyte (μ-) calpain was subject to autolysis in the presence of calcium. Aliquots of the reaction mixture were removed at various time points along the continuum of reaction and assayed for their ability to promote myoblast proliferation and differentiation. The results were similar to those observed for m-calpain. There was an initial increase in anabolic activity at 10 and 30 min that persisted (albeit to a lesser extent) at 60 min. The time course data suggested that the active fragment was an early product of autolysis. The size exclusion data suggested that a low molecular weight peptide (<10 kDa) was responsible for the observed stimulation in myoblast proliferation and differentiation. This result was consistent with reports from other laboratories that synthetic peptides corresponding to the NH_2-terminus of both calpain isozymes are chemotactic to neutrophils and immunocytes (124). The present data are the first to indicate a similar mechanism operates in skeletal muscle. Since the regulatory subunits of μ- and m-calpain are identical, both isozymes could be the source of the same active peptide. It will be interesting to isolate the active peptide, characterize it by sequence analysis and identify a potential receptor on myoblasts.

The combined findings of these studies suggest a novel and exciting consequence of calpain activation. Specifically the data suggest that the active peptide is an early product of calpain autolysis that retains its ability to stimulate myoblast proliferation and differentiation even as the catalytic (80 kDa) subunit undergoes further processing. Importantly, the anabolic properties of the active peptide were evident in both the presence and absence of myofibrils, which suggests that this is not an isolated calpain phenomenon and may be physiologically relevant in vivo. Furthermore, it lends support for the idea of a proposed link between an acute exercise stimulus and long-term adaptation in skeletal muscle.

The overall conclusion from these studies is that a soluble factor capable of stimulating myoblast proliferation and differentiation is released from skeletal muscle in response to an acute physiological stimulus (exercise). However, the active factor released following exercise was probably not the same peptide produced by calpain autolysis. Even though both factors had a common ability to stimulate myoblast proliferation and differentiation, they eluted at different molecular weights. Calpain activation occurs in response to prolonged running exercise (100,101), therefore it is possible that the autolytic fragment was present in the saline extract immediately following exercise but at too low a concentration to demonstrate any effect on myoblast proliferation and differentiation. Indeed, the autolytic fragments of both isozymes showed dose-dependent effects on both proliferation and differentiation that could be reduced to no effect at high dilutions. Further investigations are warranted to determine the precise

identity and the role of autolytic calpain fragments in vivo.

Given that autolytic fragments from both calpain isozymes can stimulate myoblast proliferation and differentiation, muscle may have the ability to adapt in a similar capacity in response to different types of stimuli. This idea is supported by the work of Sultan and co-workers (178). They show that μ-calpain activity increases following chronic low frequency stimulation while m-calpain activity increases when more widespread degeneration of myofibers occurs, as with myotoxin treatment.

Overall, these data support a relationship between the acute loss in myofibril integrity caused by calpain activation and long-term adaption. There are currently no commercially available site-directed and/or isozyme specific calpain inhibitors to appropriately test the assumption that calpain-induced changes in myofibril integrity are directly linked to long-term adaptation in skeletal muscle. Once more specific protease inhibitors are available, it will be possible to determine whether calpain activation is directly responsible for the observed changes in morphology and whether the effect of calpain autolysis on myoblast proliferation and differentiation can be extrapolated to other in vivo conditions of altered loading.

Notwithstanding, the results of these studies will be useful in understanding basic muscle function but they also have clinical utility. Calcium-dependent proteases play a key role in the pathology of various disorders including cardiac ischemia, muscular dystrophy and other genetic myopathies. Because acute muscle injury is a prerequisite for many degenerative conditions, the results of these experiments may be beneficial in developing long-term treatment strategies in muscle-wasting diseases. The results provide insight into the identity of factors which favor adaptation and repair and this will be of further importance in exercise prescription related to sport, rehabilitation and space flight.

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