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ARTICLES

Calpain II Expression Is Increased by Changes in Mechanical Loading of Muscle In Vivo

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Abstract In the present investigation, we have tested the hypothesis that calpain expression or activity in skeletal muscle is influenced by changes in mechanical loading in vivo. Muscle unloading for 10 days produced no change in the concentrations of calpain I, or II, and no change in calpain activation, as assessed by measurements of the proportion of calpain I or II isoforms that exhibited autoproteolytic modifications. However, muscle reloading for 2 days produced a 90% increase in calpain II concentration per unit wet weight of muscle relative to ambulatory controls. Although no change in the activation index for calpain I or II was identified for reloaded muscle, this index is an expression of the proportion of the total mass of each calpain isoform that is autoproteolyzed. Thus, there is also approximately a 90% increase in autolyzed calpain II in muscle experiencing increased loading than in controls. Northern analysis shows that the concentration of mRNA for calpain II is increased in reloaded muscle, but no change in calpain II mRNA concentration in unloaded muscle. In situ reverse transcription polymerase chain reaction was used to confirm that nearly all calpain II mRNA in reloaded muscle is located in muscle fibers, with very little detectable calpain II mRNA in non-muscle cells present in the tissue. Together, these findings show that increased muscle loading causes a selective increase in the expression of calpain II isoform, thereby indicating that its regulation is independent from other calpain isoforms. J. Cell. Biochem. 64:55–66.

Key words: protease; proteolysis; calcium; rat; muscle injury

Calcium-dependent proteases, or calpains, are a family of papain-like, intracellular proteases that have been associated with diverse biological functions including platelet activation, apoptosis and cell shape changes [Croall and DeMartino, 1991; Saido et al., 1993; Squier et al., 1994]. In each of these events, cytosolic calcium concentrations are elevated transiently, either by release from intracellular stores or by entry from the extracellular space [Fox et al., 1983; Fox, 1985; Baracos and Goldberg, 1986; Squier et al., 1994; Oshimi and Miyazaki, 1995]. The increase in cytosolic calcium activates calpains, resulting in their autolysis and cleavage of substrate proteins [Hathaway et al., 1982; Crawford et al., 1987; Saido et al., 1993]. Two calpain isoforms, calpain I (also called μ-calpain) and calpain II (also called m-calpain), differ functionally in their sensitivity to calcium for half maximal activation, when casein is used as a substrate in vitro [Croall, 1989; Croall and DeMartino, 1990]. No differences in substrate specificity have been identified for the two isoforms, [Takahashi, 1990] and the activities of both are inhibited by the same molecule, calpastatin, which suggests that the functions of the calpain isoforms are closely related [Maki et al., 1988]. Muscle contains both calpain I and calpain II, as well as a newly-discovered, muscle specific calpain III (also called p94) [Sorimachi et al., 1989; Sorimachi and Suzuki, 1992]. Calpain I and II are capable of cleaving many prominent proteins in muscle, including myofibrillar proteins, major Z-disk proteins, and proteins involved in myofibril linkage to the cell membrane, such as talin and vinculin [Takahashi, 1990]. Calpain III substrates have not yet been specifically identified, although the structural similarities between calpain III and the other isoforms suggest that there will be similarities in substrates and regulation [Sorimachi and Suzuki, 1992].

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Modifications in muscle loading result in dramatic remodeling of the muscle cytoskeleton by mechanisms that are unknown [Booth, 1982; Booth and Gollnick, 1983; Riley et al., 1990]. Structural rearrangements that occur during adaptation and remodeling include increased turnover of existing myofibrils [Desplanches et al., 1987; Riley et al., 1990], remodeling of associations between the cytoskeleton and the cell membrane [Tidball and Quan, 1992], and changes in expression of structural proteins in the cell [Booth and Kirby, 1992]. Several recent investigations have focused on identifying mechanisms by which mechanical loading of muscle can cause adaptive responses in the loaded cells, and shown that loading causes the generation of second messengers that mediate cellular events associated with adaptation [Vandenburgh, 1992]. For example, mechanical stretch activates several second messenger systems in cardiac myocytes that cause increased expression of c-fos, an event associated with hypertrophy [Komuro et al., 1990; Schunkert et al., 1991; Sadoshima and Izumo, 1993]. Furthermore, these same investigators [Sadoshima and Izumo, 1993] show that many of the loading-dependent responses of cardiac muscle could be inhibited by preventing increases in cytosolic [Ca^{2+}] concentration, or stimulated by treatment with [Ca^{2+}] ionophores. Thus, elevation of cytosolic [Ca^{2+}] concentrations may be a response of central importance in muscle by which the activity of proteases associated with remodeling, and the expression of genes involved with adaptation can occur. Many of the proteins that experience remodeling during modified muscle loading are calpain substrates, which suggests that calpains may participate in these cellular responses [Takahashi, 1990].

Calpain activation during normal muscle development in vitro has been inferred from observations showing that antisense oligoribonucleotides to m-calpain inhibit myoblast fusion in vitro [Balcerzak et al., 1995] and that calpain becomes redistributed in myocytes during their fusion in vitro [Schollmeyer, 1986; Schollmeyer, 1986]. This inference is based upon the observation that calpain activation in vitro is accompanied by calpain translocation to the cell membrane during platelet activation, although no direct biochemical evidence for calpain activation has been obtained in myocytes [Fox et al., 1993; Saido et al., 1993]. Calpain activation has also been inferred during several muscle pathologies in vivo, when cytosolic calcium concentrations are elevated and calpain substrates undergo proteolysis. For example, during muscle necrosis observed in the diseases Duchenne muscular dystrophy and mdx dystrophy, it has been reported that increases in cytosolic [Ca] are an early change in the disease [Emery and Burt, 1980], and this is expected to activate calpains leading to muscle catabolism. Recent observations have provided the first direct biochemical evidence for increases in calpain concentration and autolysis in dystrophic muscle in vivo [Spencer et al., 1995]. In addition, it was shown in that same investigation that the expression of calpain changed during regeneration of dystrophic muscle, which suggests that calpains may play a role in anabolic as well as catabolic processes in muscle. The possibility that calpains function in these processes has received further support recently by findings that mutations in the calpain III gene lead to severe muscle wasting in humans [Richard et al., 1995].

In the present investigation, we examine whether calpains play a role in regulating muscle remodeling during loading and unloading in vivo by testing whether these treatments influence the expression or concentration of calpain protein or mRNA, or change levels of calpain autoproteolysis. Modifications in muscle loading are achieved by subjecting rats to periods of muscle unloading, followed by return to loading, to which the muscle responds as an increased load. An activation index is determined by measuring the extent of autoproteolytic modification of calpains, using antibodies specific for propeptides that are cleaved during the autoproteolysis. Further, the identity of the cells expressing calpains during modified loading is assessed by in situ reverse transcription polymerase chain reaction (in situ RT PCR), to confirm that the cells responding to modified loading are indeed muscle fibers.

METHODS

Reagents

TAQ polymerase was purchased from Promega (Madison, WI), DNAse, random primers, restriction enzymes, FPLC, and Mono Q column were purchased from Pharmacia (Alameda, CA), digoxigenin 11-dUTP and anti-digoxigenin antibody from Boehringer Mannheim (Indianapolis, IN), keyhole limpet hemocyanin from Calbiochem (San Diego,CA), α^{32}P dCTP

from Amersham (Arlington Heights, IL). MMLV Reverse transcriptase was obtained from Gibco BRL, cDNA probes to calpain I and calpain II were generously donated from Dr. John Elce (Queen's University, Kingston, Ontario) [Samis et al., 1991], antibodies to pro-peptides of calpain I and m-calpain II were a kind gift from Dr. Dorothy Croall (University of Maine, Orono) [Croall et al., 1992], synthesis of anti-total calpain has been previously described [Spencer et al., 1995]. Oligonucleotide primers were derived from bases 1045–1064 (upstream) and bases 2043–2062 (downstream) of the rat calpain II cDNA [DeLuca et al., 1993]. All other reagents were the highest grade available from Fisher Scientific.

Animals

Modifications of muscle loading in vivo was achieved using the hindlimb suspension technique of Morey-Holton and Wronski [Morey-Holton and Wronski, 1981]. Muscle unloading was performed for 10 days followed by return to reloading for a period of 2 days of normal weight-bearing. This time course results in substantial remodeling of myofibrillar organization and of cytoskeletal associations with the membrane in soleus and plantaris muscles. In other treatments, animals were subjected to periods of muscle unloading only, with no reloading period. Control tissues were obtained from normal ambulatory animals. Soleus and plantaris muscle were removed from animals at the end of treatment, and then assayed for calpain concentration, activation, and distribution as well as for the concentration and distribution of calpain mRNA.

Assays for Calpain Concentration and Autolysis

The procedure used for assaying intact and autolyzed calpains I and II was developed because intact and autolyzed calpain I in rat muscle cannot be resolved by SDS-PAGE alone [Croall and DeMartino, 1990 and personal communication, Dr. Dorothy Croall]. Also, intact and autolyzed calpain II have not been successfully resolved by SDS-PAGE alone, for any species [Croall et al., 1992; Brown and Crawford, 1993]. In the technique employed here, calpain I and II are separated first by ion exchange chromatography after which intact and autolyzed calpains were identified in immunoblots using epitope-specific antisera.

Samples were prepared for Mono Q fractionation as follows. Plantaris muscles were homogenized with a Dounce homogenizer for 2 min in 5 vol of buffer A (20 mM Tris pH 7.6, 3 mM 2-mercaptoethanol [BME], 5 mM ethylene glycol bis N, N, N', N', tetraacetic acid [EGTA], 10 mM NaCl) and centrifuged at 10,000*g* at 4°C for 20 min. The supernatant was then filtered through a 0.22 µM filter and the protein concentrations in the samples were determined by measuring absorbance at 280 nm. The same mass of each sample was loaded to a Mono Q column in buffer A at the same concentration. Each sample was then washed to baseline in buffer A. Samples were eluted with two NaCl cuts at 300 mM NaCl (for calpain I elution) and at 550 mM NaCl (for calpain II elution). A 65 µl sample of each fraction was loaded onto SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Triplicate sets of column fractions were analysed; one for anti-total calpain analysis, one for anti-pro-calpain II analysis, and one for pro-calpain I analysis. Relative protein concentration of Mono Q separated fractions were then quantitated by densitometry of immunoblots and normalized to control values processed in parallel.

Northern Analysis

Plantaris and soleus muscles used for RNA analysis were dissected and quickly frozen in liquid nitrogen until the time of use. Total RNA was prepared according to Chomczynski [Chomczynski and Sacchi, 1987]. Total RNA (25 µg) was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde according to Sambrook et al. [1989]. Samples were transferred to uncharged nylon membranes electrophoretically in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 1 amp for 2 h at 4°C. After transfer the membrane was washed briefly in 2X SSC (0.03 M citric acid trisodium, 0.3 M sodium chloride) and crosslinked to the membrane in a UV crosslinker (Bio Rad) with 125 mJ. Transfer efficiency was checked by exposing the transferred gel to UV light and staining the membrane with methylene blue.

Nylon membranes were prehybridized in 5–7 ml of pre-hybridization fluid (50% formamide, 5X SSPE (0.05 M sodium phosphate, 0.75 M soidum chloride, 5 mM EDTA), 2X Denhardts, 0.1% SDS, 50 µg/ml tRNA, and 100 µg/ml salmon sperm DNA) for 2–4 h at 42°C. Membranes were hybridized with 2 x 10^6 cpm/ml of

probe in the pre-hybridization fluid for 15–18 h. Washing was done with 0.2X SSC, 0.1% SDS at 42°C for 2 h with at least six changes of wash buffer. Following washes, blots were exposed to Kodak autoradiographic film for 2–6 days.

In Situ RT PCR

In situ RT PCR (reverse transcription polymerase chain reaction) protocol was adapted from a previously published procedure [Nuovo et al., 1994]. Ribonuclease-free solutions were prepared according to standard procedures [Sambrook et al., 1989]. PCR conditions were initially determined by performing RT PCR of rat RNA and agarose gel electrophoresis until sufficiently stringent conditions were observed to produce one band at the correct molecular weight. Those PCR conditions were then used for the in situ RT PCR. Reactions on the tissue were covered with a glass coverslip, adhered by nail polish, and sealed with sterile mineral oil. Soleus muscles were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Paraffin sections were sectioned at 8 µm and placed on silane-coated slides, before deparaffinizing with xylene and then rehydrating. Sections were then treated with trypsin (2 mg/ml) for 20 min at RT. Trypsin treatment was inactivated by treating the sections for 1 min in DEPC (diethylpyrocarbonate) treated water, and 1 min in 100% EtOH and allowed to air dry. The sections were then DNAse treated for 4 h at 37°C prior to the RT reaction. Positive controls were not DNAse treated. The RT reaction was carried out using random pentamers (Pharmacia) and Reverse Transcriptase enzyme (RT) (Gibco BRL) under standard conditions [Sambrook et al., 1989] for 30 min at 42°C. RT was inactivated by heating the sections to 95°C for 5 min. Negative controls were not incubated in the RT enzyme. Following heat inactivation, slides were incubated in xylene for 5 min, the coverslips were removed, and the slides placed in 100% EtOH for 5 min and air dried. PCR was carried out according to standard procedures [Sambrook et al., 1989] using 0.5 µM of each primer, 4.0 mM $MgCl_2$, 200 µM of each dNTP (dinucleotide triphosphate), 10 µM digoxygenin 11-dUTP, 0.08% BSA and 0.1 U/µl TAQ polymerase in a final volume of 6 µl per section. A coverslip was placed on top of the reaction and the tissue then was placed in the thermocycler and 35 cycles of PCR were carried out (1 min 94°C, 1 min 50°C, 1.5 min 72°C). When the PCR was complete, the slide was removed from the block and immersed in xylene for 5 min to facilitate removal of coverslips. The slide was then placed in 100% EtOH, allowed to air dry, and placed in TBS (100 mM Tris pH 7.6, 150 mM NaCl) for 5 min at room temperature, then blocked in TBS with 1% BSA for 30 min at room temperature. Anti-digoxygenin conjugated to alkaline phosphatase was applied (1:100 in TBS, 1% BSA) for 1 h at room temperature in a humid chamber. The tissue was then washed three times in TBS (5 min each) and then one time in reaction buffer (100 mM Tris pH 9.5, 150 mM NaCl, 5 mM $MgCl_2$). Color reaction of the alkaline phosphatase enzyme was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt in reaction buffer for 3–5 min at room temperature.

Statistical Analysis

All quantitative data were analyzed using statistical analysis. For all experiments normalized to controls, Mann-Whitney analysis was utilized. All other experiments were analyzed by Students' *t*-test.

RESULTS

Calpain II Concentration is Significantly Increased in Reloaded Muscle

Calpain I and II were fractionated by Mono Q ion exchange chromatography of extracts of unloaded, reloaded, and control rat muscle. Using a linear NaCl gradient, calpain I elutes at approximately 180 mM NaCl and calpain II at approximately 480 mM NaCl. Samples in the present study were batch eluted with two salt cuts, 300 mM and 500 mM NaCl and analyzed in immunoblots with anti-total calpain as described previously [Spencer et al., 1995]. This antibody recognizes both calpain I and calpain II because the 20 amino acid peptide antigen is highly conserved. Immunoblot analysis of calpain isoform concentration showed no significant difference in the total mass of either calpain isoform between control and unloaded muscle samples (Fig. 1 and 2). There was also no difference in the total mass of calpain I in control and reloaded muscle (Fig. 2). However, the total mass of calpain II showed a significant increase of 57% in reloaded muscle, compared to control muscle. Calpain mass was also expressed following normalization to muscle wet weight because the mass of the soleus muscles

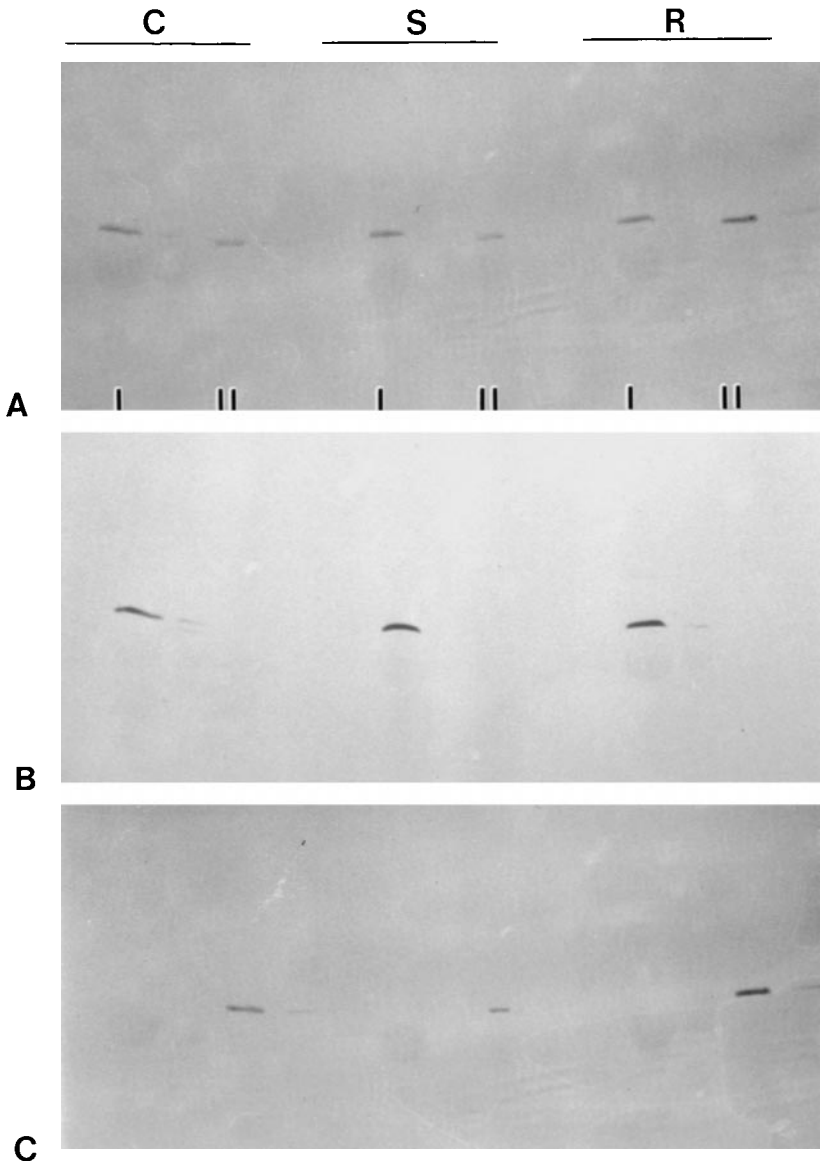

Fig. 1. Fractionation of whole muscle extracts by Mono Q ion exchange chromatography and immunoblotting for calpain I and II and pro-calpain I and II. Control and experimental muscles were fractionated by ion exchange chromatography and immunoblotted for total calpain (**A**), pro-calpain I (**B**), or pro-calpain II (**C**). Blot in (A) indicates the total amount of each isoform of calpain while blots in (B) and (C) indicate only un-autolyzed calpains. Densitometric measurements were made of all bands and the relative fraction of unautolyzed calpain (compared to total calpain) was determined. This method also allowed for the determination of the relative concentration of each isoform of calpain. C, control muscle; S, 10 day suspended (unloaded) muscle; R, 2 day reloaded muscle. I and II refer to calpain I and calpain II.

changes over the course of unloading followed by reloading. Again, no significant differences were seen between calpain concentrations of control and unloaded muscle for either calpain isoform, and no significant difference in the concentration of calpain I was identified in control and reloaded muscle. A significant, 90% increase in calpain II concentration was observed between reloaded and control muscle, following normalization for changes in muscle wet weight (Fig 2).

Muscle Reloading Does Not Affect the Activation Index of Calpains I or II

The autoproteolytic modification of calpain during muscle unloading or reloading in vivo was assayed using antibodies generated specifically against the pro-peptides of calpains I and

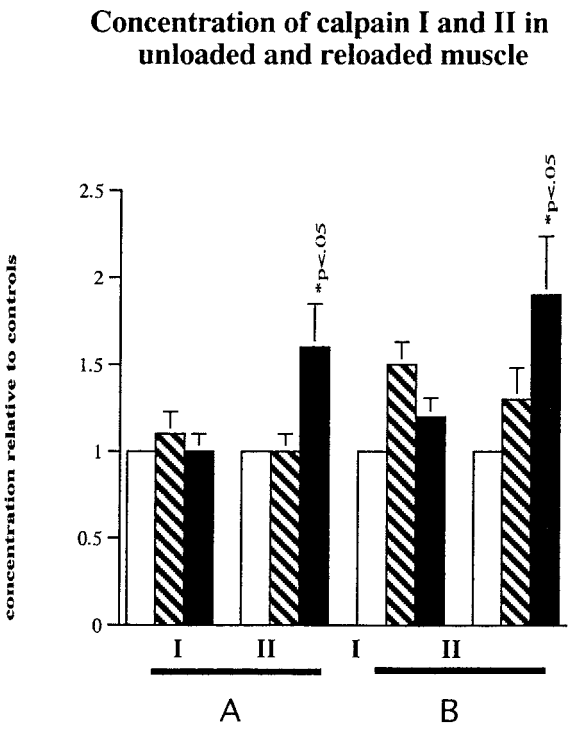

Fig. 2. Histogram of calpain I and II concentration in unloaded (striped bars) and reloaded (solid black bars) muscle relative to ambulatory controls (solid white bars). Sets labeled 'I' are calpain I values; sets labeled 'II' are calpain II values. Group A shows data expressed as calpain mass per muscle, relative to controls. Group B shows data expressed after normalization for muscle wet weight, relative to controls. *Significant to *P*

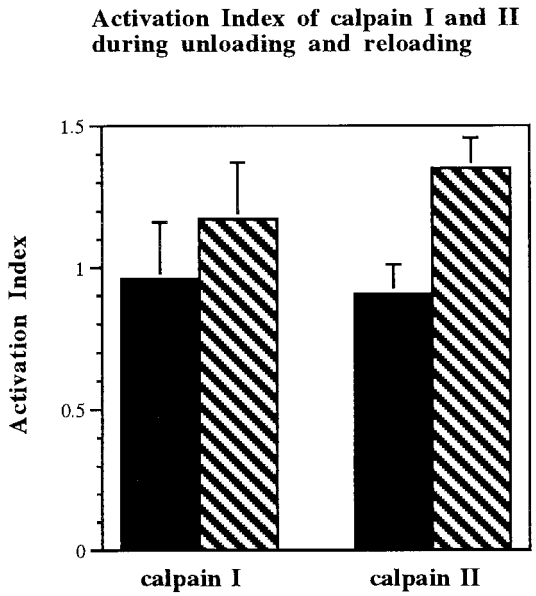

Fig. 3. Histogram of activation index of calpains I and II as determined by pro-peptide analysis of immunoblots. Densitometric measurements of calpain bands were made and the proportion of autolyzed calpain molecules was calculated. No significant difference in the proportion of calpain molecules autolyzed was observed. However, the total number of autolyzed calpain molecules increased suggesting the presence of an increase of active calpain II. Solid bars indicate 10 day unloaded muscle, striped bars indicate 2 day reloaded muscle.

II [Croall et al., 1992]. These antibodies recognize only the un-autolyzed form of calpain because the amino pro-peptide is autolytically removed prior to, or accompanying calpain exposure to calcium [Croall et al., 1992; Saido et al., 1992; Saido et al., 1993]. Because the removal of the amino terminus is believed to accompany or follow the activation of calpain, this characteristic can thus be considered an indication of activation in vivo. Thus, a decrease in the amount of pro-peptide present in the calpain molecule suggests that the protein is or has been activated.

Triplicate samples of each Mono Q fractionated sample were analyzed in immunoblots using either: 1) anti-total calpain, which recognizes the proteolytic domain (domain II) of each calpain isoform; 2) anti-pro-calpain I, which recognizes only the amino terminal propeptide of calpain I; or 3) anti-pro-calpain II, which recognizes only the propeptide of calpain II. Densitometry of the bands was performed and the activation index was derived using the formula: (experimental total calpain/control total calpain) x (control pro-calpain/experimental pro-calpain).

This index is an expression of the proportion of the total mass of calpain present that is autolyzed. Analysis of control, unloaded, and reloaded muscle was performed (Fig. 3). No significant difference was observed between either experimental condition and control muscle. Thus, the proportion of autolyzed calpains I or II, relative to total calpain concentration, did not differ between experimental and control muscles. However, calpain II concentration is significantly elevated in reloaded muscle, so that the total concentrations of both active and inactive calpain II are increased in muscle reloading. Thus a 90% increase in autolyzed calpain molecules is present in reloaded muscle.

Calpain II mRNA is Upregulated in Reloaded Muscle

Northern analysis of control, suspended, and recovery rat muscle showed an increase in calpain II, but not calpain I, mRNA in reloaded rat muscle (Fig. 4). Densitometric measurements of northern blots probed with specific calpain I and calpain II probes showed a 2.3 fold increase

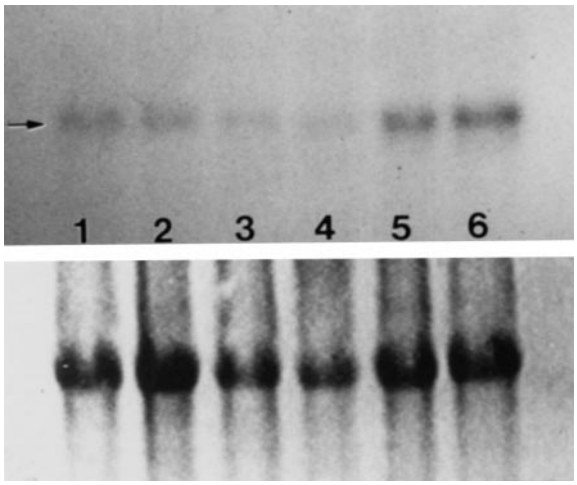

Fig. 4. Analysis of calpain mRNA in unloaded and reloaded muscle. Plantaris muscles from rats subjected to unloading and reloading were dissected and total RNA isolated for northern analysis. Shown in the top panel is a representative blot of rat total RNA probed for calpain II. Bottom panel is 18 S RNA. Lanes 1 and 2: Ambulatory control rat; Lanes 3 and 4: unloaded rat muscle; Lanes 5 and 6: 2 day reloaded rat muscle.

in specific calpain II mRNA concentration (normalized by 18S RNA) occurred during reloading (Fig. 5).

Calpain II mRNA in Muscle Tissue is Located in the Cytoplasm of Muscle Fibers

Previous investigations have shown that the modifications in muscle loading employed in this investigation cause an increase in inflam-

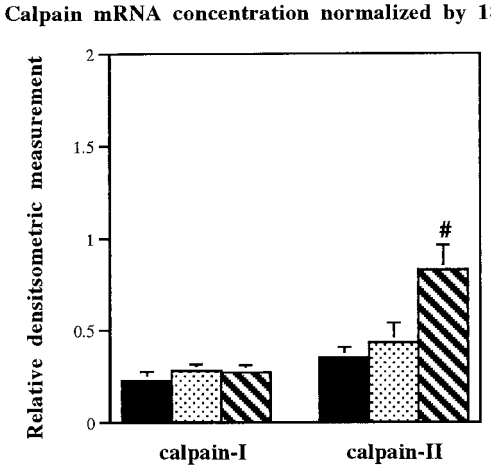

Fig. 5. Calpain II mRNA is increased in reloaded muscle. Histogram of densitometric measurements of northerns probed with specific calpain I and II cDNA probes. Solid bars, ambulatory control rat muscle RNA; Spotted bars, 10 day unloaded rat muscle RNA; Striped bars, 2 day reloaded rat muscle RNA. #Significant to $P < .01$.

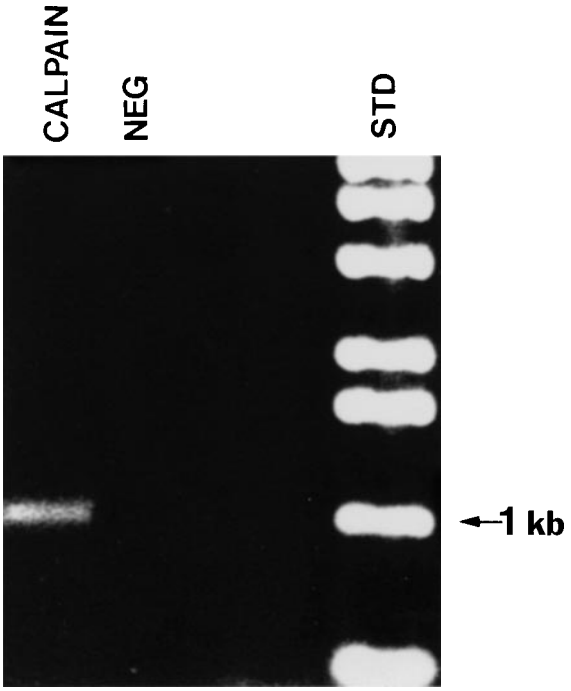

Fig. 6. PCR product from calpain II primer set using rat cDNA as template. Shown is a 1% agarose gel containing the PCR product generated by the calpain II primers utilized in the in situ RT PCR experiments. The PCR product is 1 kb which is the expected size based on the rat cDNA clone [DeLuca et al., 1993]. No other PCR products are generated using these primers. Neg is the negative control lane for the PCR.

matory cell invasion of the muscle [St. Pierre and Tidball, 1994; Tidball, 1995]. We tested whether the changes in calpain II expression identified here were attributable to changes in expression of the enzyme by muscle cells or inflammatory cells by identifying the calpain expressing cells by in situ RT PCR using primers for calpain II mRNA that amplify a 1 kb fragment from cDNA template (Fig. 6). We observed the great majority of calpain-mRNA is concentrated in muscle cells with little detectable calpain II mRNA present in inflammatory cells (Fig. 7). Thus, the changes in calpain expression that occur during muscle reloading reflect an increase in calpain II expression in muscle cells.

DISCUSSION

The results of the present study show that increases in muscle loading cause an increase in the concentration of calpain II in muscle, but no detectable change in the concentration of calpain I. Furthermore, the change of calpain II expression that is influenced by modifications

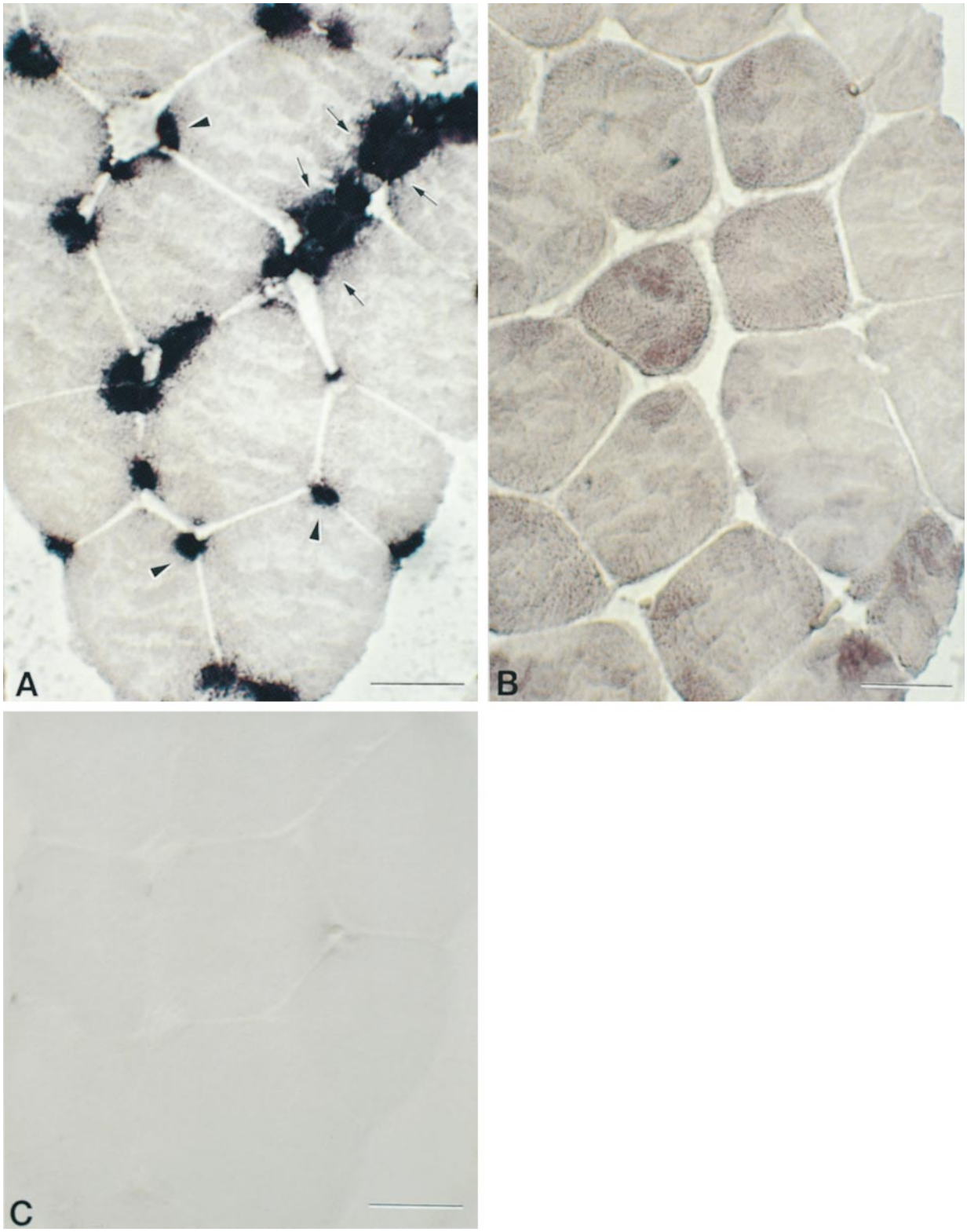

Fig. 7. In situ RT PCR of calpain II mRNA. In situ RT PCR was performed on paraffin sections of reloaded rat muscle using calpain II PCR primers. This tissue was chosen because it contains a large number of inflammatory cells. **A**: Positive control (no DNAse treatment). Arrowheads point to positive staining in what appear to be myonuclei. Arrows point to positive staining in what appear to be a cluster of inflammatory cells. **B**: Experimental tissue. The majority of calpain mRNA appears cytosolically with no positive cells apparent in the endomysium. **C**: Negative control tissue (no reverse transcriptase plus DNAse treatment). Bar is 25 µM.

in muscle loading is regulated either at the level of gene transcription or mRNA stability, because the response recorded here is also reflected in increases in calpain II mRNA concentration. Thus, these findings show that expressions of calpain isoforms in muscle are regulated independently, with the expression of calpain II influenced by increased mechanical loading or some variable associated with increased loading. The findings of the present investigation also show that reloading skeletal muscle causes no change in the activation index of either calpain I or II. However, the activation index is a measure of the proportion of autolyzed molecules in the total calpain isoform population, not a measure of the net number of autolyzed molecules. Thus, the increase in number of autolyzed calpain II molecules in reloaded muscle is proportional to the increase in calpain II concentration resulting in a 90% increase in autolyzed calpain molecules.

The involvement of calpains in other models of muscle remodeling, particularly those in which the muscle is in negative protein balance, is generally murky. The first studies of calpain in muscle [Busch et al., 1972] provided compelling evidence that underlies the current view that calpains are involved in myofibrillar degradation by showing that a calcium activated factor was involved in degradation of Z-discs. This calcium dependent activity was shown to be a protease, subsequently named calcium-activated neutral protease (CANP) or calpain, that was capable of removing alpha-actinin from Z-discs [Reddy et al., 1975; Goll et al., 1991]. The similarity between the ultrastructural appearance of muscle treated with calcium ionophore, in which calpains were presumably activated [Yoshimura et al., 1986; Duncan, 1987], and in muscle injured by exercise, injury, denervation, or disease, in which intracellular Ca^{2+} concentrations may have been elevated, [Mokri and Engel, 1975; Ogilvie et al., 1988] led to the expectation that muscle proteolysis following these perturbations in vivo was calpain-mediated. However, this view has received little unequivocal, experimental support. The most frequently used approach to examining the basis for the increase in proteolysis that occurs in muscle following experimental perturbations is to measure tyrosine release in the presence or absence of protease inhibitors [Llados, 1985; Zeman et al., 1985; Baracos et al., 1986; Turner et al., 1988], or following changes in extracellular or intracellular [Ca^{2+}] [Llados, 1985; Baracos et al., 1986; Turner et al., 1988]. Experimental perturbations that have been used most extensively in these studies are denervation, modified loading or direct trauma [Furuno and Goldberg, 1986; Furuno et al., 1990; Tischler et al., 1990]. Many of these investigations have used the cysteine protease inhibitors leupeptin or E-64 which inhibit calpains and cathepsins B and D, and found that these treatments could inhibit the increase in proteolysis that occurs following muscle unloading or trauma [Baracos et al., 1986; Furuno and Goldberg, 1986; Furuno et al., 1990; Tischler et al., 1990], but not affect the increased proteolysis occurring following denervation [Furuno et al., 1990; Tischler et al., 1990]. Inhibitors of lysosomal activity were effective in inhibiting the increase in proteolysis occurring in denervated muscle [Furuno et al., 1990; Tischler et al., 1990], indicating proteolysis mediated by lysosomal enzyme(s) other than cysteine proteases. From these results alone, it could be concluded that proteolysis following unloading or trauma could be mediated by either cytosolic cysteine proteases, such as calpains, or lysosomal cysteine proteases such as cathepsin B and D. However, inhibitors of lysosomal activity did not inhibit the elevation in proteolysis caused by unloading or trauma [Furuno and Goldberg, 1986], which argues for a key role for calpain or some other cytosolic cysteine protease.

Although the experimental approach described above has shown clearly that lysosomal and non-lysosomal proteolytic systems differ in their contributions to muscle proteolysis, depending on the perturbation, they are limited in what they reveal of the role of calpain. In addition to the non-specificity of the inhibitors used, assaying for proteolysis by measuring tyrosine release is not optimal for inferences concerning calpain-mediated proteolysis [Takahashi, 1990; Croall and DeMartino, 1991]. Although the extent to which calpains can degrade substrate proteins in vivo has been little studied, some of the best characterized calpain substrates present in skeletal muscle undergo very little proteolytic modification in vivo, and are instead cleaved at a small number of specific sites (e.g., talin) [Fox, 1985; Fox et al., 1985; O'Halloran et al., 1985; Beckerle et al., 1986]. Thus, calpain-mediated proteolysis could occur in tissue without detection by tyrosine release assays.

Direct assays of calpain concentration [Belcastro, 1993; present study] and apparent autoproteolysis in vivo [present study] indicate that increased muscle loading is a positive regulator of calpain. A single bout of exercise increases calpain I and II concentrations by approximately 25% when the muscle is assayed immediately following exercise, but their concentrations return to control levels within 24 h of the exercise [Belcastro, 1993]. Although the period of loading in the previous investigation [Belcastro, 1993] was until the animals were exhausted, rather than for a stated length of time, the increase in calpain concentration presumably occurred too rapidly to reflect transcriptional control, and more likely reflected increased translation of existing calpain mRNA. This transient increase in calpain concentration during a single bout of exercise may also reflect increased activity of the enzymes in vivo [Belcastro, 1993] although inactive procalpains and autolyzed forms were not assayed separately. The results of the present investigation show that increased muscle loading, when applied for longer periods, can selectively increase expression of calpain II, and that regulation occurs either at the transcriptional level or by increasing mRNA stability. The distinction shown here in the regulation of expression of calpain II compared to calpain I, is surprising because very few differences have been identified in vivo for the distribution, regulation, substrate specificity, or function of calpains I and II. Exceptions to this are reports of specific increases in calpain II concentration in dystrophic hamster muscle [Johnson and Hammer, 1988], and in Duchenne muscular dystrophy [Reddy et al., 1986] and mdx dystrophy [Spencer et al., 1995]. The findings of this study lead to the hypothesis that mechanical loading, or some factor associated with mechanical loading, selectively upregulates calpain II expression.

A likely candidate for the mechanical loading associated factor involved in upregulating calpain II expression is calcium. The promoter/enhancer sequence of the calpain II gene contains a TPA-response element (TRE) [Hata et al., 1992], that is involved in increased gene expression in the presence of activated protein kinase C (PKC) [Nishizuka, 1984]. However, calpain I expression is not increased in the presence of TPA, indicating a lack of a TRE. Thus, elevation of cytosolic [Ca^{2+}] would be expected to activate PKC, thereby increasing expression of calpain II, but not calpain I. Although it has not yet been shown directly that the muscle loading protocol used here causes an increase in cytosolic [Ca^{2+}], recent work has shown that unloading of rat hindlimb muscle followed by reloading introduces cell membrane lesions that are large enough to permit the transit of molecules as large as albumin [Kasper, 1995]. Such lesions would easily permit the influx of extracellular Ca^{2+}, that could enhance calpain II expression via PKC-mediated activation of the TRE.

Two other possibilities by which increased loading could result in modification of gene expression in muscle cells have recently been elucidated [Sadoshima and Izumo, 1993]. One is that mechanical stimuli activate mechanochemical transducers, such as the integrin-associated cytoskeleton, that can then activate numerous second messengers [Plopper et al., 1995]. In platelets, there is substantial evidence that binding of the GP IIb-IIIa integrin is associated with calpain activation [Fox et al., 1993] although this integrin is not present on skeletal muscle. Alternatively, mechanical loading can induce the release of factors from the muscle that can then stimulate generation of second messengers after binding to their receptors on muscle in an autocrine signaling mechanism. This second mechanism has received experimental support through investigations showing that exercise induces the release of cytosolic, basic fibroblast growth factor from muscle [Clarke et al., 1993] and that mechanically-stimulated cardiac myocytes release soluble factors that can induce c-fos expression in cardiac myocytes [Sadoshima and Izumo, 1993]. Current studies are directed toward examining the contribution of each of these signaling systems to the regulation of calpain expression.

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