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# The activation of protein degradation in muscle by $Ca^{2+}$ or muscle injury does not involve a lysosomal mechanism

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By use of different inhibitors, we distinguished three proteolytic processes in rat skeletal muscle. When soleus muscles maintained under tension were exposed to the calcium ionophore A23187 or were incubated under no tension in the presence of $Ca^{2+}$, net protein breakdown increased by 50–80%. Although leupeptin and E-64 inhibit this acceleration of protein breakdown almost completely, other agents that prevent lysosomal function, such as methylamine or leucine methyl ester, did not inhibit this effect. A similar increase in net proteolysis occurred in muscle fibres injured by cutting, and this response was also inhibited by leupeptin, but not by methylamine. In contrast, all these inhibitors markedly decreased the 2-fold increase in protein breakdown induced by incubating muscles without insulin and leucine, isoleucine and valine. In addition, the low rate of proteolysis seen in muscles under passive tension in complete medium was not affected by any of these inhibitors. Thus the basal degradative process in muscle does not involve lysosomes or thiol proteinases, and muscle can enhance protein breakdown by two mechanisms: lack of insulin and nutrients enhances a lysosomal process in muscle, as in other cells, whereas $Ca^{2+}$ and muscle injury activate a distinct pathway involving cytosolic thiol proteinase(s).

## INTRODUCTION

The overall rate of protein breakdown in skeletal muscle increases in a variety of physiological and pathological states (Wildenthal, 1980). For example, lack of amino acids and insulin (Fulks *et al.*, 1975; Tischler *et al.*, 1982; Garlick *et al.*, 1985; Millward *et al.*, 1985) stimulate proteolysis in muscle, as in other tissues (Hershko & Ciechanover, 1982; Mortimore, 1982). Studies of perfused liver (Schworer *et al.*, 1981), heart (Long *et al.*, 1983) and cultured cells (Poli *et al.*, 1981) suggest that lack of these nutrients leads to autophagic-vacuole formation and protein degradation by lysosomal enzymes. In non-muscle cells, this enhancement of protein breakdown is sensitive to inhibitors of thiol proteinases, such as leupeptin or E-64, as well as weak bases, which can raise intralysosomal pH and prevent lysosomal function (Hopgood *et al.*, 1977; Seglen *et al.*, 1979; Ward *et al.*, 1979). However, clear evidence for such a lysosomal mechanism in skeletal muscle is lacking.

In isolated skeletal muscles, protein breakdown can also be stimulated by treatments that increase intracellular $Ca^{2+}$ (Etlinger *et al.*, 1980; Baracos *et al.*, 1984, 1986). For example, exposure of the cells to the $Ca^{2+}$ ionophore A23187 (Kameyama & Etlinger, 1979; Sugden, 1980; Lewis *et al.*, 1982; Rodemann *et al.*, 1982) or membrane depolarization (Rodemann *et al.*, 1982), which should increase intracellular $Ca^{2+}$, causes a large increase in protein degradation. Furthermore, when rat muscles are incubated *in vitro*, they tend to shorten and show a large increase in protein breakdown, and this effect requires $Ca^{2+}$ in the medium (Baracos *et al.*, 1984, 1986; Baracos & Goldberg, 1985, 1986). This latter response can be prevented by maintaining the muscles under passive tension, i.e. near resting length (Baracos & Goldberg, 1985, 1986). In addition, the stimulation of muscle protein breakdown by interleukin-1, which seems responsible for muscle wasting during febrile disease (Fagan & Goldberg, 1985), requires $Ca^{2+}$ in the medium (Baracos *et al.*, 1983, 1984).

Rodemann *et al.* (1982) have reported that the activation of protein breakdown by $Ca^{2+}$ is inhibited dramatically by leupeptin or Ep-475 (E-64C). These agents can enter intact muscle cells and inhibit lysosomal thiol proteinases, such as cathepsin B (Libby & Goldberg, 1978). A lysosomal site of action appeared likely, since these inhibitors, like various lysosomotropic agents (e.g. weak bases), can decrease protein breakdown in non-muscle cells deprived of serum (Seglen *et al.*, 1979; Ward *et al.*, 1979; Poli *et al.*, 1981). An alternative possibility is that these inhibitors might be affecting some non-lysosomal thiol proteinases in the muscle, such as the $Ca^{2+}$-activated proteinases calpain I or II (Waxman, 1981). Although these enzymes have frequently been proposed to play a role in the breakdown of myofibrillar proteins, Rodemann *et al.* (1982) and Baracos *et al.* (1986) found that treatment of diaphragms with mersalyl inactivated almost completely the major $Ca^{2+}$-activated proteinases, but did not decrease the stimulation of proteolysis by $Ca^{2+}$. These findings led to the suggestion (Rodemann *et al.*, 1982; Baracos *et al.*, 1984) that a rise in cytosolic $Ca^{2+}$ promoted intralysosomal proteolysis. However, the present studies, which used other inhibitors of lysosomal function, indicate that $Ca^{2+}$ and muscle injury activate protein breakdown in muscle by a non-lysosomal mechanism.

After these studies were completed, similar conclusions on the effects of $Ca^{2+}$ were reported by Zeman *et al.* (1985).

## MATERIALS AND METHODS

Methylamine and leucine methyl ester were purchased from Sigma Chemical Co., and the calcium ionophore A23187 from Calbiochem Behring. L-[U-$^{14}$C]Phenylalanine was a product of New England Nuclear Corp. Leupeptin (Umezawa, 1976) was kindly provided by Dr. T. Aoyagi and Dr. H. Umezawa, Institute of Microbial Chemistry (Tokyo, Japan), and E-64 (Hanada *et al.*,

**Table 1. Effect of leupeptin and methylamine on the increased proteolysis induced by $Ca^{2+}$ ionophore A23187 in soleus muscles maintained under tension**

Contralateral soleus muscles were pinned at approximately their resting length on to plastic supports and incubated in the absence or presence of A23187 (10 $\mu$g/ml), which had been dissolved in dimethyl sulphoxide. The control muscles were incubated in medium containing the same amount of dimethyl sulphoxide (0.1%). Leupeptin (50 $\mu$M) and methylamine (10 mM) were added to the preincubation and final incubation media. Values represent means ± S.E.M. for muscles from five rats. Data were evaluated with Student's $t$ test for unpaired (**) and paired (*) observations: *$P < 0.001$ compared with muscle with no ionophore; **$P < 0.001$ compared with ionophore-treated muscles with no inhibitor.

| | Protein degradation (nmol of tyrosine/2 h per mg of muscle) | | | |
|---|---|---|---|---|
| Inhibitor | −A23187 | +A23187 | Difference | Increase (%) |
| None | 0.111 ± 0.004 | 0.195 ± 0.007 | 0.084 ± 0.004* | 76 |
| Leupeptin | 0.105 ± 0.005 | 0.115 ± 0.004** | 0.010 ± 0.003 | 9 |
| Methylamine | 0.108 ± 0.004 | 0.202 ± 0.009 | 0.094 ± 0.008 | 87 |

**Table 2. Effect of thiol-proteinase inhibitors and weak bases on $Ca^{2+}$-dependent proteolysis in unrestrained muscles**

Contralateral soleus and extensor digitorum longus muscles were incubated without being pinned to a support in buffer prepared without and with 2.5 mM-$CaCl_2$. Leupeptin (50 $\mu$M), E-64 (50 $\mu$M), methylamine (10 mM) and leucine methyl ester (10 mM) were added to the preincubation and final incubation media. Values represent the means ± S.E.M. of muscles from five rats. Data were evaluated with Student's $t$ test for unpaired (**) and paired (*) observations: *$P < 0.001$ for comparison of muscles with and without $Ca^{2+}$; **$P < 0.001$ compared with muscles without the inhibitor.

| | | Protein degradation (nmol of tyrosine/2 h per mg of muscle) | | | |
|---|---|---|---|---|---|
| Muscle | Inhibitor | $Ca^{2+}$-free | +$Ca^{2+}$ (2.5 mM) | Difference | Increase (%) |
| Soleus | None | 0.126 ± 0.006 | 0.219 ± 0.007 | 0.093 ± 0.012* | 74 |
| | Leupeptin | 0.118 ± 0.005 | 0.132 ± 0.004** | 0.014 ± 0.004 | |
| | Methylamine | 0.129 ± 0.009 | 0.228 ± 0.011 | 0.099 ± 0.013 | 77 |
| Extensor digitorum longus | None | 0.119 ± 0.005 | 0.181 ± 0.005 | 0.062 ± 0.005* | 52 |
| | E-64 | 0.110 ± 0.004 | 0.115 ± 0.003** | 0.005 ± 0.002 | |
| | Leucine methyl ester | 0.147 ± 0.009 | 0.285 ± 0.010** | 0.138 ± 0.006 | 94 |

1978) by Dr. K. Hanada, Taisho Pharmaceutical Co., (Saitama, Japan). All chemicals used were of reagent grade.

Young male CD strain rats from Charles River Breeding Laboratories were maintained on Purina Lab Chow and water *ad libitum*. Animals weighing 60–80 g were killed by cervical dislocation, and the extensor digitorum longus and soleus muscles were dissected rapidly. These muscles were incubated either under no tension, where they shortened spontaneously, or under passive tension at resting length (or slightly longer). In the latter case, the tendons were fixed with stainless-steel needles to plastic supports at approximately the lengths seen on full extension of the muscles *in vivo*.

The muscles were incubated at 37 °C in Krebs–Ringer bicarbonate buffer, equilibrated with $O_2/CO_2$ (19:1), as described previously (Fulks *et al.*, 1975; Tischler *et al.*, 1982). Except where indicated, this buffer was supplemented with 5 mM-glucose, 0.1 unit of insulin/ml, 0.17 mM-leucine, 0.10 mM-isoleucine and 0.20 mM-valine, which are approximately the normal plasma concentrations of these amino acids. The tissues were preincubated for 60 min and then transferred to new flasks for a final 2 h incubation. The composition of the preincubation medium, including the various inhibitors, was identical with that used in the final incubation.

Net protein degradation was estimated as described by Fulks *et al.* (1975) from the rate of production of free tyrosine from tissue proteins. Since muscle cannot synthesize or degrade tyrosine, its net production represents the net degradation of muscle proteins. Evidence documenting the validity of this approach has been reported elsewhere (Fulks *et al.*, 1975; Tischler *et al.*, 1982). Under the conditions studied here (e.g. treatment with $Ca^{2+}$ ionophores or inhibitors), changes in net protein breakdown reflected primarily changes in protein breakdown and not protein synthesis (see below). Tyrosine was assayed by the method of Waalkes & Udenfriend (1957). To measure the intracellular pools of tyrosine, the individual muscles were homogenized in 10% (w/v) trichloroacetic acid and centrifuged (1700 *g*), and the supernatant was assayed for tyrosine.

The rate of protein synthesis was determined in the muscles by incubating with L-[U-$^{14}$C]phenylalanine

**Table 3. Effect of various inhibitors on the increased proteolysis induced by lack of insulin and amino acids in muscles maintained under tension**

Contralateral soleus and extensor digitorum longus muscles were pinned at approximately their resting lengths on to plastic supports and incubated in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 5 mM-glucose with or without 0.1 unit of insulin/ml and the plasma concentrations of branched amino acids. Leupeptin (50 $\mu$M), E-64 (50 $\mu$M), methylamine (10 mM) and leucine methyl ester (10 mM) were added to the preincubation and final incubation media. Values represent means ± S.E.M. for muscles from five rats. Data were evaluated with Student's $t$ test for unpaired (**) and paired (*) observations: *$P < 0.001$ compared with muscle with insulin and amino acids; **$P < 0.001$ compared with muscle incubated without the inhibitor.

| | | Protein degradation (nmol of tyrosine/2 h per mg of muscle) | | | |
|---|---|---|---|---|---|
| Muscle | Inhibitor | Insulin and amino acids | Unsupplemented medium | Difference | Increase (%) |
| Soleus | None | 0.107 ± 0.002 | 0.216 ± 0.009 | 0.109 ± 0.009* | 102 |
| | Leupeptin | 0.104 ± 0.004 | 0.143 ± 0.004** | 0.039 ± 0.002 | 37 |
| | Methylamine | 0.110 ± 0.002 | 0.131 ± 0.007** | 0.021 ± 0.006 | 19 |
| Extensor digitorum longus | None | 0.087 ± 0.003 | 0.207 ± 0.009 | 0.120 ± 0.010* | 138 |
| | E-64 | 0.086 ± 0.005 | 0.145 ± 0.006** | 0.059 ± 0.008 | 69 |
| | Leucine methyl ester | 0.093 ± 0.006 | 0.110 ± 0.003** | 0.017 ± 0.005 | 18 |

**Table 4. Effect of leupeptin and methylamine on the increased proteolysis induced by injury to soleus muscles maintained under tension**

Contralateral soleus muscles were pinned on to plastic supports in the usual manner. Two cuts were made across the muscle. Leupeptin (50 $\mu$M) and methylamine (10 mM) were added to the preincubation and final incubation media. Values represent means ± S.E.M. for muscles from five rats. Data were evaluated with Student's $t$ test for unpaired (**) and paired (*) observations: *$P < 0.001$ compared with the uninjured muscles; **$P < 0.001$ compared with muscle with no inhibitor.

| | Protein degradation (nmol of tyrosine/2 h per mg of muscle) | | | |
|---|---|---|---|---|
| Inhibitor | Uncut muscle | Cut muscle | Difference | Increase (%) |
| None | 0.108 ± 0.002 | 0.216 ± 0.004 | 0.108 ± 0.018* | 100 |
| Leupeptin | 0.103 ± 0.004 | 0.121 ± 0.003** | 0.018 ± 0.003 | 17 |
| Methylamine | 0.112 ± 0.003 | 0.218 ± 0.017 | 0.106 ± 0.005 | 95 |

(0.5 mM, 0.05 $\mu$Ci/ml) and measuring the rate of incorporation of the radioactivity into tissue proteins, as described previously (Rodemann *et al.*, 1982).

## RESULTS

### Effects of inhibitors on $Ca^{2+}$-stimulated protein breakdown

When the soleus or extensor digitorum longus muscles were incubated under passive tension in the presence of insulin and branched-chain amino acids, net protein degradation (i.e. the net production of free tyrosine from tissue proteins) was minimal. These treatments have been shown previously (Baracos & Goldberg, 1986) to decrease maximally overall protein breakdown. Under these conditions, protein breakdown was not affected by the thiol-proteinase inhibitors leupeptin or E-64 (Tables 1 and 3), or by the presence or absence of $Ca^{2+}$ (Baracos & Goldberg, 1985, 1986). Furthermore, neither the weak base methylamine (Seglen *et al.*, 1979) nor leucine methyl ester, which can inhibit intralysosomal proteolysis (Long *et al.*, 1983), affected this basal proteolytic process (Tables 1 and 3). Therefore this process appears to occur by a non-lysosomal mechanism and does not require thiol proteinases.

Several groups have shown that $Ca^{2+}$ ionophores can stimulate protein breakdown in unrestrained muscle (Etlinger *et al.*, 1980; Sugden, 1980; Lewis *et al.*, 1982; Rodemann *et al.*, 1982). On exposure of the stretched soleus to A23187, a large increase in net proteolysis (Table 1) occurred, even though the tissue was fixed under passive tension and thus not able to shorten. This 76% increase in tyrosine release from the muscle must reflect enhanced protein degradation, since under these conditions the intracellular pool of tyrosine remained approximately constant and since overall rates of protein synthesis did not change (Rodemann *et al.*, 1982). Furthermore, this effect seems to result from a rise in cytosolic $Ca^{2+}$, since no increase in proteolysis was evident when the ionophore was added in the absence of extracellular $Ca^{2+}$ (results not shown). In accord with prior findings of Rodemann *et al.* (1982), leupeptin caused almost a complete inhibition of the enhanced

proteolysis without affecting protein synthesis in the tissue. By contrast, the weak base methylamine did not cause any inhibition of this $Ca^{2+}$-induced proteolysis (Table 2). Thus $Ca^{2+}$-activated proteolysis probably involves a non-lysosomal mechanism, in contrast with previous suggestions from our laboratory (Rodemann *et al.*, 1982; Baracos *et al.*, 1984).

Protein breakdown also rises when the incubated muscles are not restrained and shorten *in vitro*. Baracos & Goldberg (1985, 1986) have reported that this activation of protein breakdown is also dependent on extracellular $Ca^{2+}$. When rat soleus and extensor digitorum longus muscles were incubated in an unrestrained state, they shortened spontaneously (whether or not $Ca^{2+}$ was in the medium). In the presence of $Ca^{2+}$, net protein breakdown was 74% greater in the soleus and 52% greater in the extensor digitorum longus than in contralateral muscles in $Ca^{2+}$-free medium (Table 2). This enhancement in tyrosine release from the muscle must reflect increased protein degradation, since the presence of $Ca^{2+}$ does not affect the rate of protein synthesis under these conditions (Baracos *et al.*, 1984).

As expected from prior work by Baracos *et al.* (1986), addition of thiol-proteinase inhibitors blocked this acceleration of protein breakdown. Leupeptin caused an 84% and E-64 an 89% inhibition of this effect (Table 2). However, neither the weak base methylamine nor leucine methyl ester caused any significant inhibition of the increased protein degradation. Methylamine also did not have any effect on protein synthesis in the muscles, as shown by [$^{14}$C]phenylalanine incorporation into muscle protein (0.169 nmol/2 h per mg of muscle with methylamine and 0.159 nmol/2 h per mg of muscle in its absence). Thus the $Ca^{2+}$-dependent proteolysis that accompanies muscle shortening, like that induced with the ionophore, does not appear to involve the lysosomal apparatus. On the contrary, leucine methyl ester consistently increased protein breakdown in the shortened muscles, but not in ones under maintained tension (Table 3). The reasons for this interesting effect are unclear.

**Enhanced protein degradation upon amino acid and insulin deprivation**

In the isolated muscles, protein breakdown rises, and protein synthesis falls, when the medium lacks insulin or branched-chain amino acids (Fulks *et al.*, 1975; Tischler *et al.*, 1982; Garlick *et al.*, 1985; Millward *et al.*, 1985). Accordingly, a 2-fold rise in net proteolysis was observed when the soleus was incubated without these anabolic factors, but was maintained under passive tension. This increase in net proteolysis (Table 3) is much greater than the increase (about 25%) reported previously in muscles lacking these factors (Fulks *et al.*, 1975; Tischler *et al.*, 1982; Zeman *et al.*, 1985). Presumably, our maintaining passive tension kept proteolysis low in the control muscles in these studies. The increase in net degradation on deprivation of insulin and amino acids was blocked almost completely by the addition of either methylamine or leucine methyl ester. This response was also markedly inhibited by leupeptin or E-64. Under these conditions, the rates of protein synthesis and the intracellular pool of tyrosine were not changed by methylamine or leupeptin (results not shown).

A similar decrease in protein breakdown was observed (results not shown) in muscles treated with ammonia, another weak base known to decrease lysosomal function. These results are consistent with a role for the lysosome in the activation of protein breakdown in poor media. The degree of inhibition by methylamine or leupeptin resembles that observed previously in various non-muscle cells deprived of amino acids and insulin (Seglen *et al.*, 1979; Ward *et al.*, 1979; Poli *et al.*, 1981; Gronostajski *et al.*, 1985). These observations suggest that skeletal muscle also has an autophagic degradation mechanism, although it is not involved in $Ca^{2+}$-induced proteolysis.

**Activation of protein breakdown by muscle injury**

Seider *et al.* (1980) have shown that deliberate injury to skeletal-muscle fibres by cutting leads to a pronounced activation of protein breakdown. Such a process could be important in various types of muscle disease or trauma and may contribute to the high rate of proteolysis seen when muscles are incubated without intact tendons. Because this degradative process also may be initiated by the increased entry of $Ca^{2+}$ into the cut fibres, we studied the injury-induced degradative process and its inhibitor-sensitivity in muscles maintained under passive tension. A 2-fold rise in net tyrosine production occurred after two horizontal cuts were made across the muscle fibres with scissors (Table 4). This response to the injury was much larger (3-fold) if the muscles were allowed to shorten than if they were maintained at resting length. In these same muscles, overall protein synthesis decreased after cutting by only 15%, which is much smaller than the decrease observed by Seider *et al.* (1980) in cut muscles allowed to shorten. Surprisingly, when the injured muscles were maintained under tension, protein breakdown appeared to be independent of $Ca^{2+}$ (results not shown), i.e. the injury-induced proteolysis was not affected by $Ca^{2+}$ removal from the medium and treatment with EDTA and dantrolene, an inhibitor of $Ca^{2+}$ release from sarcoplasmic reticulum (Ebashi, 1976).

Incubation of the injured muscle with leupeptin caused a large inhibition of this increased net protein breakdown, but the inhibitor of lysosomal acidification, methylamine, did not decrease this injury-induced proteolysis (Table 4). Thus muscle injury, like $Ca^{2+}$, seems to activate a non-lysosomal pathway for protein breakdown that requires thiol proteinases.

## DISCUSSION

In accord with prior findings (Baracos & Goldberg, 1985), muscles maintained under passive tension in the presence of insulin and amino acids show very low rates of protein breakdown, and this basal process is independent of extracellular $Ca^{2+}$ and does not require thiol proteinases. It thus resembles the basal protein breakdown seen in cultured hepatocytes (Hershko & Ciechanover, 1982; Mortimore, 1982) or fibroblasts during growth (Gronostajski *et al.*, 1984, 1985). The nature of the proteinases responsible for this basal process in muscle, as in other cells, is an important unanswered question. Muscle cells contain several non-lysosomal proteinases (Pennington, 1977; Wildenthal, 1980; Kay *et al.*, 1985). Several studies (Etlinger *et al.*, 1981; Libby & Goldberg, 1981) suggest the presence within muscle of the soluble ATP-dependent pathway (Hershko & Ciechanover, 1982) as well as a high-$M_r$ multifunctional proteinase (Dahlmann *et al.*, 1985). Since neither of these activities is sensitive to leupeptin or

E-64 (Tanaka *et al.*, 1984), they may possibly contribute to the basal degradative process in the muscle.

The ability of the weak base leucine methyl ester and thiol-proteinase inhibitors to block the enhancement of protein breakdown upon nutritional deprivation strongly suggests the involvement of the lysosome (Table 3). Under these incubation conditions, leupeptin and Ep-475 can inactivate intralysosomal cathepsin B and presumably other thiol proteinases, such as cathepsin H or L, within the muscle (Libby & Goldberg, 1978; Baracos & Goldberg, 1986). It is noteworthy that inactivation of these enzymes with leupeptin or E-64 did not decrease proteolysis as completely as did methylamine (Table 3). By raising the pH within the organelle, methylamine should inhibit nearly all acid hydrolases within the lysosome, and not just the thiol proteinases.

By contrast, these findings strongly argue that the effect of $Ca^{2+}$ does not involve the lysosomal apparatus, as had been suggested previously. Etlinger and co-workers have come to a similar conclusion in analogous experiments (Zeman *et al.*, 1985). The exact mechanism by which $Ca^{2+}$ activates proteolysis *in vitro*, and the physiological significance of this effect *in vivo*, are uncertain. Under these incubation conditions, energy levels fall in the shortened muscles (Baracos & Goldberg, 1986), and microscopic studies by Harris *et al.* (1985) suggest ischaemic damage to internal regions of the muscle. Perhaps, therefore, $Ca^{2+}$ permeability into these parts of the tissue may rise. It is noteworthy that passive tension prevents these biochemical and morphological changes, and prevents the stimulation of proteolysis by extracellular $Ca^{2+}$ (K. Furuno, T. Okada, M. Karnovsky & A. L. Goldberg, unpublished work).

One simple attractive explanation of these effects would be that intracellular $Ca^{2+}$ enhances protein breakdown in these muscles through an effect on the cytosolic thiol proteinases calpain I or calpain II. However, our prior experiments indicated that these proteinases could be selectively inactivated by mersalyl without any inhibition of the stimulation of proteolysis by A23187 (Rodemann *et al.*, 1982) or extracellular $Ca^{2+}$ (Baracos *et al.*, 1984). Thus the actual mechanism of $Ca^{2+}$-induced breakdown remains uncertain. This issue is of particular interest, because similar $Ca^{2+}$-dependent phenomena may occur in certain diseased states. For example, protein breakdown appears to increase in dystrophic (Goldspink & Goldspink, 1977) and denervated muscles (Goldspink, 1976), which have been reported to show increased concentrations of $Ca^{2+}$ (Engel *et al.*, 1977; Emery & Burt, 1980; Jaffe *et al.*, 1981). In addition, interleukin-1 and endotoxin-induced fever make protein breakdown in the tissue more sensitive to extracellular $Ca^{2+}$ (Baracos *et al.*, 1983; Fagan & Goldberg, 1985).

The accelerated degradation of muscle proteins seen on injury to the fibres was also sensitive to leupeptin, but not to the weak base (Table 4). This effect thus resembles that induced by a rise in cytosolic $Ca^{2+}$, and damage to the muscle membrane should cause a large influx of $Ca^{2+}$ and $Na^{+}$ ions. Although $Ca^{2+}$ influx appears important in the response to injury in muscles allowed to shorten (K. Furuno, unpublished work), in muscles at resting length, cutting caused a similar increase in protein degradation in normal medium and in medium containing EGTA and dantrolene. Nevertheless, this type of tissue injury seems to activate a non-lysosomal proteolytic system (Table 4) similar to that stimulated by $Ca^{2+}$ (Tables 1 and 2).

The finding that $Ca^{2+}$-induced and injury-induced proteolysis are susceptible to thiol-proteinase inhibitors, but not to other lysosomotropic agents, emphasizes that caution is necessary in interpreting experimental data obtained with these inhibitors in intact cells. Generally, inhibition by such agents has been taken as evidence for lysosomal involvement in protein breakdown. This conclusion seems valid for cultured cells or muscles during nutrient deprivation, but does not appear to be applicable to conditions in which protein breakdown may be activated by another mechanism.

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