Effects of Chymostatin and other Proteinase Inhibitors on Protein Breakdown and Proteolytic Activities in Muscle

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To learn more about the enzymes involved in protein catabolism in skeletal and cardiac muscle and to identify selective inhibitors of this process, we studied the effects of proteinase inhibitors on protein turnover in isolated muscles and on proteolytic activities in muscle homogenates. Chymostatin (20 μM) decreased protein breakdown by 20–40% in leg muscles from normal rodents and also in denervated and dystrophic muscles. These results are similar to our previous findings with leupeptin. The related inhibitors pepstatin, bestatin, and elastatinal did not decrease protein breakdown; antipain slowed this process in rat hind-limb muscles but not in diaphragm. Chymostatin did not decrease protein synthesis and thus probably retards proteolysis by a specific effect on cell proteinase(s). In homogenates of rat muscle, chymostatin, in common with leupeptin and antipain, inhibits the lysosomal proteinase cathepsin B, and the soluble Ca^{2+} -activated proteinase. In addition, chymostatin, but not leupeptin, inhibits the chymotrypsin-like proteinase apparent in muscle homogenates. In muscles depleted of most of this activity by treatment with the mast-cell-degranulating agent 48/80, chymostatin still decreased protein breakdown. Therefore inhibition of this alkaline activity probably does not account for the decrease in protein breakdown. These results are consistent with a lysosomal site of action for chymostatin. Because of its lack of toxicity, chymostatin may be useful in maintaining tissues *in vitro* and perhaps in decreasing muscle atrophy *in vivo*.

The proteins of muscle, in common with those of other tissues, undergo constant degradation and replacement (Schimke, 1970; Goldberg & Dice, 1974). Changes in the rate of protein breakdown may contribute to normal muscle growth and the atrophy resulting from denervation, dystrophy and thyrotoxicosis (Goldspink, 1976; Goldspink & Goldspink, 1977; Goldberg *et al.*, 1977). Our knowledge of the factors influencing the rates of this process exceeds our understanding of its mechanism. Muscle contains many proteinases, both lysosomal and non-lysosomal, that could degrade cell protein *in vivo* (Pennington, 1977; Bird, 1975). Both lysosomal and non-lysosomal proteinases seem to participate in breakdown of proteins in other cells (Knowles & Ballard, 1976; Hopgood *et al.*, 1977; Etlinger & Goldberg, 1977; Neff *et al.*, 1979). However, in muscle the role of any given proteinase or organelle in the breakdown of average cell protein is unknown.

One approach to identification of the enzymes responsible for intracellular protein catabolism uses proteinase inhibitors of restricted specificity as

probes. For example, leupeptin, a peptide-aldehyde produced by actinomycetes that inhibits the lysosomal proteinase cathepsin B in intact muscles, slows proteolysis in isolated muscles (Libby & Goldberg, 1978). The present study investigates the effects of the related proteinase inhibitor chymostatin on proteolysis in intact muscles and on proteinases in muscle homogenates. One goal of the present study was to identify additional agents that can inhibit protein breakdown in intact cells without producing toxic effects. Such compounds might be useful in retarding muscle atrophy *in vivo* and in helping to maintain tissues in culture. In fact, when cultured with leupeptin, dystrophic muscle cells appear to degenerate more slowly (McGowan *et al.*, 1976; Stracher *et al.*, 1978), and foetal mouse hearts undergo atrophy to a lesser extent (Libby *et al.*, 1979).

Materials and Methods

The antibiotic proteinase inhibitors used in the present studies were the gifts of Professor H.

Umezawa (Institute of Microbial Chemistry, Shinagawa-Ku, Tokyo, Japan) and the U.S.-Japan Cancer program (c/o Dr. Walter Troll, New York University Medical Centre, New York, NY, U.S.A.). Chymostatin and pepstatin were dissolved in dimethyl sulphoxide and subsequently diluted with buffer before use. Controls always contained the same concentration of solvent (0.2%). [^{14}C]phenylalanine (460 mCi/mmol) was obtained from Schwartz-Mann (Orangeburg, NY, U.S.A.). The cathepsin B substrate was purchased from Enzyme Systems Products (Livermore, CA, U.S.A.). Male rats (strain CD) were obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.). Male C57 Bl6 mice homozygous for the *dy*² allele were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.), and were studied 5–7 weeks after birth.

Studies on protein turnover in intact muscles

Rodent muscles were incubated at 37°C in Krebs-Ringer bicarbonate (Umbreit *et al.*, 1964) buffer equilibrated with O₂/CO₂ (19:1), supplemented with glucose (10 mM), insulin (0.1 unit/ml) and in some cases amino acids at five times their normal rat plasma concentration (Fulks *et al.*, 1975). Under these conditions, rates of protein synthesis and degradation are constant for the period of study (Fulks *et al.*, 1975). Rates of protein breakdown were determined by measuring the net release of tyrosine from tissue protein with a fluorimetric assay (Fulks *et al.*, 1975; Waalkes & Udenfriend, 1957). This amino acid is neither synthesized nor degraded in muscle cells. Chymostatin cross-reacted weakly with tyrosine in this assay, and the fluorescence due to chymostatin was determined by blanks in each experiment and was subtracted from values reported. To measure protein degradation as an isolated process, we included cycloheximide (0.5 mM) in the medium to block protein synthesis. Net protein breakdown (i.e. the rate of protein degradation minus the rate of protein synthesis) was measured by tyrosine release in the absence of cycloheximide. These studies compared data obtained from five to seven paired contralateral muscles or from portions of the same atrium or diaphragm taken from littermate animals and incubated in parallel. Data obtained in this way showed much less variation than comparisons between groups of animals studied on different days. For reasons that are still unclear, rat muscles show significant day-to-day variations in rates of protein breakdown (Fulks *et al.*, 1975). The one-tailed Student *t* test was used for statistical comparisons with paired analysis when contralateral muscles or portions of the same muscle were studied.

Rates of protein synthesis were determined by measuring the incorporation of [^{14}C]phenylalanine

(200 $\mu\text{Ci}/\text{mmol}$) into material insoluble in 0.2 M-HClO₄. Non-radioactive phenylalanine (0.5 mM) was included in the medium to minimize the contribution of unlabelled phenylalanine generated by protein breakdown to the precursor pool for protein synthesis. Under these conditions the specific radioactivity of the precursor pool should approximate that of the medium (Li *et al.*, 1973; McKee *et al.*, 1978). Radioactivity was measured by liquid-scintillation spectroscopy with an automatic external standard to correct for quenching.

Preparation of muscle extracts and enzyme preparations

Crude homogenates of mixed hind-limb muscle of rats were prepared by washing the freshly excised tissues with NaCl solution (154 mM), placing them in buffer and disrupting them with four 30 s bursts of a Teckmar homogenizer at 70% full speed. The homogenates were then dialysed at least overnight. These procedures were performed at 4°C. For studies at acid pH, a phosphate/citrate buffer [100 mM (K₂HPO₄, 76 mM; citric acid, 24 mM), pH 6] containing 2-mercaptoethanol (5 mM) or dithiothreitol (0.5 mM) and EDTA (1 mM) was used. For studies at alkaline pH, we used a potassium phosphate buffer (50 mM, pH 7.8) containing KCl (100 mM). The Ca²⁺-activated proteinase was partially purified from rat skeletal muscle by the method of Dayton *et al.* (1976). In some experiments, rats were treated with the mast-cell-degranulating agent 48/80 (Pastan & Almqvist, 1966).

Enzyme and protein assays

Hydrolysis by dialysed preparations of endogenous proteins and casein was measured at 37°C by the production of primary amino groups soluble in trichloroacetic acid (10%, w/v) reactive with fluorescamine in borate/phosphate buffer (100 mM, pH 9), with leucine as a standard (Bohlen *et al.*, 1973). Cathepsin B was measured by monitoring the hydrolysis of the substrate benzyloxycarbonylalaninyl-arginylarginyl-4-methoxy- β -naphthylamine fluorimetrically; the phosphate/citrate buffer described above was used (Smith & Van Frank, 1975). The Ca²⁺-activated proteinase was assayed in Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (100 mM, pH 7.5, with 100 mM-KCl and 1 mM-NaN₃ at 25°C). The release of primary amines soluble in trichloroacetic acid (10%, w/v) was measured as described above during incubation with α -casein (20 mg/ml). Hydrolysis of acetyltyrosine ethyl ester was measured by the technique of Roberts (1958). Protein was determined by the biuret reaction or by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Effects of proteinase inhibitors on protein turnover in intact muscles

Chymostatin (20μM) decreased the rate of protein breakdown in the red soleus muscle, the predominantly white extensor-digitorum-longus muscle, the diaphragm, which contains a mixture of fibre types, and in atrial strips (Table 1a). The extent of the inhibition of proteolysis in the soleus muscle increased over the concentration range 0–20μM (Table 1b). In muscles incubated without cycloheximide, chymostatin also decreased net protein breakdown (i.e. the rate of protein breakdown minus the rate of protein synthesis; Table 2a). To evaluate possible non-specific toxic effects of chymostatin, we measured directly the rates of protein synthesis in rat hind-limb muscles incubated with this agent. Chymostatin did not alter the rate of [¹⁴C]phenylalanine incorporation (Table 2b). Since this agent decreased protein breakdown without decreasing protein synthesis it probably acts specifically by inhibiting one or more cell proteinases.

Chymostatin also decreased protein breakdown in muscles atrophying due to section of the sciatic nerve compared with the contralateral muscle denervated at the same time (Table 3a). The rate of protein breakdown increases in these muscles as a consequence of denervation (Goldspink, 1976). In muscles of mice affected by genetic muscular dystrophy (*dy*²), this agent also inhibited protein breakdown (Table 3b). In studies reported elsewhere, leupeptin produced comparable decreases in proteolysis in skeletal and atrial muscles from normal animals as well as denervated and dystrophic skeletal muscle (Libby & Goldberg, 1978).

Antipain also decreased protein breakdown in rat hind-limb muscles. In the extensor-digitorum-longus muscle, antipain (20μM) decreased the rate of this process from 0.249±0.014 to 0.189±0.014 nmol of tyrosine/2h per mg of tissue

Table 2. Effect of chymostatin on net protein breakdown and protein synthesis in muscle

Net protein breakdown was measured by net release of tyrosine from cell protein (in the absence of cycloheximide). Rates of protein synthesis were calculated from the rates of incorporation of [¹⁴C]-phenylalanine into material insoluble in HClO₄ (0.2M) in a medium containing unlabelled phenylalanine (0.5 mM). Molar rates of phenylalanine incorporation were calculated by using the specific activity of the medium as an estimate of the precursor-pool specific activity. Abbreviation used: N.S., not significant. Values are means ± S.E.M. for five to seven individual measurements.

Muscle	(a) Net protein breakdown (nmol of tyrosine/2h per mg)		
	Control	20μM-Chymo- statin-treated	P
Soleus	0.255 ± 0.011	0.143 ± 0.014	<0.01
	(b) Protein synthesis (nmol of phenylalanine/2h per mg)		
	Control	20μM-Chymo- statin-treated	P
Soleus	0.280 ± 0.018	0.286 ± 0.018	N.S.
Extensor-digitorum- longus muscle	0.239 ± 0.017	0.250 ± 0.015	N.S.

Table 1. Effect of chymostatin on protein breakdown in various rat muscles

Protein breakdown was measured by release of tyrosine from cell protein in the presence of cycloheximide (0.5 mM). (a) Similar results were observed in independent experiments in the diaphragm, soleus and extensor-digitorum-longus muscle. (b) Since each rat hind-limb contains one soleus muscle, we used contralateral solei from one group of seven animals for 0 and 8μM-chymostatin and paired solei from another group of seven animals for the other concentrations. The statistical analysis compared each group of inhibitor-treated muscles to the control group, the Student *t* test for independent observations being used. Values are means ± S.E.M. **P* < 0.01; ***P* < 0.05.

	Muscle	Chymostatin (μM)	Protein degradation (nmol of tyrosine/2h per mg)		Inhibition (%)
			Control	Chymostatin-treated	
(a)	Diaphragm	20	0.345 ± 0.016	0.256 ± 0.010*	25
	Soleus	20	0.348 ± 0.021	0.221 ± 0.027**	34
	Extensor-digitorum- longus muscle	20	0.271 ± 0.010	0.169 ± 0.014*	37
	Atrium	20	0.351 ± 0.014	0.248 ± 0.028*	29
(b)	Soleus	2	0.481 ± 0.031	0.407 ± 0.027	15
		8	0.481 ± 0.031	0.397 ± 0.024*	21*
		20	0.481 ± 0.031	0.304 ± 0.027**	37**

Table 3. *Effect of chymostatin on protein breakdown in denervated and dystrophic muscles*

(a) Bilateral sciatic-nerve division was performed on rats 24 h before measurements of protein breakdown in the paired contralateral denervated muscles, one incubated with chymostatin, the other serving as a control. These data are combined from two independent experiments each comparing seven pairs of extensor-digitorum-longus muscles and seven pairs of solei. (b) Dystrophic muscles were obtained from eight C57 Bl6J mice homozygous for the *dy*² allele. Values are means $\pm$ s.e.m. * $P < 0.01$; ** $P < 0.05$

	Protein degradation (nmol of tyrosine/2h per mg)		Change (%)
	Control	20 μ M-Chymostatin-treated	
(a) Denervated muscles (rat)			
Soleus	0.397 $\pm$ 0.034	0.307 $\pm$ 0.013*	23
Extensor-digitorum-longus muscle	0.405 $\pm$ 0.020	0.218 $\pm$ 0.011**	46
(b) Dystrophic muscles (mouse)			
Extensor-digitorum-longus muscle	0.521 $\pm$ 0.012	0.383 $\pm$ 0.019**	27
Plantaris	0.541 $\pm$ 0.007	0.441 $\pm$ 0.021*	19

($P < 0.05$). In another experiment a higher concentration of antipain (50 μ M) decreased this process from 0.362 ± 0.022 to 0.151 ± 0.018 in extensor-digitorum-longus muscle ($P < 0.01$) and from 0.581 ± 0.061 to 0.349 ± 0.053 nmol of tyrosine/2h per mg in solei ($P < 0.01$). However, this agent failed to slow proteolysis in rat diaphragms even at 100 μ M (results not shown). Since antipain lowered proteolysis in hind-limb muscles but not diaphragm, was less potent than leupeptin and chymostatin, and inhibits the same endoproteinases as leupeptin (Umezawa, 1976; Toyo-Oka *et al.*, 1978), we did not study it further. Other proteinase inhibitors including pepstatin (20–100 μ M), elastatinal (100 μ M) and bestatin (50 μ M), did not diminish protein breakdown in rat muscles (results not shown). Even when dimethyl sulphoxide (final concn. 2%, v/v) was included to facilitate its penetration, pepstatin failed to inhibit protein breakdown (results not shown). It was noteworthy that the dimethyl sulphoxide itself decreased this process by about 15% ($P < 0.05$). This effect may not reflect a specific action on the proteolytic process, since under these conditions, the solvent (2%, v/v) concomitantly decreased the rate of protein synthesis ($P < 0.05$; results not shown).

Effects of inhibitors on proteinase activities in cell-free preparations

Chymostatin, leupeptin and antipain have been reported to inhibit the lysosomal proteinase cathepsin B from certain other tissues (Umezawa, 1976). Since this lysosomal proteinase was a likely target for inhibition by these agents, we measured its activity with the specific substrate, benzyloxycarbonylalanylarginylarginyl-4-methoxy- β -naphthylamine (Smith & Van Frank, 1975). Muscle extracts prepared in phosphate/citrate buffers cleaved this substrate with an optimum around pH 6 (results not shown). This activity was stimulated by

Table 4. *Effects of modifiers on cathepsin B activity of skeletal-muscle homogenate (pH 6)*

Cathepsin B activity was measured by using the fluorogenic substrate benzyloxycarbonylalanylarginylarginyl-4-methoxy- β -naphthylamine in phosphate/citrate buffer (100 mM, pH 6, containing 1 mM-EDTA and 0.5 mM-dithiothreitol). Under the last two listed conditions, dithiothreitol was removed from the muscle extracts by dialysis, and this agent was omitted from the buffer used for the assay.

Agent	Concn. (μ M)	Percentage of control activity
Leupeptin	1	2
	0.03	66
Chymostatin	2	43
Antipain	2	9
Elastatinal	2	47
Pepstatin	1.5	100
Phosphoramidon	2	100
Minus dithiothreitol		34
Minus dithiothreitol, plus iodoacetate	10	3

dithiothreitol (1 mM) and inhibited by iodoacetate (10 μ M, Table 4); thus it resembled cathepsin B from other sources (Barrett, 1977). Leupeptin (30 nM) inhibited this activity by 66% and virtually completely at 1 μ M (Table 4). Chymostatin, antipain and elastatinal inhibited cathepsin B in this cell-free preparation less effectively than leupeptin (Table 4).

Other experiments tested the effects of chymostatin and leupeptin on hydrolysis of endogenous proteins in cell-free homogenates of muscle at various pH values. At pH 5, chymostatin (3 μ M) did not affect this process in extracts of cardiac or skeletal muscle (results not shown). Under these conditions, leupeptin inhibited proteolysis in extracts of both cardiac and skeletal muscle by up to 40%, but this effect was variable and not present in all

preparations (results not shown). Failure of these agents to diminish acid proteolysis in muscle homogenates consistently, even though they inhibit cathepsin B, might be due to the presence of various amounts of endogenous macromolecular inhibitors of cathepsin B (Schwartz & Bird, 1977; Lenny *et al.*, 1979). Such inhibitors may prevent hydrolysis of protein substrates without interfering with cleavage of small synthetic substrates (Starkey & Barrett, 1973).

A Ca^{2+} -activated proteinase exists in muscle that catalyses removal of the Z-disc from the sarcomere *in vitro* (Reddy *et al.*, 1975; Dayton *et al.*, 1976). An endogenous inhibitor also prevents assay of this enzyme in crude muscle homogenates, and no specific substrate for this enzyme is currently known (Dayton *et al.*, 1976; Waxman & Krebs, 1978). To determine whether chymostatin inhibits this activity, we partially purified the enzyme from rat skeletal muscle (Dayton *et al.*, 1976). Ca^{2+} and dithiothreitol enhanced, and iodoacetate inhibited, this activity (Table 5), which thus resembled the enzyme found in muscles of other species. Both chymostatin ($2\mu\text{M}$) and leupeptin ($2\mu\text{M}$) inhibited most of this activity (Fig. 1). Toyo-Oka *et al.* (1978) have since reported that antipain and leupeptin also inhibit the Ca^{2+} -activated proteinase from bovine heart. Therefore this calcium-activated proteinase is another possible site of action of these agents.

Rat skeletal-muscle homogenates exhibit appreciable proteolytic activity at alkaline-pH values (Koszalka & Miller, 1960). Several groups have proposed that this alkaline activity, which may consist of one or more enzymes, plays a role in breakdown of myofibrillar protein (Noguchi & Kandatsu, 1971; Mayer *et al.*, 1974; Dahlmann & Reinauer, 1978). The so-called 'group-specific proteinase' in skeletal muscle resembles this activity as well (Banno *et al.*, 1975). Leupeptin, antipain,

pepstatin and phosphoramidon had little or no effect on hydrolysis of endogenous proteins in skeletal-muscle homogenates at pH 7.8 (results not shown). However, chymostatin ($3\mu\text{M}$) inhibited alkaline autolysis of muscle by 60–80% (Fig. 2). To define this activity further we tested its sensitivity to a number of other protease inhibitors of known specificity. Toluenesulphonyl (tosyl) (1 mM) inhibited most of this alkaline proteolysis (Fig. 2). Therefore this activity probably contains a serine residue in its active site. The site-directed affinity inhibitor 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK'; 1 mM) decreased alkaline autolysis more than 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'; 10 mM; Fig. 2). Thus this activity shows more affinity for aromatic than basic residues, a property shared by chymotrypsin. Soya-bean trypsin inhibitor ($50\mu\text{g/ml}$) inhibited proteolysis by muscle homogenates at pH 7.8 as well (Fig. 2). Muscle extracts also exhibited chymostatin-sensitive cleavage of acetyltyrosine ethyl ester, a chymotrypsin substrate (Table 6). These properties are similar to

Table 5. Effect of modifiers on the Ca^{2+} -activated proteinase of rat skeletal muscle

This activity was measured by monitoring hydrolysis of α -casein (20 mg/ml) at pH 7.5 by a fraction prepared from rat skeletal muscle by differential centrifugation and isoelectric precipitation (Dayton *et al.*, 1976).

Presence (+) or absence (–) of:				Relative activity
Modifiers	Ca ²⁺ (5 mM)	Dithio- threitol (1.4 mM)	Iodo- acetate (1 mM)	
...	–	–	–	1
	+	–	–	2.2
	+	+	–	6.0
	–	+	–	1.8
	+	–	+	0.9

Fig. 1. Effect of proteinase inhibitors on Ca^{2+} -activated neutral-proteinase activity in rat muscle

The fraction studied was prepared by differential centrifugation and isoelectric precipitation under the conditions used by Dayton *et al.* (1976). The casein-hydrolytic activity in this fraction was activated by Ca^{2+} and by a thiol-reducing agent (Table 5). The concentration of chymostatin and leupeptin was $3\mu\text{M}$. An independent experiment gave similar results.

Fig. 2. Effect of proteinase inhibitors on alkaline autolysis by skeletal-muscle homogenate

Autolysis was measured at pH 7.8. The concentration of 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (TLCK), 1-chloro-4-phenyl-3-L-tosylamido-butan-2-one (TPCK) and tosyl fluoride (TSF) was 1 mM. Soya-bean trypsin inhibitor (STI) was studied at 50 µg/ml. The concentration of chymostatin (CH) was 2 µM. 1-Chloro-4-phenyl-3-L-tosylamido-butan-2-one, tosyl fluoride and chymostatin were dissolved in dimethyl sulphoxide before dilution to a final concentration of 1% (v/v). This concentration of dimethyl sulphoxide did not itself affect the rate of autolysis. An independent experiment on the same preparation yielded similar results.

those described for the alkaline proteolytic activity in rat muscle by others (Noguchi & Kandatsu, 1971; Holmes *et al.*, 1971; Mayer *et al.*, 1974; Banno *et al.*, 1975).

This chymotrypsin-like activity in muscle may occur predominantly in mast cells rather than in myocytes. Park *et al.* (1973) showed that treatment of rats with the mast-cell-degranulating agent 48/80 (a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde) substantially decreased this activity. We confirmed this result, and showed that this treatment *in vivo* diminished casein hydrolysis at pH 7.8 by homogenates of both red and white skeletal muscle of rats (Table 6). Furthermore, treatment with agent 48/80 *in vivo* decreased the chymostatin-sensitive alkaline autolytic and esterolytic activity in muscle homogenates (Table 6). This treatment did not, however, decrease the specific activity of the lysosomal proteinase cathepsin B (results not shown). We then tested whether

chymostatin could still decrease protein breakdown in muscles from agent-48/80-treated animals. Despite marked depletion of chymostatin-sensitive proteinase (Table 6), protein breakdown still occurred at normal rates and the inhibitor still decreased protein breakdown in the isolated muscles. For example, chymostatin (20 µM) decreased this process in extensor-digitorum-longus muscle from agent-48/80-treated rats from 0.247 ± 0.010 to 0.176 ± 0.007 nmol of tyrosine/2h per mg (data combined from 17 pairs of extensor-digitorum-longus muscle studied in three independent experiments; $P < 0.01$). These effects are very similar to those observed in muscles of control rats (Table 1).

Discussion

One goal of the present study was to identify enzymes responsible for protein breakdown in mammalian muscle. We found that chymostatin inhibits this process without altering the rate of protein synthesis. Since this agent did not seem to be toxic, it probably decreased protein breakdown by inhibiting one or more cell proteinases. Both chymostatin and leupeptin inhibited the lysosomal proteinase cathepsin B in muscle extracts (Table 4). This result, and those of other studies, are consistent with a role for this or related lysosomal proteinases such as cathepsin L in protein breakdown in intact muscles (Libby & Goldberg, 1978; Kirschke *et al.*, 1977). Chymostatin, leupeptin and antipain also inhibited the Ca^{2+} -activated proteinase found in rat skeletal muscle (Fig. 1; Toyo-Oka *et al.*, 1978). Inhibition of this enzyme may also contribute to the effects of these agents on protein breakdown in intact cells.

In other experiments in cultured embryonic chick muscle, we found that leupeptin, chymostatin and antipain inhibited degradation of a specific muscle protein, the acetylcholine receptor (Libby *et al.*, 1980). These effects of chymostatin and leupeptin were not additive, which indicates that these agents may act on the same enzyme or on enzymes in the same pathway. Observations in myotubes strongly suggested a lysosomal site of action of these inhibitors, since degradation of the acetylcholine receptor probably occurs in the structure (Devreotes & Fambrough, 1976; Libby *et al.*, 1980).

Chymostatin, unlike leupeptin or antipain, is also a potent inhibitor of most of the alkaline proteolytic activity apparent in rat muscle homogenates. We considered the possibility that this agent might also decrease protein breakdown in the intact muscle by inhibiting this activity. Many investigators have characterized alkaline proteinase(s) in muscle (Koszalka & Miller, 1960; Noguchi & Kandatsu, 1971; Holmes *et al.*, 1971; Mayer *et al.*, 1974;

Table 6. *Effect of treatment in vivo with agent 48/80 on chymotrypsin-like activity in rat muscles*

Rats were treated with intraperitoneal injections of agent 48/80 in increasing doses over 5 days before the rats were killed (Pastan & Almqvist, 1966). The muscles were then excised and homogenized. This homogenate was dialysed and the indicated assay performed was measured. Autolysis (hydrolysis of endogenous proteins) and acetyltyrosine ethyl ester hydrolysis were measured in Hepes buffer (pH 7.8, 20mM) containing 1mM-EDTA. Hydrolysis of α -casein (plasmin-free, 5mg/ml) was measured in Hepes buffer (pH 7.8, 50mM). Proteolysis occurred at a constant rate under the conditions of these experiments. All of the values below are means $\pm$ s.e.m. for duplicate determinations.

		Autolysis (nmol of leucine equivalents/ 2 h per mg of protein)	
		Control	Agent-48/80-treated
Pooled hindquarter muscles	—Chymostatin	47 $\pm$ 1	13 $\pm$ 0.4
	+Chymostatin (3 μ M)	18 $\pm$ 0.1	6 $\pm$ 0.1
		Acetyltyrosine ethyl ester hydrolysis (μ mol/h per mg of protein)	
		Control	Agent-48/80-treated
Pooled hindquarter muscles	—Chymostatin	0.59 $\pm$ 0.10	0.20 $\pm$ 0.01
	+Chymostatin (3 μ M)	0.22 $\pm$ 0.05	0.13 $\pm$ 0.02
		Casein hydrolysis (nmol of leucine equivalents/ 4 h per mg of protein)	
		Control	Agent-48/80-treated
Soleus	—Chymostatin	114 $\pm$ 21	49 $\pm$ 3
Extensor-digitorum-longus muscle	—Chymostatin	97 $\pm$ 1	26 $\pm$ 2

Banno *et al.*, 1975; Dahlmann & Reinauer, 1978; Woodbury *et al.*, 1978*b*; Pennington, 1977). Several groups have suggested an important role for these alkaline activities in intracellular protein breakdown, since they have activity at physiological pH, and are found associated with myofibrils in muscle homogenates. However, there is increasing evidence that the chymotrypsin-like activity in muscle occurs in mast cells rather than myocytes. As shown by Park *et al.* (1973) and confirmed in the present experiments (Table 6), treatment of rats with an agent that discharges mast-cell granules lowered this activity. Immunofluorescent and biochemical data also support a mast-cell origin and localization of this enzyme (Woodbury *et al.*, 1978*a,b*). Isolated cardiac myocytes contain little of this enzyme (Clark *et al.*, 1980).

We found that muscles largely depleted of this activity after treatment with agent 48/80 still exhibit decreased protein breakdown when treated with chymostatin. For example, in the extensor-digitorum-longus muscle depleted of 70% of its alkaline-proteinase activity (Table 6), chymostatin still decreased protein breakdown by about 30%, which is similar to the inhibition seen in muscles from control rats (Table 1). One cannot exclude the possibility that a minor pool of chymostatin-sen-

sitive alkaline proteinase could exist within the myocyte and play an important role in protein breakdown. However, the simplest interpretation of these various results (Table 6; Park *et al.*, 1973; Woodbury *et al.*, 1978*a,b*; Clark *et al.*, 1980) would be that the muscle chymotrypsin-like activity does not contribute significantly to breakdown of average proteins in muscle, and that chymostatin, in common with leupeptin, decreases protein breakdown by inhibiting lysosomal enzyme(s) and/or the Ca²⁺-activated proteinase.

Since chymostatin does not seem to damage muscles at concentrations that inhibit overall protein breakdown, it may be of value in future studies of the mechanism of this process in other tissues as well. For example, one could use this or related agents as a probe to study the pathway of degradation of specific proteins. Previous work has used related proteinase inhibitors to study the pathway of degradation of proteins with normal or abnormal structures (Knowles & Ballard, 1976; Hopgood *et al.*, 1977; Neff *et al.*, 1979). Since chymostatin and leupeptin retard the breakdown of protein in muscles atrophying after denervation or as a result of genetic muscular dystrophy, further studies should evaluate their potential for slowing atrophy of muscles *in vitro* and *in vivo*.

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