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Differential effects of acute changes in cell Ca^{2+} concentration on myofibrillar and non-myofibrillar protein breakdown in the rat extensor digitorum longus muscle *in vitro*

Assessment by production of tyrosine and N^{τ}-methylhistidine

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The influence of Ca^{2+} on myofibrillar proteolysis was evaluated in the isolated extensor digitorum longus muscle incubated *in vitro* with agents previously shown to increase the intracellular concentration of Ca^{2+}. Myofibrillar proteolysis was evaluated by measuring the release of N^{τ}-methylhistidine, and total proteolysis was evaluated by measuring tyrosine release by incubated muscles after the inhibition of protein synthesis with cycloheximide. Incubated muscles released measurable quantities of N^{τ}-methylhistidine, and muscle contents of the amino acids remained stable over 2 h of incubation. The release of N^{τ}-methylhistidine by incubated muscles was similar to its release by perfused rat muscle in response to brief starvation, indicating the integrity of the incubated muscles. Ca^{2+} ionophore A23187, dibucaine, procaine, caffeine and elevated K^+ concentration increased lactate release by incubated muscles and decreased tissue contents of ATP and phosphocreatine to varying degrees, indicating the metabolic effectiveness of the agents tested. Only A23187 and dibucaine increased total cell Ca^{2+}, and they increased tyrosine release. Caffeine and elevated [K^+] increased neither cell Ca^{2+} nor tyrosine release; however, only A23187 and dibucaine increased tyrosine release significantly. On the other hand, these agents were without effect on myofibrillar proteolysis as assessed by N^{τ}-methylhistidine release by incubated muscles and changes in tissue contents of the amino acid. In fact, some of the agents tested tended to decrease myofibrillar proteolysis slightly. These results indicate that acute elevation of intracellular Ca^{2+} is associated with increased breakdown of non-myofibrillar but not myofibrillar proteins. Because of this, the role of elevated Ca^{2+} in muscle atrophy in certain pathological states is questioned. The data also indicate that the breakdown of myofibrillar and non-myofibrillar proteins in muscle is regulated independently and by different pathways, a conclusion reached in previous studies with perfused rat muscle.

INTRODUCTION

Insulin, amino acids and agents that inhibit lysosomal proteinase activity can attenuate the breakdown of protein in skeletal muscle (Fulks *et al.*, 1975; Jefferson *et al.*, 1977; Libby & Goldberg, 1978; Jenkins *et al.*, 1979; Tischler, 1981; Clark *et al.*, 1984). Previous studies have assessed the effects of these agents on muscle proteolysis by measuring the release of either tyrosine or phenylalanine by perfused or incubated muscles. Although this procedure is a good reflection of total protein breakdown, it does not distinguish between the breakdown of myofibrillar and non-myofibrillar proteins. Because of this, we re-evaluated the effects of these agents on myofibrillar and non-myofibrillar proteolysis by measuring simultaneously the release of both tyrosine and N^{τ}-methylhistidine by perfused rat muscle of fed and starved rats (Lowell *et al.*, 1986*a*,*b*). When this procedure is used, the release of tyrosine in the presence of cycloheximide reflects total proteolysis, whereas that of N^{τ}-methylhistidine reflects the breakdown of myofibrillar proteins, i.e. actin and myosin (Lowell *et al.*, 1986*b*). We found that although insulin, insulin plus amino acids or lysosomal proteinase inhibitors (i.e. leupeptin, chloroquine and NH_4Cl) decreased tyrosine release by perfused rat muscle, neither attenuated the release of N^{τ}-methylhistidine. These results suggested that the breakdown of myofibrillar and non-myofibrillar proteins can be regulated independently and that the breakdown of myofibrillar proteins may be regulated by a non-lysosomal pathway. They also suggested that Ca^{2+} or a Ca^{2+}-activated proteinase may not regulate myofibrillar protein breakdown, since leupeptin, which inhibits not only lysosomal cathepsins B and L (Kirschke *et al.*, 1976) but also a cytosolic Ca^{2+}-activated neutral proteinase (Toyo-Oka *et al.*, 1978), failed to decrease N^{τ}-methylhistidine release under stimulated conditions. However, we could not rule out a role of Ca^{2+}.

The aim of the present work was to test further the influence of Ca^{2+} on myofibrillar protein breakdown in skeletal muscle. For this purpose, Ca^{2+} flux in muscle was perturbed by using Ca^{2+} ionophore A23187, local anaesthetics, caffeine or elevation of extracellular K^+ concentration, manipulations that have been shown previously to increase or change the redistribution of intracellular Ca^{2+} (Lewis *et al.*, 1982; Sugden, 1980). Previous studies have reported that acute increases of intracellular Ca^{2+} in skeletal muscle can increase proteolysis (Kameyama & Etlinger, 1979; Sugden, 1980; Lewis *et al.*, 1982; Rodemann *et al.*, 1982; Zeman *et al.*, 1985). However, it has not been determined if this response reflects an increased breakdown of all classes of proteins, as past studies measured only release of

tyrosine or phenylalanine. To evaluate this, we have measured the release of both tyrosine and N^{τ}-methylhistidine from skeletal muscle after inhibition of protein synthesis with cycloheximide in the presence or absence of agents that modify Ca^{2+} flux. In past studies, we have evaluated myofibrillar proteolysis by measuring the release of N^{τ}-methylhistidine by perfused rat muscle (Lowell *et al.*, 1986*b*). This preparation could not be used in the present study, since agents such as Ca^{2+} ionophore A23187 increase vascular resistance and diminish blood flow to muscles. Therefore the present studies utilized muscles from young rats incubated *in vitro*. The first part of this paper characterizes the use of this preparation for studies of N^{τ}-methylhistidine release, and the second part describes the effects of Ca^{2+}-mobilizing agents on myofibrillar as well as total proteolysis.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (60–70 g), obtained from Charles River Breeding Laboratory, Willmington, MA, U.S.A., were used. The rats were either fed on Purina Laboratory chow *ad libitum* or deprived of food overnight (16 h) before isolation of muscles. All rats were allowed water *ad libitum*. Animals were housed in animal quarters maintained at 22 °C with a 12 h-light/12 h-dark cycle.

Dissection procedures

The dissection and isolation of the extensor digitorum longus muscle was carried out under anaesthesia with pentobarbital (5–7 mg/100 g body wt., intraperitoneally) as described in detail previously (Maizels *et al.*, 1977). The isolated extensor digitorum longus muscle was fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating to resting length) during the incubation. Such muscles are able to maintain normal ATP and phosphocreatine concentrations during a 2–3 h incubation (see Table 2).

Incubations

Once attached to the clips, muscles were transferred to 20 ml glass scintillation vials which contained 3 ml of Krebs–Henseleit (1932) bicarbonate solution with 2.5 mM-Ca^{2+} [previously gassed with O_2/CO_2 (19:1), pH 7.4], 0.13% albumin, 6 mM-glucose and 0.5 mM-cycloheximide. Other additions are described in Table legends. One muscle was placed in each vial so that control and experimental incubations could be compared from the same rat. (As these studies were conducted during the period of 1 year, this approach minimized differences in both tyrosine and N^{τ}-methylhistidine release that were encountered at different times of the year. This approach also minimized any differential effects of overnight starvation on individual rats.) After addition of the muscles to the vials, they were flushed with O_2/CO_2 (19:1), re-stoppered and placed in a Dubnoff metabolic shaker set at 35 °C and a shaking rate of 80 cycles/min. After preincubation for 1 h, muscles were removed, blotted and transferred to a second set of vials containing fresh media identical in composition and volume, and incubated for a further 2 h. All measurements were made during this incubation.

At the end of each incubation, muscles (while still attached to the clips) were removed from the incubation flasks, quickly rinsed in cold saline (0.9% NaCl) and blotted and then dropped into liquid N_2. Muscles were stored in liquid N_2 until analysis. Samples of incubation medium (2.5 ml) were added to 0.2 ml of ice-cold 30% (v/v) $HClO_4$ for deproteinization. The samples were then centrifuged (4 °C) at 200 *g* for 20 min and the supernatants frozen at −20 °C until analysed.

Analytical methods

Tissue specimens were removed from the clips, quickly weighed in a test tube pre-cooled in a methanol/solid-CO_2 bath and homogenized in 3 ml of ice-cold 3% (v/v) $HClO_4$. The muscle samples were then centrifuged (4 °C) at 2000 *g* for 20 min. A portion of the resulting supernatant was neutralized with a solution of 2 M-KOH containing 0.5 M-triethanolamine, and the precipitated $KClO_4$ was removed by centrifugation (4 °C) at 2000 *g* for 20 min. A portion of the $HClO_4$ extract of the incubation medium was also neutralized similarly. ATP, phosphocreatine and lactate in the neutralized tissue extracts and lactate in the neutralized incubation-medium extracts were determined as described previously (Maizels *et al.*, 1977). Tyrosine in $HClO_4$ extracts of tissue or incubation-medium samples was determined by the method of Waalkes & Udenfriend (1957). Muscle Ca^{2+} content was measured by atomic-absorption spectroscopy as described by Sugden (1980).

The concentration of N^{τ}-methylhistidine in $HClO_4$ extracts of incubation medium and tissue was assayed by h.p.l.c., by a modification of the procedure described by Wassner *et al.* (1980). To enhance its sensitivity and reproducibility, their method was altered by changing the concentration of the borate buffer and by modifying the amounts of other reagents used. Briefly, 500 μl of sodium borate (0.2 M, pH 10) was added to a glass screw-top vial containing 200 μl of standard or $HClO_4$ extracts of medium or tissue. With continuously vortex-mixing, 500 μl of acetonitrile containing fluorescamine (1.6 mg/ml) was added slowly, and 5 min later, 175 μl of 60% $HClO_4$ was added, vortex-mixed, and the vials were placed into an 80 °C water bath for 1 h. After cooling, the mixtures were neutralized to pH 5.5–6.5 with 3 M-NaOH/0.5 M-Mops reagent. Immediately before analysis, the assay mixtures were passed through a 0.45 μm Millex Millipore filter. Samples (50 or 100 μl) of the fluorescamine derivatives were separated isocratically on an ASI (Santa Clara, CA, U.S.A.) C-18 column with 23% (v/v) acetonitrile in water. With a flow rate of 1.5 ml/min, N^{τ}-methylhistidine was eluted after 14 min. Concentrations of N^{τ}-methylhistidine were determined by comparing peak areas of samples with that of an external standard (0.2 μM). A Farrand Ratio Fluorometer with a 100 μl flow cell was used for detection. Excitation and emission wavelengths were 365 and 460 nm respectively. Routinely, 0.2–0.5 pmol of N^{τ}-methylhistidine was assayed against a standard of 1 pmol. When the precision of the assay was tested, samples assayed in duplicate were within 3% of each other.

Materials

Chemicals were reagent grade and were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., or Fisher Scientific Co., Boston, MA, U.S.A. Enzymes were obtained from Boehringer–Mannheim, Indianapolis, IN,

U.S.A. Bovine serum albumin was obtained from Miles Laboratories, Kankakee, IL, U.S.A., and was dissolved in Krebs–Henseleit (1932) bicarbonate medium and dialysed against 4 litres of the same solution for 24 h. Calcium ionophore A23187 (Sigma) was dissolved in ethanol before addition (5 μl) to the incubation medium. Control vials received 5 μl of ethanol. Other agents were added in powdered form directly to batches of incubation medium.

Statistics

Student's *t* test (paired) was used for statistical analysis of the data.

RESULTS

N^{τ}-Methylhistidine release by incubated muscles

Myofibrillar protein breakdown has been evaluated previously by measuring the release of N^{τ}-methylhistidine by perfused rat skeletal muscle (Wassner & Li, 1982; Lowell *et al.*, 1986*a*,*b*). No previous report has described the use of individual muscles incubated *in vitro* for this purpose. Therefore, our initial studies focused on characterizing the use of incubated muscles for measurement of myofibrillar proteolysis and comparison of these results with those reported previously with perfused rat muscle. The initial incubation medium contained no detectable amounts of N^{τ}-methylhistidine. After incubation for 2 h in the absence of cycloheximide with one extensor digitorum longus muscle from a fed or overnight-starved rat ($n = 6$), the concentration of N^{τ}-methylhistidine in the incubation medium rose to an average of 27 ± 2 or 52 ± 4 nM respectively. As shown in Table 1, overnight starvation resulted in a 135% increase in N^{τ}-methylhistidine release. A similar result was obtained when 0.5 mM-cycloheximide was present in the medium, indicating that at this concentration it did not alter the rate of myofibrillar proteolysis during a 3 h incubation. In these same experiments, ovenight starvation also increased tyrosine release by an amount which depended on whether or not cycloheximide was included in the medium. The release of N^{τ}-methylhistidine (as well as tyrosine) was not due simply to a wash-out from the free intracellular pool within the muscle, as its concentration remained reasonably stable during the 2 h incubation. These results compare qualitatively with those from perfused muscle of young 100 g rats (Table 1), in which starvation for 24 h increased N^{τ}-methylhistidine release by 320%.

Metabolic effectiveness of Ca^{2+}-mobilizing agents in incubated muscles

Ca^{2+} ionophore A23187 and the local anaesthetic dibucaine have been reported to increase intracellular Ca^{2+} in incubated muscles (Lewis *et al.*, 1982; Sugden, 1980). This has been confirmed in the present study. A23187 and dibucaine increased cell Ca^{2+} content by 50–60%, whereas caffeine and KCl did not produce a detectable increase in total cell Ca^{2+} [control, A23187 (10 μM), dibucaine (500 μM), caffeine (4 mM), KCl (15 mM): 1.52 ± 0.15, 2.28 ± 0.23, 2.43 ± 0.24, 1.50 ± 0.15 and 1.45 ± 0.13 μmol/g wet wt. respectively ($n = 4$)]. Although caffeine and KCl failed to increase total cell Ca^{2+}, it is likely that they changed the redistribution of Ca^{2+} within the cell so as to increase the free cytosolic concentration (Clusin, 1985). To ascertain further the metabolic effectiveness of the various agents, we reasoned that if the free concentration of cellular Ca^{2+} was increased it would (1) activate myosin ATPase, leading to a decrease in muscle ATP and phosphocreatine, and/or (2) activate phosphorylase, resulting in glycogen breakdown, enhanced glycolysis and formation of lactate. As shown in Table 2, all of the agents tested, as well as the omission of Ca^{2+}, increased lactate formation by 11–72%, with ionophore A23187 being the most and dibucaine the least effective. These treatments, except for procaine addition and Ca^{2+} omission, also decreased tissue ATP and phosphocreatine to varying degrees, with ionophore A23187 and dibucaine producing the most marked changes. Although each agent increased lactate formation, and most decreased tissue ATP and phosphocreatine, there was no high correlation between the two. For instance, dibucaine produced the most marked decrease in tissue ATP and

Table 1. N^{τ}-Methylhistidine release by incubated extensor digitorum longus muscles of fed and overnight-starved rats

Results are means ± S.E.M. for the numbers of observations in parentheses. Muscles were incubated as described in Methods. Data for perfused rat muscle are taken from Lowell *et al.* (1986*a*). For measurement of tissue content, one muscle was removed after 1 h of preincubation (0 h) and the contralateral muscle was removed after 3 h of incubation (2 h). $*P < 0.05$ compared with appropriate fed group; $**P < 0.05$ compared with appropriate starved group.

Group	Cycloheximide	N^{τ}-Methylhistidine release (nmol/2 h per g wet wt.)	Tyrosine release (nmol/2 h per g wet wt.)	Tissue content (nmol/g wet wt.)			
				N^{τ}-Methylhistidine		Tyrosine	
				0 h	2 h	0 h	2 h
1. Incubated muscle							
Fed rats (6)	–	2.20±0.18	134±10	—	—	—	—
(6)	+	2.34±0.20	208±16	2.05±0.16	1.93±0.15	107±9	110±8
Overnight-starved rats							
(6)	–	5.18±0.21*	246±20*	—	—	—	—
(6)	+	5.20±0.25*	306±24*	5.53±0.44	5.10±0.41	114±9	132±11
2. Perfused muscle							
Fed rats (4)	+	1.62±0.20	308±20	—	—	—	—
24 h starved rats (4)	+	6.84±0.34	500±50	—	—	—	—

Table 2. Effect of Ca^{2+}-mobilizing agents on lactate release and tissue contents of ATP, phosphocreatine and lactate in incubated extensor digitorum longus muscles of fed and overnight-starved rats

Results are means ± S.E.M.; n = no. of rats per group. Muscles were incubated in the presence of cycloheximide as described in the Materials and methods section. From each rat, one muscle served as control, and the contralateral muscle served as the experimental. Since lactate release and tissue metabolites for control muscles were similar for each group, they were combined. †Ca^{2+} was omitted from and 1 mM-EGTA was added to the Krebs–Henseleit buffer. *$P < 0.05$ versus control group. ATP and phosphocreatine (μmol/g wet wt.) in muscles ($n = 6$) freeze-clamped *in situ* are respectively: fed rats, 5.05 ± 0.40 and 15.25 ± 1.22; overnight-starved rats, 5.76 ± 0.46 and 16.52 ± 1.32.

Group	Addition	n	Lactate release (nmol/2 h per g wet wt.)	Tissue content (μmol/g wet wt.)		
				Lactate	ATP	Phospho-creatine
1. Fed rats	None	12	11.0 ± 0.8	1.96 ± 0.16	4.71 ± 0.38	13.96 ± 1.12
	A23187 (10 μM)	6	15.4 ± 1.2*	3.92 ± 0.32*	3.75 ± 0.30*	9.09 ± 0.72*
	Dibucaine (500 μM)	6	13.2 ± 1.0	2.52 ± 0.20*	1.17 ± 0.09*	2.79 ± 0.22*
2. Overnight-starved rats	None	36	15.2 ± 1.2	1.78 ± 0.14	5.26 ± 0.26	15.02 ± 1.20
	EGTA (1 mM)†	6	21.2 ± 1.7*	2.40 ± 0.19*	5.47 ± 0.44	16.64 ± 1.33
	A23187 (10 μM)	6	26.2 ± 2.0*	4.24 ± 0.34*	1.74 ± 0.14*	2.68 ± 0.21*
	Dibucaine (500 μM)	6	16.8 ± 1.4	1.96 ± 0.16	1.16 ± 0.09*	2.52 ± 0.20*
	Procaine (500 μM)	6	20.4 ± 1.6*	2.39 ± 0.19*	5.94 ± 0.48	14.37 ± 1.15
	Caffeine (4 mM)	6	21.8 ± 1.6*	2.97 ± 0.24*	3.24 ± 0.26*	8.12 ± 0.65*
	KCl (15 mM)	6	22.8 ± 1.8*	2.61 ± 0.21*	4.38 ± 0.21*	12.38 ± 0.61*

Table 3. Effect of Ca^{2+}-mobilizing agents on release of N^{τ}-methylhistidine and tyrosine by incubated extensor digitorum longus muscles of fed and overnight-starved rats

Results are means ± S.E.M., for six rats per group. Muscles were incubated in the presence of cycloheximide as described in the Materials and methods section. From each rat, one muscle served as control, and the contralateral muscle served as the experimental. *$P < 0.05$ versus control group. †Ca^{2+} was omitted from and 1 mM-EGTA was added to the Krebs–Henseleit buffer.

Group		Tyrosine release (nmol/2 h per g wet wt.)	N^{τ}-Methylhistidine release (nmol/2 h per g wet wt.)	Tissue content (nmol/g wet wt.)	
				Tyrosine	N^{τ}-Methylhistidine
1. Fed rats	(*a*) Control	196 ± 10	2.10 ± 0.11	107 ± 5	1.76 ± 0.09
	A23187 (10 μM)	246 ± 12*	2.02 ± 0.10	126 ± 6*	1.61 ± 0.08
	% change	+26	−4	+18	−9
	(*b*) Control	242 ± 12	2.12 ± 0.11	124 ± 6	1.93 ± 0.10
	Dibucaine (500 μM)	358 ± 18*	2.72 ± 0.14*	205 ± 10*	1.21 ± 0.06*
	% change	+48	+28	+65	−37
2. Starved rats	(*a*) Control	346 ± 17	4.50 ± 0.23	103 ± 8	4.84 ± 0.24
	EGTA (1 mM)†	280 ± 14*	4.40 ± 0.22	108 ± 9	4.60 ± 0.23
	% change	−19	−2	+5	−5
	(*b*) Control	302 ± 15	5.54 ± 0.28	125 ± 10	4.50 ± 0.36
	A23187 (10 μM)	364 ± 18*	5.38 ± 0.27	130 ± 11	4.35 ± 0.35
	% change	+21	−3	+4	−3
	(*c*) Control	424 ± 21	4.66 ± 0.23	133 ± 7	4.70 ± 0.23
	Dibucaine (500 μM)	628 ± 31*	6.82 ± 0.34*	258 ± 13*	2.79 ± 0.14*
	% change	+48	+46	+94	−41
	(*d*) Control	299 ± 15	5.43 ± 0.27	116 ± 9	4.51 ± 0.25
	Procaine (500 μM)	210 ± 11*	5.31 ± 0.42	124 ± 10	4.76 ± 0.30
	% change	−30	−2	+7	+6
	(*e*) Control	300 ± 15	4.92 ± 0.25	—	—
	Caffeine (4 mM)	320 ± 16	4.26 ± 0.21	—	—
	% change	+7	−13		
	(*f*) Control	272 ± 14	5.40 ± 0.27	—	—
	KCl (15 mM)	298 ± 15	5.31 ± 0.40	—	—
	% change	+10	−2		

phosphocreatine, but it was the least effective in increasing lactate formation. Overall, these results indicate that each agent tested was metabolically effective, presumably due, in part, to a change in intracellular Ca^{2+}. It is also possible that changes in the flux of other ions may be responsible for some of these changes.

Influence of Ca^{2+}-mobilizing agents on tyrosine and N^{τ}-methylhistidine release by incubated muscles

As shown in Table 3, Ca^{2+} ionophore A23187, dibucaine, caffeine and KCl increased the release of tyrosine (i.e total proteolysis) by incubated muscles by 7–48%; however, only A23187 and dibucaine increased tyrosine release significantly, which correlated with the increase in cell Ca^{2+} that they induced. The addition of procaine decreased tyrosine release, as did the omission of calcium from the incubation buffer. Myofibrillar proteolysis, on the other hand, was unrelated to tyrosine release, for, when the release of N^{τ}-methylhistidine by incubated muscles and tissue changes were accounted for, there were no significant differences in any of the groups. It was of interest that the significant release of N^{τ}-methylhistidine in the presence of dibucaine could be accounted for by a similar decrease in the free tissue pool of the amino acid. Presumably, dibucaine increased specifically the transport (efflux) of N^{τ}-methylhistidine by incubated muscles. The transport of N^{τ}-methylhistidine by muscle, and the system by which it is translocated, have yet to be examined.

Influence of inhibition of energy metabolism on protein degradation in incubated muscles

The increase in total proteolysis (i.e. tyrosine release) in incubated muscles in the presence of A23187 and dibucaine was associated with both an increase in total cell Ca^{2+} and a marked decrease in ATP. To determine if a decrease in tissue ATP alone could reproduce the effects of A23187 and dibucaine on total proteolysis, muscles were incubated in the presence of 2,4-dinitrophenol. As shown in Table 4, dinitrophenol decreased tissue ATP as well as phosphocreatine to near-undetectable values after incubation for 3 h. When Ca^{2+} was present in the incubation medium, dinitrophenol increased tyrosine production by 89%, but no significant change was evident for N^{τ}-methylhistidine production. When Ca^{2+} was omitted from the medium, the effect of dinitrophenol on tyrosine production was markedly attenuated (23% increase). These results suggest that the large increase in tyrosine production in the presence of dinitrophenol is most likely due to changes in cell Ca^{2+} mediated by the marked decrease in ATP (Clusin, 1985). By inference, the effects of A23187 and dibucaine are most likely also to be due predominantly to the change in cell Ca^{2+} and not the decrease in ATP.

DISCUSSION

N^{τ}-Methylhistidine release by incubated muscles

Several approaches have been used to assess myofibrillar protein breakdown in rat skeletal muscle. In the intact animal the free concentration of N^{τ}-methylhistidine in muscles (Li & Wassner, 1981; Lowell *et al.*, 1986*b*), as well as its excretion in urine (Young & Munro, 1978), have been used as indices of myofibrillar proteolysis. Evaluation by these procedures has several limitations in that intracellular content of N^{τ}-methylhistidine in muscle may reflect, in addition to proteolysis,

Table 4. Effect of 2,4-dinitrophenol on tissue ATP and phosphocreatine and on tyrosine and N^{τ}-methylhistidine production by incubated extensor digitorum longus muscles of overnight-starved rats

Muscles were incubated in the presence of cycloheximide as described in the Materials and methods section. 2,4-Dinitrophenol (0.1 mM) was added to the initial incubation medium. Both muscles from the same rat were initially placed in 6 ml of buffer (control or experimental) and preincubated for 1 h. One muscle was then removed quickly, rinsed, blotted and frozen immediately in liquid N_2, and the other muscle was removed, rinsed in buffer, blotted and transferred to 3 ml of fresh medium and incubated for an additional 2 h. After incubation, the muscles were quick-frozen as above. Production of tyrosine and N^{τ}-methylhistidine was calculated as follows: total production (nmol/2 h per g wet wt.) = amino acid release (nmol/2 h per g) + change in tissue content (nmol/g) at 120 min minus that at 0 min. ATP and phosphocreatine were measured on muscles incubated for 2 h. Results are means ± S.E.M. with four rats per group: $*P < 0.01$ and $**P < 0.05$ versus respective control. ‡$CaCl_2$ was replaced by 1 mM-EGTA.

Group	ATP (μmol/g)	Phospho-creatine (μmol/g)	Release (nmol/2 h per g)	Tissue content (nmol/g) 0 min	Tissue content (nmol/g) 120 min	Total production (nmol/2 h per g)
			Tyrosine			
Control	5.12±0.41	15.25±1.22	258±21	144±12	160±13	274±22
Dinitrophenol	0.56±0.04*	0.40±0.03*	394±32	171±14	296±24	519±42*
Control (−Ca^{2+})‡	5.00±0.40	14.56±1.16	220±18	130±10	135±11	225±11
Dinitrophenol (−Ca^{2+})‡	0.36±0.03*	0.55±0.04*	242±19	140±11	175±14	277±14**
			N^{τ}-Methylhistidine			
Control	5.12±0.41	15.25±1.22	4.82±0.39	6.23±0.50	4.67±0.37	3.26±0.26
Dinitrophenol	0.56±0.04*	0.40±0.03*	6.58±0.53	5.12±0.41	2.10±0.17	3.56±0.28
Control (−Ca^{2+})‡	5.00±0.40	14.56±1.16	4.40±0.22	5.84±0.47	4.84±0.24	3.40±0.27
Dinitrophenol (−Ca^{2+})‡	0.36±0.03*	0.55±0.04*	6.00±0.48	4.74±0.40	2.39±0.19	3.65±0.30

changes in transport, and its excretion in urine may originate from non-muscle sources (Rennie & Millward, 1983). An alternative approach in evaluating myofibrillar proteolysis has been to measure the release of N^{τ}-methylhistidine over several hours by perfused rat muscle under defined conditions (Wassner & Li, 1982; Lowell *et al.*, 1986*a*,*b*). Although the latter is an approach *in vitro*, studies have shown that the metabolism of N^{τ}-methylhistidine by perfused rat muscle reflects the situation *in vivo* (Lowell *et al.*, 1986*b*). Evaluation of myofibrillar proteolysis with perfused rat muscle, however, is not without limitations. The large volume of perfusion medium (100–200 ml) required per animal often limits the routine use of certain test compounds that are available in limited quantities as well as being costly. Also compounds that have vasoactive properties are often excluded from use, since they may diminish perfusate flow to perfused muscles. For these reasons, the present study evaluated the use of incubated muscles for assessing myofibrillar proteolysis. The major limitation in this approach, that of quantification of small amounts of N^{τ}-methylhistidine, was overcome by use of an extremely sensitive h.p.l.c. system. The results indicated that under normal conditions individual muscles released measurable quantities of N^{τ}-methylhistidine during several hours of incubation and that tissue contents of the amino acid remained reasonably stable. In addition, the release of N^{τ}-methylhistidine by incubated muscles in response to brief starvation was qualitatively similar to results obtained with perfused muscle of young rats (Lowell *et al.*, 1986*a*). The use of incubated muscles from small rats for metabolic studies has received some criticism on the grounds that diffusion of oxygen and substrates to the core of the muscle is limited (Maltin & Harris, 1985). This did not appear to present a major problem in the present study, since incubated muscles responded in a similar manner to perfused muscle, where problems of diffusion should be very minimal.

Influence of Ca^{2+} on myofibrillar proteolysis

Ca^{2+} is known to be involved in the regulation of many cellular functions. Its role in the degradation of myofibrillar proteins in skeletal muscle gained importance after the isolation from muscle of a Ca^{2+}-activated proteinase that could completely remove Z lines, and could selectively degrade troponin, tropomyosin, C-protein and M-protein (Bird *et al.*, 1980). The Ca^{2+} requirement for activation of the proteinase is high (in the millimolar range), but a proteinase activated by more physiological concentrations has also been found (Mellgren, 1980). For these reasons, the increase in muscle cell Ca^{2+} in some pathological states (Emery & Burt, 1980) has been linked to atrophy and necrosis owing to increased protein breakdown. Indeed, acute experimental elevation of cell Ca^{2+} concentration has been shown to increase proteolysis in incubated muscles (Kameyama & Etlinger, 1979; Sugden, 1980; Lewis *et al.*, 1982; Rodemann *et al.*, 1982; Zeman *et al.*, 1985). Those studies did not determine, however, whether the increase in proteolysis was due to the breakdown of myofibrillar or non-myofibrillar proteins or both.

The present study has confirmed that acute increases in cell Ca^{2+} or a change in its cellular distribution can increase total proteolysis in incubated muscles as assessed by tyrosine release and tissue content of the amino acid. On the other hand, these changes in total cell proteolysis were not associated with similar changes in myofibrillar proteolysis when assessed by both N^{τ}-methylhistidine release and changes in tissue contents of the amino acid. It thus appears that increases in cell Ca^{2+} in muscle augment the breakdown of non-myofibrillar, but not myofibrillar, proteins. This is in keeping with results from a previous study where leupeptin, an inhibitor of Ca^{2+}-activated proteinase (Toyo-Oka *et al.*, 1978) as well as lysosomal cathepsins B and L (Kirschke *et al.*, 1976), failed to diminish the release of N^{τ}-methylhistidine by perfused rat muscle, but was effective in curtailing the release of tyrosine (Lowell *et al.*, 1986*a*). Since muscle mass is regulated primarily by the turnover of myofibrillar proteins, the results of the present study question the role of Ca^{2+} in muscle atrophy in certain pathological states. The present study, however, cannot completely rule out a role of Ca^{2+}, since incubations were of only short duration. It remains possible that increased muscle Ca^{2+} for upwards of several hours may lead to increased myofibrillar proteolysis. However, Smith (1985) reported that exposure of cultured muscle cells to A23187 for 30 h decreased total proteolysis.

The present study also revealed that marked changes in tyrosine release by incubated muscles occurred in situations where the energy status of the muscle cell was greatly decreased. For instance, in overnight starved rats Ca^{2+} ionophore A23187 and dibucaine decreased muscle ATP by 67 and 78% respectively, yet increased tyrosine release by 21 and 48% respectively. Previous studies have reported proteolysis to diminish when energy metabolism is inhibited and ATP concentrations fall (Reijngoud & Tager, 1973; Etlinger & Goldberg, 1977; Sugden, 1980). In experiments with Ca^{2+} ionophore A23187 and dibucaine (as well as with dinitrophenol), the increase in proteolysis was more than likely to be dependent on the increase in muscle Ca^{2+}, rather than on the energy status of the cell, a point suggested also by Sugden (1980) previously. Also, the present study revealed that some of the agents tested actually decreased N^{τ}-methylhistidine release by incubated muscles to a small extent. Whether changes in muscle Ca^{2+} or changes in Na^{+} (Bittar *et al.*, 1974) or K^{+} (Reed & Lardy, 1972) flux sometimes induced by these agents were responsible for this remains to be determined. The notion that an increase in muscle cell Ca^{2+} may be associated with a decrease in myofibrillar proteolysis is not inconceivable, since N^{τ}-methylhistidine release by perfused rat muscles undergoing electrically induced contractions has been reported to decrease (Bylund-Fellenius *et al.*, 1984). Other cellular events in addition to an increase in cell Ca^{2+} that could result in this response would be a change in Na^{+} and/or K^{+} flux, a decrease in ATP or possibly a decrease in intracellular pH associated with muscular contractions.

Finally, the results of the present as well as our past studies (Lowell *et al.*, 1986*a*,*b*) re-emphasize that the breakdown of myofibrillar and non-myofibrillar proteins in muscle is regulated independently and by different pathways. Because the turnover of myofibrillar proteins is several orders of magnitude slower than that of non-myofibrillar proteins (Bates & Millward, 1983), changes in total cellular proteolysis (i.e. tyrosine release) may not always reflect the status of myofibrillar proteolysis; likewise, changes in myofibrillar proteolysis

can occur in situations where total cellular proteolysis is not revealing. Future studies must take this into account.

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