ATP depletion stimulates calcium-dependent protein breakdown in chick skeletal muscle

JULIE M. FAGAN, EWA F. WAJNBERG, LAURA CULBERT, AND LLOYD WAXMAN
Department of Animal Sciences, Rutgers University, New Brunswick, New Jersey 08903

Fagan, Julie M., Ewa F. Wajnberg, Laura Culbert, and Lloyd Waxman. ATP depletion stimulates calcium-dependent protein breakdown in chick skeletal muscle. *Am. J. Physiol.* 262 (*Endocrinol. Metab.* 25): E637–E643, 1992.—The contribution of metabolic energy to the degradation of intracellular proteins in skeletal muscle was investigated. Isolated chick skeletal muscles deprived of oxygen and muscles incubated in buffer under nonphysiological conditions containing inhibitors of glycolysis and mitochondrial respiration had lower concentrations or undetectable levels of ATP and faster rates of proteolysis. Both total protein breakdown and the breakdown of myofibrillar proteins were stimulated 35–124% in ATP-depleted tissues. However, ATP-depleted muscles incubated in buffer to which no Ca^{2+} was added showed slower rates of total protein breakdown and no significant change in myofibrillar proteolysis compared with control muscles. *Trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), a compound that inhibits the calpains and the lysosomal cysteine proteases, completely blocked the Ca^{2+}-stimulated breakdown of nonmyofibrillar and myofibrillar proteins in ATP-depleted muscles. However, Ca^{2+}-stimulated proteolysis was not inhibited in ATP-depleted muscles incubated with weak bases to prevent lysosome function. These data suggest that intracellular proteins can be degraded in skeletal muscle in the absence of metabolic energy and that the calpains play a major role in the enhanced proteolysis in skeletal muscles depleted of ATP.

adenosine 5′-triphosphate; intracellular proteins; calpains; metabolic energy; proteases

IT HAS BEEN RECOGNIZED for some time that the synthesis and degradation of proteins in mammalian and bacterial cells requires metabolic energy. Although the energy requirement for protein synthesis is now well understood, the role of energy in intracellular proteolysis is less clear.

Early studies investigated the apparent energy requirement for protein degradation using compounds that inhibit glycolysis and mitochondrial respiration and using conditions, such as anoxia, to deplete cellular ATP, creatine phosphate, and total adenine nucleotides. Investigations by Simpson (23) suggested that cellular energy may be necessary for the breakdown of proteins to amino acids. Incubation of rat liver and kidney slices under conditions that limit the supply of ATP (anaerobiosis, cyanide, 2,4-dinitrophenol) resulted in a decreased release of amino acids from radiolabeled endogenous proteins (23). Anaerobiosis and 2,4-dinitrophenol also inhibited the release of proteins in rat and liver slices incubated in a Ca^{2+}- and Mg^{2+}-free buffer (24). The metabolic inhibitors, fluoride and iodoacetate, reduced the cellular ATP levels in rat fibroblasts and inhibited the rate at which proteins with both long and short half-lives were degraded (21). As was shown by Hershko and Tompkins (17), a 60–70% reduction in ATP content in hepatoma cells incubated with 2-deoxyglucose to inhibit glycolysis had little or no effect on the degradation of tyrosine aminotransferase, whereas a further reduction in ATP by the addition of dinitrophenol completely blocked degradation of this enzyme. In growing fibroblasts, it was reported that intracellular ATP levels must fall 90% to inhibit proteolysis by 50% (15). This suggests that the proteolytic steps requiring energy have a high affinity for ATP. On the other hand, a drop of only 15% in intracellular ATP levels slowed the rate of protein synthesis by 50% in fibroblasts (15).

Although the rate of protein synthesis generally slows when levels of ATP are reduced, evidence for a causative relationship between the rate of proteolysis and levels of ATP in various tissues is less convincing. In rat skeletal muscles deprived of oxygen, levels of ATP and other phosphorylated compounds were decreased while rates of proteolysis in these tissues were significantly elevated (4). Higher rates of proteolysis were also observed in muscle incubated in buffer containing dinitrophenol, whereas a decrease in the release of tyrosine from protein was observed in skeletal muscles incubated in Ca^{2+}-free buffer containing dinitrophenol (14, 25). Thus the rate at which proteins are degraded may be critically dependent on the concentration of both Ca^{2+} and ATP. In this report we incubated skeletal muscles completely depleted of ATP in Ca^{2+}-containing and Ca^{2+}-free buffers and measured rates of proteolysis. We found that metabolic energy is not necessarily required for the breakdown of all proteins, since protein degradation was greatly stimulated in ATP-depleted muscles via an ATP-independent nonlysosomal process that required calcium.

METHODS

The calcium ionophores A23187 and ionomycin were purchased from Calbiochem (La Jolla, CA). Leupeptin and *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) were obtained from Cambridge Research Biochemicals (Wilmington, DE). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Male single-comb White Leghorn chicks (Avian Services, Frenchtown, NJ) were obtained on the day of hatch and were housed in wire-floored 32–35°C brooder chambers with a 12:12-h light-dark cycle in an environmentally controlled room. Chicks were provided with food (Country Broiler Maker Agway, Flemington, NJ) and water ad libitum.

Chicks (10–18 days old) were killed by cervical dislocation, and the extensor digitorum communis muscles were rapidly excised with tendons intact and were blotted and weighed. Muscles were incubated at their approximate resting length by pinning the tendons with stainless steel needles (30-gauge 0.5 in.) to inert plastic supports (25 × 4 × 4). Muscles weighing 10–25 mg were incubated in 4 ml of Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with all the essential and nonessential amino acids (except tyrosine, which was omitted) at concentrations found in rat plasma (20). Other additions are described (see Tables 1–5). Results are expressed as means ± SE and the significance of differences tested by the paired or unpaired Student's *t* test.

In some experiments, muscles were incubated with glucose (5 mM) in buffer equilibrated with O_2/CO_2 (19:1) or N_2/CO_2 (19:1) to examine the effects of oxygen deprivation (4). To study the effects of ATP depletion on protein degradation, muscles were incubated in buffer equilibrated with O_2/CO_2 (19:1) containing either 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM; ATP-depleted condition) or glucose (5 mM; control condition). ATP content in control muscles preincubated for 1.5 h was 4.8 ± 0.4 nmol/mg muscle and was not significantly different from control muscles incubated for an additional 2 h (4.7 ± 0.3 nmol/mg muscle). ATP was not detectable in muscles that were either preincubated for 1.5 h in buffer containing 5 mM 2-deoxyglucose and 0.2 mM 2,4-dinitrophenol or preincubated for 1.5 h and incubated for 2 h in the same buffer containing inhibitors of energy metabolism. However, these ATP-depleted muscles were capable of generating measurable amounts of ATP (0.156 ± 0.014, 0.291 ± 0.027, and 0.55 ± 0.06 nmol/mg muscle wet wt after 30, 60, and 120 min, respectively) when incubated with glucose but without inhibitors of energy metabolism. When allowed to recover on incubation under aerobic conditions, these ATP-depleted muscles oxidized [^{14}C]glucose to $^{14}CO_2$ at a rate (0.217 ± 0.022 and 0.503 ± 0.062 nmol/mg muscle for 1 and 2 h, respectively) not significantly different from control muscles (0.302 ± 0.047 and 0.592 ± 0.073 nmol/mg muscle for 1 and 2 h, respectively). Tissue concentrations of lactate dehydrogenase in ATP-depleted muscles were also not different from control muscles after a 2-h incubation with glucose but without inhibitors of energy metabolism. By these criteria, muscles depleted of ATP appear to be viable and capable of recovery after removal of inhibitors of energy metabolism.

Muscles were routinely preincubated at 37°C for 1.5 h either in the presence of 5 mM glucose or in buffer containing 5 mM 2-deoxyglucose and 0.2 mM 2,4-dinitrophenol and then transferred to fresh media and incubated for 2 h. Incubations were terminated by removing the tissue and heating the medium in boiling water for 3 min. Muscles were blotted and homogenized in ice-cold 0.2 M $HClO_4$ (0.75 ml), centrifuged for 10 min at 2,000 *g* and 4°C, and the supernatant was adjusted to pH 5.5–6.5 with 2.5 M KOH-0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid). $KClO_4$ was then removed by centrifugation (10 min at 2,000 *g*, 4°C).

ATP was determined in the neutralized muscle extracts by a spectrophotometric (or fluorometric) assay (19) and by a chemiluminescence assay (2) using firefly luciferase (Analytical Luminescence Laboratory, San Diego, CA). Both methods for measuring ATP content gave similar results. For determining low levels of ATP, the firefly luciferase assay was found to be less time consuming than the fluorometric hexokinase assay (19).

The breakdown of total muscle protein (sarcoplasmic, myofibrillar, and stromal proteins) was estimated by the amount of tyrosine released from cell protein in the presence of 0.25 mM cycloheximide, an inhibitor of protein synthesis (10). Breakdown of myofibrillar proteins was determined by the release of 3-methylhistidine from muscle actin and myosin. Protein degradation was calculated as the amount of tyrosine or 3-methylhistidine in the sample incubation medium after correcting for the change in the tissue pool. Tissue pools of tyrosine and 3-methylhistidine were determined as the mean difference after incubation and after preincubation (or death if muscles were not preincubated) in a separate group of muscles. Generally, the changes in the tissue pool of tyrosine and 3-methylhistidine had little effect on the calculated rates of proteolysis. Tyrosine was measured fluorometrically (28), and 3-methylhistidine was assayed by high-performance liquid chromatography (HPLC) by a modification of the method of Wassner et al. (30). Before assay for 3-methylhistidine, the neutralized extracts were lyophilized and redissolved in water. Because the 2,4-dinitrophenol contained substances that coeluted with the fluorescamine derivative on HPLC, all samples were first passed through C_{18} Sep-Pak columns (Millipore) equilibrated in 0.1% (vol/vol) trifluoroacetic acid-5% acetonitrile (vol/vol). Columns were washed with 2 ml of this solvent, and the eluate was lyophilized and dissolved in 0.5 ml water. Aliquots (100–500 μl) were then derivatized with fluorescamine and analyzed for 3-methylhistidine content on HPLC.

RESULTS

Stimulation of proteolysis by anoxia and by inhibitors of energy metabolism. Compared with oxygenated control muscles, rat skeletal muscles incubated in buffer equilibrated with nitrogen were previously shown to have similar rates of proteolysis during the first hour of incubation and significantly higher rates of proteolysis during the second hour of incubation (4). Similarly, rates of protein breakdown did not differ in chick extensor digitorum communis muscles incubated for 1.5 h in buffer equilibrated with nitrogen or oxygen (Fig. 1). After a 3- to 5-h incubation, proteolysis was significantly increased in the anoxic muscles compared with the oxygenated controls (Fig. 1). Throughout the incubation, tissue ATP in oxygenated muscles was maintained at levels (4–6 nmol/mg) measured in muscles immediately after death (Figs. 1 and 2). ATP content in the oxygen-deprived muscles dropped to 1.08 ± 0.25 nmol/mg muscle wet wt after 1.5 h of incubation and to 0.45 ± 0.12 nmol/mg muscle wet wt after 5 h of incubation.

To examine the rate of protein breakdown in muscles completely depleted of ATP, chick extensor digitorum communis muscles were incubated in Krebs-Henseleit buffer containing 2-deoxyglucose (5 mM) to inhibit glycolysis and 2,4-dinitrophenol (0.2 mM) to block oxidative phosphorylation. Such treatment reduced intracellular

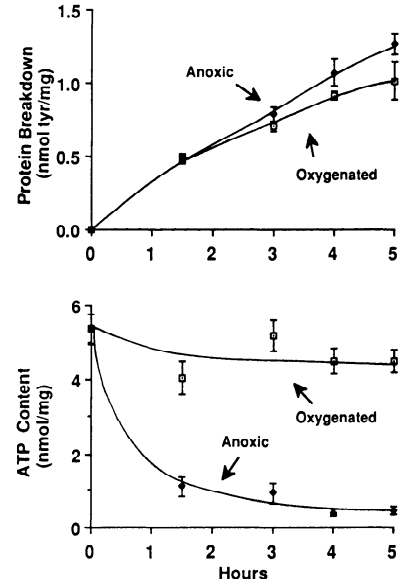

Fig. 1. Time course of protein breakdown and ATP levels in muscles incubated in oxygenated and anoxic conditions. Contralateral chick muscles were incubated in oxygenated or nitrogenated Krebs-Henseleit (pH 7.4) bicarbonate buffer containing 2.5 mM Ca^{2+} for 1.5, 3, 4, and 5 h. Tissue ATP and tyrosine pools were determined in blotted muscles immediately after death (0 h) and after incubation at times indicated. Protein degradation was calculated as release of tyrosine from muscle into media minus net change (from 0 h) in tissue pool of tyrosine. Points represent means ± SE for 7 observations.

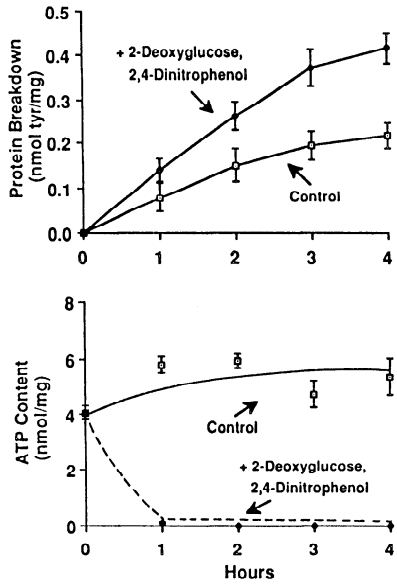

Fig. 2. Time course of ATP depletion and protein degradation in muscles incubated with 2-deoxyglucose and 2,4-dinitrophenol. Contralateral chick muscles were incubated in Krebs-Henseleit bicarbonate buffer containing Ca^{2+} (2.5 mM) and either glucose (5 mM) or 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM). At times indicated and after death (0 h), protein degradation and amount of ATP were determined as described in legend to Fig. 1. Points represent means ± SE for 6 observations.

ATP levels to 0.12 ± 0.04 nmol/mg muscle after 1 h of incubation and to 0 nmol/mg muscle after 2–4 h of incubation (Fig. 2). These energy-depleted muscles showed faster rates of proteolysis than control muscles incubated in media containing 5 mM glucose. Compared with the controls, ATP-depleted muscles released approximately twice the amount of tyrosine from muscle protein throughout the 4-h incubation. This marked increase in proteolysis in skeletal muscles completely devoid of ATP indicates that muscles contain a proteolytic pathway that is independent of metabolic energy.

Proteolysis in energy-depleted muscles requires Ca^{2+}. To investigate the role of Ca^{2+} in the increase in proteolysis in energy-depleted muscles, muscles were incubated in media prepared either with Ca^{2+} (2.5 mM) or without added Ca^{2+}. To ensure that muscles were depleted of ATP before determining rates of proteolysis, muscles were preincubated for 1.5 h in buffer containing inhibitors of energy production. ATP could not be detected in these muscles using either the hexokinase or firefly luciferase assay. Energy-depleted muscles incubated for a further 2 h in buffer containing Ca^{2+} had greatly elevated rates of proteolysis compared with control muscles incubated in buffer containing glucose and Ca^{2+} (Table 1). However, energy-depleted muscles incubated in buffer prepared without added Ca^{2+} showed a 36% slower rate of proteolysis compared with control muscles incubated without added Ca^{2+} (Table 1). Addition of Ca^{2+} to the incubation mixture stimulated protein breakdown in ATP-depleted muscles by 294% and caused a significant but much smaller increase (+13%) in protein breakdown in control muscles (Table 1). Thus Ca^{2+} appears to be required for the increased degradation of proteins in ATP-depleted muscle.

Table 1. *Protein degradation in control and ATP-depleted muscles incubated in Ca^{2+}-free medium*

Muscle Condition	Protein Degradation pmol Tyr·mg^{-1}·2 h^{-1}		
	$-Ca^{2+}$	$+Ca^{2+}$	Difference
Control	112±9	127±8	+15(+13%)*
ATP-depleted	72±12	284±34	+212(+294%)*
Difference	−40(−36%)†	+157(+124%)†	

Values are means ± SE. Contralateral chick muscles were preincubated for 1.5 h and then incubated for 2 h in Krebs-Henseleit buffer containing glucose (5 mM) prepared in presence (2.5 mM) or absence of Ca^{2+}. In another group of 7 chicks, contralateral muscles were preincubated for 1.5 h and incubated for 2 h in buffer prepared with or without Ca^{2+} (2.5 mM) but containing 2-deoxyglucose (5 mM) and 2,4-dinitriophenol (0.2 mM). Protein degradation was determined as described in Fig. 1, except that tissue pools of tyrosine were determined after preincubation and after incubation. Both absolute difference and percent difference (in parentheses) from control are calculated; plus denotes an increase and minus denotes a decrease relative to control. Data on effects of incubation in Ca^{2+}-free medium were treated by paired Student's t test, where * $P \leq 0.025$. Effects of ATP depletion were tested by unpaired Student's t test, where † $P \leq 0.005$.

Ca^{2+} ionophores are frequently used to examine the effects of elevated levels of intracellular Ca^{2+} on physiological processes. In isolated skeletal muscle, Ca^{2+} ionophores have been reported to increase protein breakdown (18, 25). Accordingly, the Ca^{2+} ionophores A23187 and ionomycin stimulated protein breakdown by 17–29% in both control and ATP-depleted muscles (Table 2). However, in the presence of Ca^{2+} ionophore, the amount of tyrosine released by the energy-depleted muscles was still 1.6- to 1.9-fold higher than for control muscles. This suggests that the enhanced proteolysis observed in ATP-depleted muscle may not solely be due to an influx of Ca^{2+} into the muscle and that additional factors must be involved in stimulating proteolysis in energy-depleted muscles.

To examine whether the release of Ca^{2+} from the sarcoplasmic reticulum plays a role in the Ca^{2+}-dependent

Table 2. *Effect of Ca^{2+} ionophores on protein degradation in control and ATP-depleted chick muscles*

Additions	Protein Degradation pmol Tyr·mg^{-1}·2 h^{-1}		
	Control	ATP depleted	Difference
None	240±22	502±31	+262(109%)†
+A23187	306±25	592±14	+286(+93%)†
Difference	+66 (+28%)*	+90 (+18%)*	
None	302±29	516±27	+214(+71%)†
Ionomycin	389±29	604±41	+215(+55%)†
Difference	+87(+29%)*	+88(+17%)*	

Values are means ± SE for 7–8 observations and were obtained as in Table 1. Contralateral chick muscles were preincubated for 1.5 h and then incubated for 2 h in Ca^{2+}-containing (2.5 mM) buffer with and without the Ca^{2+} inophores A23817 or ionomycin (5 μg/ml) in media containing either glucose (5 mM) or 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM). Ionophores were dissolved in dimethyl sulfoxide. Control and ionophore-containing incubation media contained dimethyl sulfoxide at a concentration of 0.07%. Data on effects of Ca^{2+} ionophores were evaluated with paired Student's t test, where * $P \leq 0.05$. Effects of ATP depletion were tested by unpaired Student's t test, where † $P \leq 0.005$.

increase in protein degradation in energy-depleted muscles, dantrolene was added to the incubation media. Dantrolene has been shown to inhibit excitation-contraction coupling by preventing the release of Ca^{2+} from the sarcoplasmic reticulum (26). Dantrolene decreased the rate of protein breakdown by 15% in control muscles and did not significantly alter the stimulated rate of proteolysis in energy-depleted muscles (Table 3). Thus sufficient intracellular Ca^{2+} appears to be available to support the increase in protein degradation in energy-depleted muscles, even when the release of Ca^{2+} from the sarcoplasmic reticulum is blocked.

Previous studies have shown an increase in protein breakdown in rat skeletal muscle exposed to arachidonic acid or prostaglandin E_2, a metabolite of arachidonic acid (22). This rise in protein breakdown by prostaglandin E_2 was reported to require Ca^{2+} (22). To examine whether prostaglandin E_2 plays a role in the Ca^{2+}-dependent increase in protein breakdown in ATP-depleted muscles, indomethacin (5 μM), an inhibitor of the prostaglandin synthetase, was added to the incubation media. Indomethacin had no effect on the rate of protein breakdown in muscles depleted of energy or in control muscles (Table 3). However, it is likely that prostaglandin production was already inhibited by cycloheximide (3), which was added to the preincubation and the incubation media to block protein synthesis. Thus prostaglandin E_2 does not play a crucial role in the Ca^{2+}-stimulated proteolysis in skeletal muscles depleted of energy.

Inhibitors of cysteine proteases block Ca^{2+}-stimulated proteolysis in ATP-depleted skeletal muscle. Because the observed increase in proteolysis in muscles depleted of ATP was Ca^{2+} dependent, the effect of inhibitors of the Ca^{2+}-activated proteases (calpains) were tested. To our knowledge, no specific synthetic inhibitors of the calpains are available. One compound reported to specifically inhibit the calpains, acetyl-Leu-Leu-norleucinal (29), was found also to inhibit cathepsin B (data not shown). Leupeptin and E-64, which are low-molecular-weight inhibitors of cysteine proteases and which enter muscle cells, will inhibit the calpains and cathepsins B, H, and L (1, 27). Leupeptin was found to inhibit protein degradation in control muscles by 10% and in ATP-depleted muscles by 67% (Table 4). Leupeptin completely abolished the increase in protein degradation in muscles depleted of ATP and reduced the rates of proteolysis to levels below that observed in control muscles.

To determine whether leupeptin blocked the rise in proteolysis in ATP-depleted muscles by inhibiting the lysosomal cysteine proteases (cathepsins B, H, and L), muscles were exposed to the lysosomotropic agents methylamine (10 mM) and ammonium chloride (10 mM). These weak bases prevent the digestion of proteins within the lysosome by increasing intralysosomal pH. Methylamine inhibited protein degradation in control muscles by 21% and in energy-depleted muscles by 10% (Table 4). Similar results were obtained with ammonium chloride (data not shown). However, the weak bases did not block the increase in the degradation of proteins in energy-depleted muscles, suggesting that these proteins were degraded by a nonlysosomal proteolytic pathway. Possibly, leupeptin suppressed proteolysis in ATP-depleted tissues, not by inhibiting cathepsins B, H, and L, but by inhibiting the calpains. E-64, when added together with methylamine, decreased proteolysis in energy-depleted muscles by 76% to levels below that found in control muscles incubated with methylamine and E-64 (Table 4). Thus the calpains, and not the cathepsins, appear to play a role in the enhanced degradation of proteins in energy-depleted muscles.

The increased release of tyrosine by muscles depleted of

Table 3. *Effect of dantrolene and indomethacin on protein degradation in control and ATP-depleted muscles*

Additions	Protein Degradation, pmol Tyr·mg^{-1}·2 h^{-1}		
	Control	ATP depleted	Difference
Expt 1			
None	261±10	353±23	+92(+35%)*
Dantrolene	222±12	330±16	+108(+49%)*
Difference	−39(−15%)†	NS	
Expt 2			
None	322±32	551±52	+229(+71%)*
Indomethacin	308±13	493±63	+185(+60%)*
Difference	NS	NS	

Values are means ± SE for 7 observations and were obtained as in Table 1. Contralateral chick muscles were preincubated for 1.5 h and then incubated for 2 h in Ca^{2+}-containing (2.5 mM) buffer in presence and absence of indomethacin or dantrolene (1 mg/100 ml buffer) in media containing glucose (5 mM) or 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM). Indomethacin (solubilized in ethanol) was added to incubation buffer at a final concentration of 5 μM. All incubations contained ethanol at a concentration of 0.1%. Effects of ATP depletion were tested by unpaired Student's t test where * $P \leq 0.01$. Effects of dantrolene and indomethacin were tested by paired Student's t test, where † $P \leq 0.05$ and NS (not significant) = $P > 0.05$.

Table 4. *Effect of methylamine and cysteine protease inhibitors leupeptin and E-64 on protein degradation in control and ATP-depleted muscles*

Additions	Protein Degradation, pmol Tyr·mg^{-1}·2 h^{-1}		
	Control	ATP depleted	Difference
Expt 1			
None	266±10	369±22	+103(+39%)†
Leupeptin	240±12	122±12	−118(−49%)†
Difference	−26(−10%)*	−247(−67%)*	
Expt 2			
None	314±7	572±23	+258(+82%)†
Methylamine	248±9	515±16	+267(+108%)†
Difference	−66(−21%)	−57(−10%)*	
Methylamine	216±18	408±74	+192(+89%)†
Methylamine + E-64	169±15	99±7	−70(−41%)†
Difference	−47(−22%)*	−309(−76%)*	

Values are means ± SE for 7 observations and were obtained as in Table 1. Contralateral chick muscles were preincubated for 1.5 h and then incubated for 2 h in Ca^{2+}-containing (2.5 mM) buffer in presence and absence of methylamine (10 mM), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64; 100 μM), or leupeptin (100 μM) in media containing glucose (5 mM) or 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM). Data on effects of methylamine, leupeptin, and E-64 were evaluated with paired Student's t test, where * $P \leq 0.05$. Effects of ATP depletion were tested by unpaired Student's t test, where † $P \leq 0.05$.

energy reflects a faster rate of breakdown of muscle proteins to amino acids. Tyrosine may be released from the breakdown of specific muscle proteins and may not necessarily reflect the degradation of the soluble sarcoplasmic proteins, the stromal proteins, and the myofibrillar proteins. In fact, several physiological and experimental conditions have been shown to change the rate of tyrosine release from muscle protein with little or no change in the rate of myofibrillar protein breakdown (or vice versa). To examine specifically the effect of ATP depletion on myofibrillar protein breakdown and the role of Ca^{2+} and the cysteine proteases in this process, muscles were incubated in the presence or absence of Ca^{2+}, E-64, and inhibitors of energy metabolism, and the release of 3-methylhistidine from actin and myosin was measured. In the absence of added Ca^{2+} or in the presence of E-64 with Ca^{2+}, the rate of breakdown of myofibrillar proteins in muscles depleted of ATP did not differ from control muscles (Table 5). This indicates that myofibrillar proteins are hydrolyzed via an ATP-independent proteolytic pathway by proteases other than the calpains. Under the same conditions, the breakdown of total muscle proteins in energy-depleted tissues was significantly decreased compared with controls (Table 5). These results suggest that, in the absence of extracellular Ca^{2+}, an energy-requiring process may be involved in the degradation of nonmyofibrillar proteins. In the presence of Ca^{2+}, depletion of muscles of ATP significantly increased the release of both tyrosine (by 73%) and of 3-methylhistidine (by 43%) from muscle protein (Table 5). This stimulation of myofibrillar and total muscle proteolysis in ATP-depleted muscles was inhibited by E-64 and after incubation in the absence of added Ca^{2+} (Table 5). Thus total muscle protein breakdown and myofibrillar proteolysis are enhanced in ATP-depleted muscles by a Ca^{2+}-dependent process involving cysteine proteases.

DISCUSSION

Exposure of various animal and bacterial cells to anaerobic conditions or inhibitors of energy production have been shown to inhibit protein degradation (12, 16, 23). These observations led to the conclusion that intracellular protein breakdown requires metabolic energy. More recently, it was shown that erythroid cells also contain a nonlysosomal proteolytic pathway that is independent of ATP (8). Hemoglobin damaged by oxidants was rapidly degraded within erythrocytes and reticulocytes even after depleting the cells of ATP with inhibitors of glycolysis and mitochondrial respiration (8). The rapid degradation of oxidatively damaged hemoglobin in red blood cells appears not to involve membrane proteases or to require Ca^{2+} and is unaffected by inhibitors of aspartic and serine proteases and by leupeptin and E-64, inhibitors of cysteine proteases (5). The hydrolysis of oxidatively damaged hemoglobin was enhanced by divalent cations, and therefore the protease responsible for the rate-limiting cleavage of oxidatively damaged proteins may involve a cytoplasmic metalloprotease (7). Alternatively, a calcium-requiring cysteine protease may be involved in the ATP-independent degradation of endogenous red blood cell proteins that have not been treated with oxidants. The addition of Ca^{2+} (10–1,000 μM) to dialyzed erythrocyte homogenates containing membranes stimulated by 3- to 7-fold the cleavage of endogenous proteins to amino acids, and this enhanced degradation was blocked by E-64 (unpublished observations). Thus several proteases may function to hydrolyze specific substrates via proteolytic pathways that do not require ATP.

Skeletal muscle contains a lysosomal proteolytic pathway, an energy-requiring proteolytic system involving an ATP-dependent ubiquitin-dependent (9) and an ATP-dependent ubiquitin-independent enzyme (6), and an

Table 5. *Effect of Ca^{2+} and E-64 on release of tyrosine and 3-methylhistidine in control and ATP-depleted muscles*

Incubation	Additions	Tissue Content, pmol/mg		Release, pmol·mg^{-1}·2 h^{-1}	Corrected Release, pmol·mg^{-1}·2 h^{-1}
		0 h	2 h		
			Tyrosine		
Control	$-Ca^{2+}$	113±25	79±4	176±13	142±14
ATP depleted	$-Ca^{2+}$	149±5	90±7	81±6	22±7*†
Control	$+Ca^{2+}$	97±5	88±12	162±6	153±14
ATP depleted	$+Ca^{2+}$	231±11	206±12	290±19	265±11*
Control	$+Ca^{2+}$ + E-64	81±5	87±16	144±10	150±13
ATP depleted	$+Ca^{2+}$ + E-64	110±12	64±8	70±5	24±7*†
			3-Methylhistidine		
Control	$-Ca^{2+}$	2.35±0.21	2.27±0.45	2.17±0.39	2.09±0.39†
ATP depleted	$-Ca^{2+}$	2.41±0.15	2.08±0.38	2.60±0.22	2.37±0.22†
Control	$+Ca^{2+}$	2.59±0.17	2.59±0.38	3.13±0.20	3.13±0.20
ATP depleted	$+Ca^{2+}$	2.74±0.26	2.58±0.29	4.65±0.33	4.49±0.33*
Control	$+Ca^{2+}$ + E-64	2.75±0.11	2.22±0.23	1.96±0.15	1.43±0.15†
ATP depleted	$+Ca^{2+}$ + E-64	2.86±0.22	2.34±0.22	2.38±0.31	1.86±0.31†

Values are means ± SE for 6 observations. Contralateral chick muscles were preincubated for 1.5 h and then incubated for 2 h in presence or absence of 2.5 mM Ca^{2+} and E-64 (100 μM) in media containing glucose (5 mM) or 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM). Tissue pools of tyrosine and 3-methylhistidine were determined after preincubation (0 h) and after incubation (2 h). Release of tyrosine and 3-methylhistidine was corrected for changes in tissue pool. Effects of ATP depletion were tested by paired Student's t test, where * $P \leq 0.05$. Data on effects of incubation in Ca^{2+}-free and E-64-containing media (compared with buffer containing 2.5 mM Ca^{2+}) were tested by unpaired Student's t test, where † $P \leq 0.05$.

ATP-independent proteolytic process (this report). We found that isolated chick skeletal muscles depleted of metabolic energy had greatly increased rates of protein degradation. The stimulated breakdown of endogenous proteins in ATP-depleted muscles required extracellular Ca^{2+}. The lysosomotropic agents methylamine and ammonium chloride did not block the rise in proteolysis in ATP-depleted muscles, whereas inhibitors of cysteine proteases inhibited completely the increased release of 3-methylhistidine and tyrosine from muscle protein. Incubation of skeletal muscle with 0.1 mM 2,4-dinitrophenol was previously shown to increase the release of tyrosine but not 3-methylhistidine from muscle protein (14). It is not clear why an increase in the release of 3-methylhistidine was not observed in this prior report, but it may have been due to the different experimental conditions and the possibility that ATP was present at some time during the incubation.

Our data suggest that the calcium-activated proteases (calpains) are involved in the enhanced degradation of total muscle protein in ATP-depleted tissue. Incubation of muscles with dinitrophenol and 2-deoxyglucose to deplete them of ATP may alter Ca^{2+} homeostasis and even damage some of the cells. The breakdown of proteins in cells that are no longer able to exclude extracellular Ca^{2+} may be due to proteolytic attack by the calpains that would be activated under these conditions. The stimulated degradation of total muscle proteins (tyrosine release) induced by thyroxine (31) and by muscle injury (11) also may involve the calpains, since the increased proteolysis was inhibited by leupeptin but not by lysosomotropic agents. The calpains have been implicated in initiating the turnover of myofibrillar proteins in skeletal muscle (13). The calcium-dependent thiol-sensitive activation of myofibrillar protein degradation in ATP-depleted muscle (this paper) provides further evidence supporting this hypothesis. Whether calpains are responsible for hydrolyzing myofibrillar proteins under physiological conditions or primarily under conditions that may lead to irreversible damage or disassembly of the myofibrils is not clear. We also show that myofibrillar proteins are hydrolyzed by other proteases via proteolytic pathways that do not require ATP, whereas an energy-dependent pathway participates in hydrolyzing nonmyofibrillar muscle proteins. The identification of the specific muscle proteins that are substrates for the calpains as well as other proteolytic enzymes and what modifications must occur to a protein to render it a substrate for the ATP-independent vs. the ATP-dependent or lysosomal proteolytic pathways are important questions currently under investigation in several laboratories.

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Address for reprint requests: J. M. Fagan, Dept. of Animal Sciences, Rutgers Univ., PO Box 231, New Brunswick, NJ 08903.

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