Acute alterations in sodium flux *in vitro* lead to decreased myofibrillar protein breakdown in rat skeletal muscle

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Myofibrillar protein breakdown was evaluated by measuring the release of *N*³-methylhistidine by isolated rat skeletal muscles or perfused rat muscles in the presence of a variety of agents known to affect Na⁺ flux. Total cell proteolysis was evaluated simultaneously by measuring tyrosine release by muscles after the inhibition of protein synthesis with cycloheximide. Treatment of muscles with the Na⁺ ionophore monensin or inhibitors of Na⁺-K⁺ ATPase (ouabain, digoxin or vanadate) decreased *N*³-methylhistidine release by muscles by 21–35%. A phorbol ester (phorbol 12-myristate 13-acetate) as well as a synthetic diacylglycerol known to activate protein kinase C and a Na⁺/H⁺ antiport also decreased *N*³-methylhistidine release by muscles. Removal of extracellular Na⁺ blocked the ability of these agents to attenuate *N*³-methylhistidine release by muscles, suggesting that their effectiveness required a change in Na⁺ flux. In contrast with *N*³-methylhistidine release by muscles, these agents, except for monensin, did not effect the release of tyrosine, suggesting that they attenuate specifically the breakdown of myofibrillar proteins. Overall these results indicate a link between Na⁺ and the regulation of protein breakdown in rat skeletal muscle, whereby an influx of Na⁺ can result in a decrease in myofibrillar proteolysis. Left unresolved is whether phospholipid hydrolysis is involved in this scheme.

INTRODUCTION

The amount of protein within skeletal muscle is regulated by a balance between the rate of protein synthesis and the rate of protein degradation. Although much has been learned about mechanisms regulating protein synthesis in muscle (Pain, 1986), less information is available concerning intracellular mechanisms that regulate protein breakdown (Goldberg & St. John, 1976; Bird *et al.*, 1980; Tischler, 1981; Hershko & Ciechanover, 1982; Fagan *et al.*, 1986). It is widely accepted that insulin and amino acids, as well as 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; Clark *et al.*, 1984; Lowell *et al.*, 1986*a*). It has also been assumed that these factors regulate the breakdown of both myofibrillar and non-myofibrillar proteins in muscle. However, we reported that these factors modulate the breakdown predominantly of non-myofibrillar proteins, as they did not effect the release of *N*³-methylhistidine by perfused rat muscle (Lowell *et al.*, 1986*a,b*). *N*³-Methylhistidine is a minor amino acid found exclusively in actin and myosin and, upon its release during proteolysis, it is neither re-incorporated into protein nor catabolized (Young & Munro, 1978). Thus its release from muscle should reflect the breakdown of myofibrillar proteins (i.e. actin and myosin). These results also suggested that the breakdown of myofibrillar and non-myofibrillar protein can be regulated independently and that the breakdown of myofibrillar proteins may be regulated by a non-lysosomal pathway (Lowell *et al.*, 1986*a,b*).

Previous studies have assessed the effects of regulatory factors on muscle proteolysis by measuring the release of

either tyrosine or phenylalanine by perfused or incubated muscles after the inhibition of protein synthesis with cycloheximide (Fulks *et al.*, 1975; Jefferson *et al.*, 1977; Libby & Goldberg, 1978; Jenkins *et al.*, 1979; Clark *et al.*, 1984; Lowell *et al.*, 1986*a,b*). Although this procedure is a good reflection of total protein breakdown, it does not distinguish between the breakdown of myofibrillar and non-myofibrillar proteins. It has also been reported that the rate of turnover of myofibrillar proteins in skeletal muscle is approximately half that for non-myofibrillar proteins (Bates & Millward, 1983). Because of this, significant changes in myofibrillar proteolysis can occur under situations where total proteolysis (i.e. tyrosine or phenylalanine release) is not changed (Lowell *et al.*, 1986*a,b*). This distinction can now be made by measuring simultaneously *N*³-methylhistidine release by muscle using very sensitive liquid-chromatography techniques (Lowell *et al.*, 1986*b*; Wassner *et al.*, 1980). Future studies attempting to delineate regulatory mechanisms of proteolysis in muscle must give this consideration.

As regulatory factors previously shown to decrease total proteolysis in muscle were ineffective in modulating myofibrillar proteolysis (Lowell *et al.*, 1986*a,b*), we have continued to probe for modulators affecting the breakdown of myofibrillar proteins. The purpose of the present paper is to describe novel findings on the effects of agents that modify Na⁺ flux, as well as those that stimulate protein kinase C activity on attenuating myofibrillar proteolysis. For this purpose, protein degradation in muscle was evaluated by measuring the release of both tyrosine (i.e. total proteolysis) and *N*³-methylhistidine (i.e. myofibrillar proteolysis) by perfused or incubated rat muscles in the presence or absence of agents previously shown to alter Na⁺ flux.

EXPERIMENTAL

Animals

Male Sprague-Dawley rats (50–60 g for incubation studies and 200–225 g for perfusion studies), obtained from Charles River Breeding Laboratory, Wilmington, MA, U.S.A., were used. They were housed in animal quarters maintained at 22 °C with a 12 h-light/12 h-dark cycle. They were allowed water and Purina Laboratory Chow *ad libitum*. Rats used for incubation studies had food removed at 17:00 h the evening before study, and rats used for perfusion studies had food removed at 09:00 h the morning before study.

Dissection procedures for incubation studies

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 muscle was fixed to a stainless-steel clip, 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 (Maizels *et al.*, 1977).

Muscle incubations

Once attached to the clips, muscles were transferred to 20 ml glass scintillation vials, which contained 3 ml of Krebs-Henseleit (Maizels *et al.*, 1977) bicarbonate solution with 2.5 mM-Ca²⁺ (previously gassed with O₂/CO₂, 19:1; pH 7.4), 0.12% albumin, 6 mM-glucose and 0.5 mM-cycloheximide. Other additions are described in legends to Tables. One muscle was placed in each vial, so that control and experimental incubations could be compared from the same rat. After addition of the muscles to the vials, they were flushed with O₂/CO₂ (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 an additional 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 0.9% NaCl and bottled, and then dropped into liquid N₂. Muscles were stored in liquid N₂ until analysis. Samples of incubation medium (2.5 ml) were added to 0.2 ml of ice-cold 30% (v/v) HClO₄ for deproteinization. The samples were then centrifuged (4 °C) at 2000 *g* for 20 min, and the supernatants were frozen at –20 °C until analysed.

Perfused-hindquarter experiments

Details of perfusion apparatus and the operative preparation of the hindquarter were as described previously (Lowell *et al.*, 1986a,b). The standard perfusion medium consisted of Krebs-Henseleit solution (pH 7.4), 4% (w/v) bovine serum albumin, 6 mM-glucose, 1 mM-lactate, 0.1 mM-pyruvate, and washed rejuvenated aged human erythrocytes (haematocrit 22%). During the experiment the perfusion medium was continuously gassed with O₂/CO₂ (19:1), and its temperature was maintained at 37 °C. The initial volume of perfusate was 150 ml; the first 25 ml that passed through

the tissues was discarded, and thereafter the medium was recycled. Perfusate flow rate was maintained at 12 ml/min. As described in detail previously (Lowell *et al.*, 1986a,b), both total and myofibrillar protein degradation in the perfused hindquarter were estimated by measuring the release of tyrosine and *N*⁷-methylhistidine, respectively, in the presence of 200 μ M-cycloheximide. At this concentration of cycloheximide, protein synthesis is inhibited by at least 90% within 30 min of perfusion. Tyrosine and *N*⁷-methylhistidine were assessed between 30 and 90 min of perfusion. After a pre-perfusion of 30 min, 5 ml of perfusion medium was taken, followed by another 5 ml after an additional 60 min of perfusion. The perfusate was deproteinized with 0.25 ml of ice-cold 60% (v/v) HClO₄, centrifuged at 4 °C for 30 min (2500 *g*) and the supernatants were frozen at –20 °C until analysed. At the end of each perfusion, the entire gastrocnemius muscle was freeze-clamped and stored in liquid N₂ until analysed.

Use of cycloheximide in measurement of protein degradation

The studies described in the present paper were conducted in the presence of cycloheximide. One reason for its inclusion was to enable simultaneous measurement of both myofibrillar (i.e. *N*⁷-methylhistidine release) and total cellular (i.e. tyrosine release) proteolysis after the inhibition of protein synthesis. In the absence of cycloheximide, *N*⁷-methylhistidine release by muscle would still reflect myofibrillar protein breakdown, but tyrosine release would reflect net protein breakdown (i.e. a balance between protein synthesis and protein breakdown). Some of the agents to be tested in the present study may alter muscle protein synthesis, and because of this tyrosine release by muscle may change independently of a change in proteolysis. Another reason for the inclusion of cycloheximide was to rule out this possibility. One shortcoming of its use is that it may actually decrease proteolysis. However, studies by Tischler *et al.* (1982) have shown that rates of proteolysis by muscles incubated for several hours were similar, whether conducted in the presence or the absence of cycloheximide.

Analytical methods

Frozen incubated muscles were removed from the clips, quickly weighed in a test tube pre-cooled in a methanol/solid-CO₂ bath and homogenized in 3 ml of ice-cold 3% (v/v) HClO₄. A similar procedure for frozen perfused muscle was followed once the tissue was pulverized in a stainless-steel mortar at liquid N₂ temperatures. Homogenized muscle samples were centrifuged (4 °C) at 2000 *g* for 30 min. A portion of the supernatant was neutralized with 2 M-KOH containing 0.5 M-triethanolamine, and the precipitated KClO₄ was removed by centrifugation (4 °C) at 2000 *g* for 30 min. A portion of the HClO₄ extract of the incubation medium was also neutralized in a similar manner. ATP and phosphocreatine in the neutralized tissue extracts were determined as described previously (Maizels *et al.*, 1977). Tyrosine in HClO₄ extracts of tissue, incubation medium or perfusion medium was determined fluorimetrically (Lowell *et al.*, 1986a,b). The concentration of *N*⁷-methylhistidine in HClO₄ extracts of tissue, incubation medium or perfusion medium was assayed by h.p.l.c. as described in detail previously (Lowell *et al.*, 1986b;

Table 1. Effect of monensin, ouabain, digoxin or vanadate on protein degradation in skeletal muscle

Results are means $\pm$ S.E.M.; n = number of observations. Protein degradation was evaluated in incubated extensor digitorum longus (EDL) muscles or perfused muscle in the presence of cycloheximide as described in the Experimental section. Test compounds were added to the initial medium as described in the Experimental section. Control values for incubated muscles were similar and were combined. Tissue metabolites were measured on quick-frozen muscles at the end of incubation or perfusion. * $P < 0.05$. Release of tyrosine and N^7 -methylhistidine is expressed as nmol/2 h per g of muscle for group 1, and as nmol/h per g of muscle for group 2.

Group	<i>n</i>	Tyrosine release	<i>N</i> ⁷ -Methyl-histidine release	Tissue content			
				Tyrosine (nmol/g)	<i>N</i> ⁷ -Methyl-histidine (nmol/g)	ATP (μmol/g)	Phospho-creatine (μmol/g)
1. Incubated EDL muscle							
Control	36	305 ± 24	5.79 ± 0.29	125 ± 6	4.85 ± 0.24	5.26 ± 0.26	15.02 ± 1.20
Monensin (0.5 μM)	12	183 ± 15*	3.76 ± 0.19*	100 ± 8*	3.88 ± 0.19*	4.46 ± 0.36	12.77 ± 1.02
Ouabain (100 μM)	12	275 ± 21	4.05 ± 0.20*	120 ± 10	4.15 ± 0.21*	4.95 ± 0.40	14.80 ± 1.18
Digoxin (50 μM)	6	295 ± 24	4.63 ± 0.23*				
Vanadate (100 μM)	6	270 ± 21	4.40 ± 0.22*				
2. Perfused muscle							
Control	6	165 ± 8	2.91 ± 0.15			5.77 ± 0.29	14.05 ± 0.89
Monensin (0.5 μM)	6	124 ± 6*	1.94 ± 0.10*			6.06 ± 0.30	14.13 ± 0.71
Ouabain (100 μM)	6	160 ± 8	2.30 ± 0.12*			6.35 ± 0.32	16.82 ± 0.84

Wassner *et al.*, 1980). Routinely, for incubation-medium samples 0.2–0.5 pmol of N^7 -methylhistidine was assayed against a standard of 1 pmol, whereas for perfusion-medium samples 5–10 pmol was assayed against a standard of 5 pmol.

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 and cofactors were obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Bovine serum albumin (fraction V) was obtained from Miles Laboratories (Kankakee, IL, U.S.A.), and was dissolved in Krebs-Henseleit buffer (Lowell *et al.*, 1986b; Maizels *et al.*, 1977) and dialysed against 4 litres of the same solution for 24 h. Monensin and 1-oleoyl-2-acetyl-*sn*-glycerol (DAG) were dissolved in ethanol before addition to incubation or perfusion medium; phorbol 12-myristate 13-acetate (PMA) and 4 α -phorbol were dissolved in dimethyl sulphoxide before addition to incubation medium, and vasopressin and angiotensin II were dissolved in water before addition to incubation medium. All other compounds were added in powdered form directly to batches of incubation or perfusion medium. Ethanol or dimethyl sulphoxide alone did not significantly alter rates of proteolysis.

Statistics

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

RESULTS

Protein degradation in perfused and incubated muscles

The use of perfused (Lowell *et al.*, 1986a,b; Wassner & Li, 1982; Smith & Sugden, 1986) or incubated (Goodman, 1987b) muscles to study myofibrillar protein

breakdown has been described previously. After a pre-perfusion or preincubation period, the release of N^7 -methylhistidine by muscle is linear for several hours, and tissue concentrations of the amino acid remain reasonably stable. In the present work, myofibrillar proteolysis was studied in muscles from rats starved overnight (incubated) or for 1 day (perfused). Brief starvation has been shown previously to increase the release of N^7 -methylhistidine by muscle severalfold (Lowell *et al.*, 1986a,b; Li & Wassner, 1984; Smith & Sugden, 1986). For example, the release of N^7 -methylhistidine by perfused muscle of a group ($n = 4$) of fed or 1-day-starved rats were 0.85 ± 0.07 and 2.13 ± 0.17 nmol/h per g of muscle, respectively, whereas its release by incubated muscles ($n = 6$) of fed or overnight-starved rats was 2.10 ± 0.17 and 5.25 ± 0.42 nmol/2 h per g of muscle respectively. Thus, in the present work, myofibrillar proteolysis was evaluated under stimulated conditions. For comparison, in some studies experiments were conducted on muscles from fed rats to determine effects on proteolysis under basal conditions.

Effect of monensin, ouabain, digoxin or vanadate on protein degradation in skeletal muscle

Initial studies were conducted with the sodium ionophore monensin (Pressman, 1968) and, as shown in Table 1, it diminished N^7 -methylhistidine release by both incubated or perfused muscles by 35 or 33 % respectively. Na^+ flux was next altered by inhibiting membrane-bound Na^+/K^+ ATPase activity, and, as shown in Table 1, incubations or perfusions in the presence of ouabain, digoxin or vanadate reproduced those of monensin on N^7 -methylhistidine release, although the inhibition of amino acid release (21–30 %) was slightly less than that produced by monensin. The effect of these agents was not simply due to inhibition of N^7 -methylhistidine efflux from muscle, as tissue concentrations of the amino acid were also decreased in muscles incubated with either

Table 2. Effect of PMA, 4 α -phorbol, DAG or vasopressin plus angiotensin II on protein degradation in incubated extensor digitorum longus muscles from overnight-starved rats

Results are means $\pm$ S.E.M.; n = number of observations. Muscles were incubated in the presence of cycloheximide as described in the Experimental section. Test compounds were added to the initial incubation medium. Control values for each group were similar and were combined. * P < 0.05 versus control.

Group	n	Release (nmol/2 h per g of muscle) of:	
		Tyrosine	N^7 -Methylhistidine
Control	20	298 $\pm$ 24	5.65 $\pm$ 0.28
PMA (200 ng/ml)	6	275 $\pm$ 22	4.25 $\pm$ 0.21*
4 α -Phorbol (200 ng/ml)	4	300 $\pm$ 24	5.60 $\pm$ 0.28
DAG (500 μ M)	6	295 $\pm$ 25	4.52 $\pm$ 0.23*
Vasopressin (1 μ M) plus angiotensin II (2 μ M)	4	280 $\pm$ 21	4.86 $\pm$ 0.24*

monensin or ouabain (Table 1). They were also not due to a non-specific toxic effect on muscle, as ATP and phosphocreatine concentrations were not significantly altered (Table 1). When muscles from fed rats were incubated with monensin and ouabain, they produced effects similar to those in overnight-starved rats, except that the inhibition of N^7 -methylhistidine release averaged 15–20% (results not shown).

Whereas all of the agents tested diminished myofibrillar proteolysis (i.e. N^7 -methylhistidine release), only monensin diminished tyrosine release significantly (Table 1). The effect of monensin on total proteolysis was most probably due to its inhibition of lysosomal function, as the ionophore has been shown to inhibit lysosomal cathepsin activity, presumably by raising intralysosomal pH (Maxfield, 1982). We reported previously that inhibition of lysosomal cathepsin activity with NH_4Cl , chloroquine or leupeptin inhibited tyrosine release by perfused rat muscle, but did not likewise alter N^7 -methylhistidine release (Lowell *et al.*, 1986a). In the present study, inhibitors of lysosomal cathepsin activity were also shown to decrease tyrosine release by incubated muscles by 20–25%, whereas they were without effect on N^7 -methylhistidine release (results not shown). It appears likely then that monensin, ouabain, digoxin and vanadate inhibited specifically the breakdown of myofibrillar proteins, and that this occurred through a non-lysosomal pathway. In addition, these results also re-emphasize that significant changes in myofibrillar proteolysis can occur in situations where total proteolysis is not changed (Lowell *et al.*, 1986a,b).

Effect of phorbol ester (PMA), DAG and vasopressin plus angiotensin II on protein degradation in incubated muscles

PMA, a tumour-promoting agent, has been shown to activate protein kinase C and enhance Na^+ entry into cells in exchange for H^+ (Na^+/H^+ antiport) (Moolenaar *et al.*, 1984; Aronson, 1985). As shown in Table 2, PMA, like monensin and inhibitors of Na^+/K^+ ATPase activity,

Table 3. Effect of monensin, ouabain or PMA on N^7 -methylhistidine release by incubated extensor digitorum longus muscles of overnight-starved rats in buffer lacking Na^+

Results are means $\pm$ S.E.M.; n = number of observations. Muscles were incubated in the presence of cycloheximide as described in the Experimental section. Buffer lacking Na^+ consisted of Hepes (20 mM), choline chloride (130 mM), CaCl_2 (2.5 mM), KCl (5 mM), MgSO_4 (1.2 mM), KH_2PO_4 (1.2 mM), glucose (5 mM), pH 7.4 (adjusted with 1 M-Tris base), equilibrated with 100% O_2 ; *NaCl (130 mM) substituted for choline chloride. † P < 0.05 versus control.

Group	n	N^7 -Methylhistidine release (nmol/2 h per g of muscle)
Control	12	5.25 $\pm$ 0.26
Monensin (0.5 μ M)	4	4.98 $\pm$ 0.25
Ouabain (100 μ M)	4	5.00 $\pm$ 0.17
PMA (200 ng/ml)	4	5.15 $\pm$ 0.19
Monensin (0.5 μ M)*	4	3.41 $\pm$ 0.17†
PMA (200 ng/ml)*	4	4.20 $\pm$ 0.21†

decreased (by 25%) the release of N^7 -methylhistidine by incubated muscles. The inactive phorbol ester, 4 α -phorbol, was without effect. Like PMA, a similar but smaller response was reproduced by DAG, an endogenous activator of protein kinase C (Moolenaar *et al.*, 1984; Berridge & Irvine, 1984), as well as by vasopressin plus angiotensin II, which have been shown to activate phospholipase C and generate DAG (Berridge & Irvine, 1984). These agents were without significant effects on tyrosine release, indicating that they altered specifically the breakdown of myofibrillar proteins.

Effect of monensin, ouabain or PMA on N^7 -methylhistidine release by incubated muscles: requirement for Na^+ and/or Ca^{2+}

To determine if the effects of monensin, ouabain or PMA were associated with alterations in the flux of extracellular Na^+ , incubations were done in buffer lacking Na^+ . As shown in Table 3, these agents were ineffective in attenuating N^7 -methylhistidine release by incubated muscles when Na^+ was removed from the buffer. In another series of experiments (results not shown), CaCl_2 was omitted from the Krebs–Henseleit buffer and replaced by 1 mM-EGTA. Omission of Ca^{2+} from the buffer did not modify, however, the effects of these agents on myofibrillar proteolysis. Thus effectiveness of these agents required extracellular Na^+ , but not Ca^{2+} .

DISCUSSION

Myofibrillar and non-myofibrillar protein breakdown

Information concerning the regulation of the breakdown of myofibrillar proteins in skeletal and cardiac muscle has been aided greatly by the use of N^7 -methylhistidine release by muscle as a specific marker of actin and myosin breakdown (Young & Munro, 1978). Evidence has now accumulated from several laboratories that the breakdown of myofibrillar and non-myofibrillar proteins is regulated independently and by different

pathways (Li & Wassner, 1984; Lowell *et al.*, 1986a,b; Smith & Sugden, 1986). In addition, previous known modulators of muscle proteolysis, such as insulin, amino acids and inhibitors of lysosomal proteinases, have been shown to be ineffective in modulating the breakdown of myofibrillar proteins (Li & Wassner, 1984; Lowell *et al.*, 1986a,b; Smith & Sugden, 1986; Goodman, 1987a,b; Goodman & Gomez, 1987). The ability of these agents to attenuate muscle proteolysis seems to be restricted to their ability to curtail the breakdown of the non-myofibrillar proteins. It has also been reported that the administration of catabolic doses of glucocorticoids to rats augments acutely the breakdown of myofibrillar proteins without affecting apparently the breakdown of non-myofibrillar proteins (Kayali *et al.*, 1987). Because of this, future studies must keep in mind that significant changes in the breakdown of myofibrillar proteins can occur in situations where total proteolysis is not revealing and, likewise, significant changes in total cell proteolysis can occur that bear no relationship to the breakdown of myofibrillar proteins.

Modulation of myofibrillar proteolysis by Na⁺ or activation of protein kinase C

Previous studies concerning perturbations in ion flux and proteolysis have dealt largely with Ca²⁺. It is now well documented that an increase in cell Ca²⁺ produced by Ca²⁺ ionophores, local anaesthetics or high KCl can increase skeletal-muscle proteolysis (Kameyama & Etlinger, 1979; Sugden, 1980; Lewis *et al.*, 1982; Rodemann *et al.*, 1982; Zeman *et al.*, 1985). There is some evidence that the Ca²⁺-induced rise in proteolysis is mediated by prostaglandin E₂ generation, although this remains an unresolved issue (Rodemann *et al.*, 1982; Zeman *et al.*, 1985). I have shown that Ca²⁺ ionophore A23187 and dibucaine increased tyrosine release by incubated muscles, confirming previous results; on the other hand, the release of *N*⁷-methylhistidine was not likewise affected (Goodman, 1987b). These results suggested that Ca²⁺ increased the breakdown of non-myofibrillar, but not myofibrillar, proteins.

In contrast with studies involving changes in cell Ca²⁺, in the present study alterations in Na⁺ flux with monensin, inhibitors of Na⁺-K⁺ ATPase, or activation of protein kinase C resulted in a significant attenuation of myofibrillar proteolysis (i.e. *N*⁷-methylhistidine release) in incubated muscles. The changes in myofibrillar proteolysis were not associated with similar changes in total proteolysis (i.e. tyrosine release), indicating that these agents affected specifically the breakdown of myofibrillar proteins.

These results represent the first successful attempt to modulate myofibrillar proteolysis *in vitro*. These findings were not due simply to non-specific toxic effects, as several unrelated agents which alter Na⁺ flux produced like effects. In addition, muscle ATP and phosphocreatine contents were not significantly altered, and positive effects were confined to changes in only myofibrillar proteolysis. The attenuation of myofibrillar proteolysis induced by these various agents appeared dependent on changes in Na⁺ flux, as they were eliminated when extracellular Na⁺ was absent from the incubation medium. On the other hand, these agents were quite effective when extracellular Ca²⁺ was omitted from the incubation medium. However, a role of Ca²⁺ cannot be

excluded, since changes in Na⁺ flux may enhance Ca²⁺ exchange from a pool within or bound to the plasma membrane or from an intracellular pool (Philipson, 1985).

Although the mechanism by which these agents attenuate myofibrillar proteolysis remains to be resolved, decreased activity of a non-lysosomal ATP-dependent (Goldberg & St. John, 1976; Hershko & Ciechanover, 1982) or -independent (Bird *et al.*, 1980; Fagan *et al.*, 1986) proteinase deserves consideration. In addition, a messenger role for Na⁺ ions in the regulation of myofibrillar proteolysis must also be considered. One possibility is that Na⁺ ions modulate the proteolytic system responsible for the breakdown of myofibrillar proteins. Since the effects of monensin and inhibitors of Na⁺-K⁺ ATPase were reproduced by agents that activate protein kinase C, it is also intriguing to speculate that Na⁺ influx into muscle can stimulate the hydrolysis of phospholipids, generating inositol phosphates, which can mobilize Ca²⁺ from internal stores, and diacylglycerols, which can activate protein kinase C (Berridge & Irvine, 1984). This action of Na⁺ is not inconceivable, since it has been reported that enhanced Na⁺ flux can stimulate the breakdown of phosphatidylinositol in neuronal tissue (Gusovsky *et al.*, 1986). The involvement of protein kinase C activation in proteolysis awaits further study, but in all likelihood it may involve phosphorylation and inactivation of a specific proteinase(s) in the pathway of myofibrillar protein breakdown.

To link a change in Na⁺ flux and/or an increase in cell Na⁺ to changes in muscle proteolysis is not without physiological relevance. There are several physiological situations where a change in Na⁺ flux may be associated with decreased myofibrillar proteolysis. Exercise (i.e. muscle activity), which can enhance Na⁺ flux into muscle by neurotransmitter release or by decreased activity of membrane Na⁺-K⁺ ATPase, has been reported to decrease the concentration of *N*⁷-methylhistidine in human muscles (Rennie *et al.*, 1981) and to decrease its release by perfused rat muscle subjected to electrical stimulation (Bylund-Fellenius *et al.*, 1984). Diabetes has also been shown to decrease Na⁺-K⁺ ATPase activity and to increase intracellular Na⁺ in skeletal muscle, as well as in neural tissue, from rats (Moore *et al.*, 1979; Greene & Lattimer, 1986; MacGregor & Matschinsky, 1986); furthermore, myofibrillar proteolysis is diminished in skeletal muscle from diabetic rats (Goodman, 1987a). Myofibrillar proteolysis has been reported to decrease during prolonged starvation in both humans (Young *et al.*, 1973) and animals (Lowell *et al.*, 1986b). This response has been linked to the availability and usage by muscles of circulating lipid fuels (Lowell *et al.*, 1986b), but it is also conceivable that increased non-esterified fatty acids can interact with muscle membrane lipids and thereby induce changes in Na⁺ flux (Ashavaid *et al.*, 1985). Like prolonged starvation, the continued administration of catabolic doses of glucocorticoids to rats causes an early increase in myofibrillar proteolysis, followed later by a decrease (Kayali *et al.*, 1987). Whether this adaptation is coincidentally related to a change in Na⁺ flux, as has been demonstrated in other cell types in response to glucocorticoids, remains to be seen (Rayson & Gupta, 1985). It is also noteworthy that there are digitalis-like factors present in plasma of normal, hypertensive and uraemic humans that have

been shown to inhibit Na⁺-pump activity in erythrocytes (Kelly *et al.*, 1985). The role of such endogenous factors in muscle proteolysis remains speculative, however, since in uraemic rats (Mitch & Clark, 1983) muscle proteolysis has been reported to be increased. Finally, the attenuation of myofibrillar proteolysis induced via activation of protein kinase C with PMA (and presumably with DAG and vasopressin plus angiotensin II) may be linked to the ability of mitogens and serum to decrease proteolysis in cultured muscle cells (Berridge & Irvine, 1984; Ballard & Gunn, 1982). Activation of protein kinase C in muscle as well as other cells leads to increased activity of a Na⁺/H⁺ antiport and cell alkalization (Moolenaar *et al.*, 1984; Aronson, 1985; Berridge & Irvine, 1984). It is noteworthy that insulin, like PMA and monensin, has been reported to increase the activity of a Na⁺/H⁺ antiport in muscles, resulting in cytoplasmic alkalization (Klip *et al.*, 1986); however, unlike PMA and monensin, insulin does not attenuate myofibrillar proteolysis (Lowell *et al.*, 1986a,b).

In conclusion, the present study has shown that, under certain conditions *in vitro*, acute changes in Na⁺ flux and/or intracellular Na⁺ can result in attenuation of myofibrillar proteolysis. However, these results should not be taken out of context to imply that every situation in which the entry of Na⁺ into muscle is increased or the activity of the Na⁺-K⁺ pump is decreased will result in decreased myofibrillar proteolysis. Such is likely not to be the case, as pointed out for insulin. On the other hand, the link between Na⁺ and myofibrillar proteolysis must be considered of relevance, since previous modulators of muscle proteolysis have failed to alter the breakdown of myofibrillar proteins. The involvement of phospholipids in this scheme awaits clarification.

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