Biochem. J. (1980) **190**, 593–603
Printed in Great Britain

The effects of calcium ions, ionophore A23187 and inhibition of energy metabolism on protein degradation in the rat diaphragm and epitrochlearis muscles *in vitro*

Peter H. SUGDEN
Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K.

(*Received 12 March 1980*)

1. The effects of external Ca^{2+}, EGTA, ionophore A23187, CN^-, dinitrophenol and iodoacetamide on the rate of protein degradation in the rat diaphragm and epitrochlearis muscles *in vitro* were investigated. 2. External Ca^{2+} increased protein degradation when compared with external EGTA. Protein degradation was further increased by Ca^{2+} + ionophore A23187. 3. EGTA and ionophore A23187 decreased ATP and phosphocreatine concentrations and the ATP/ADP ratio. 4. CN^-, dinitrophenol and iodoacetamide decreased protein degradation, presumably by interfering with energy metabolism. 5. The effects of EGTA may be caused by disturbances in energy metabolism. The effects of ionophore A23187 cannot be readily explained by disturbances in energy metabolism. 6. Incubation of diaphragms with Ca^{2+} causes a rapid increase in whole-tissue Ca content. This is further stimulated by ionophore A23187. The uptake of Ca^{2+} may be, at least in part, into the cytoplasm because an increase in the glycogen phosphorylase activity ratio is observed. 7. A Ca^{2+}-activated proteinase is present in rat heart and diaphragm. This enzyme may mediate in part the effects of Ca^{2+} described above. The apparent K_A of this enzyme for Ca^{2+} is about 0.25 mM. 8. Because effects of ionophore A23187 cause a large increase in whole-tissue Ca content and because the Ca^{2+}-activated proteinase has a relatively low affinity for Ca^{2+}, it is felt that the effects of Ca^{2+} upon muscle proteolysis are unlikely to be of importance in steady-state protein turnover *in vivo*. The mechanism may, however, be important in breakdown of necrotic tissue in the living animal.

Muscle proteins are continuously being synthesized and degraded (for a review, see Rannels *et al.*, 1976). The balance between the rates of these two processes determines whether net synthesis or net degradation of muscle protein occurs. The regulation of the synthesis and degradation of muscle proteins is therefore important in understanding muscle hypertrophy and atrophy. However, relatively little is known of the regulation and enzymology of muscle protein degradation. It has been proposed that various hormonal and nutritional factors (e.g. insulin, branched-chain amino acids) alter the rate of protein degradation in the isolated rat diaphragm (Fulks *et al.*, 1975). The way in which these factors affect the rate of protein degradation is poorly understood.

There have been reports indicating a possible role for Ca^{2+} in regulation of protein breakdown in the frog cutaneous pectoris and mouse diaphragm muscles (Statham et al., 1976; Publicover *et al.*, 1978). Based mainly on ultrastructural evidence, these authors have shown that treatment of muscles with the divalent-cation ionophore A23187 (Reed & Lardy, 1972) leads to rapid dissolution of the myofilaments. It has been known for many years that muscle tissues contain a Ca^{2+}-activated proteinase (Drummond & Duncan, 1968; Huston & Krebs, 1968; Busch *et al.*, 1972; Okitani *et al.*, 1974; Dayton *et al.*, 1976*a*,*b*). Several authors have proposed that this enzyme may be important in the turnover of muscle proteins. The proteinase is active at neutral pH and appears to be cytoplasmic. In view of the possible importance of Ca^{2+} in the regulation of muscle protein degradation, it seemed important to investigate whether manipulation of intracellular Ca^{2+} concentrations in intact muscle preparations could affect the rate of protein degradation.

Although there appears to be evidence that muscle protein degradation may involve neutral proteolytic systems, there is also evidence for the involvement of

0306-3283/80/090593-11$01.50/1

lysosomal systems (for a review, see Goldberg & St. John, 1976). The so-called 'energy requirement' of protein degradation in animal cells may be related to the necessity for ATP hydrolysis to maintain a relatively low intralysosomal pH (Reijngoud & Tager, 1973). There has also been a report of an ATP-requiring proteinase in reticulocyte preparations (Etlinger & Goldberg, 1977). In the present paper, the effects of some inhibitors of energy metabolism on protein degradation in muscle preparations are also reported.

Experimental

Materials

Male Wistar rats (80–110 g) were obtained from Olac (1976), Bicester, Oxon. OX6 0TP, U.K. and were kept in the laboratory animal house for at least 48h before use. They were allowed free access to food (Oxoid 41B diet) and water. Chemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. or from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Ionophore A23187 was a gift from Dr. R. L. Hamill and Dr. Mary Root of Eli Lilly & Co., Indianapolis, IN, U.S.A. Bovine insulin was a gift from Boots Pure Drug Co., Nottingham, U.K. Sagatal (sodium pentobarbitone, 60 mg/ml) was from May & Baker, Dagenham, Essex RM10 7XS, U.K. DEAE-cellulose was obtained from Whatman Biochemicals, Springfield Mill, Maidstone, Kent, U.K. Calmodulin was a gift from Dr. P. Cohen, Department of Biochemistry, University of Dundee, U.K.

Incubation of muscle preparations

For the removal of diaphragms, male rats (80–110 g) were killed either by a blow on the head and decapitation or by anaesthetization by intraperitoneal injection of 0.2 ml of Sagatal and decapitation. The carcasses were bled prior to dissection. For the epitrochlearis muscles, dissection was carried out under Sagatal anaesthesia as above. Muscles were removed as rapidly as possible and placed in freshly prepared Krebs–Henseleit (1932) bicarbonate solution at 25°C, equilibrated with O_2/CO_2 (19:1) and containing 5.5 mM-glucose. In some cases, $CaCl_2$ was omitted (see the Results and Discussion sections). Muscles were blotted lightly on filter paper and weighed into 25 ml Erlenmeyer flasks usually containing 3 ml of Krebs–Henseleit bicarbonate solution at 25°C supplemented with 5.5 mM-glucose and 0.5 mM-cycloheximide. Ionophore A23187 (when added) was present at a final concentration of 10 μg/ml and was added in 30 μl of ethanol. In such incubations, control incubations contained 30 μl of ethanol. However, it was also shown in separate experiments that this concentration of ethanol did not affect the rate of tyrosine release from muscles when compared with incubations in the absence of ethanol. The contents of the flasks were re-equilibrated with O_2/CO_2 and placed in a shaking water bath for 30 min at 37°C and 50 shaking cycles/min. After this time, muscles were transferred to fresh media, re-gassed, and incubated as before for various intervals.

Estimation of protein degradation

Protein degradation was measured by the release of tyrosine into the medium. Since this amino acid is neither synthesized nor metabolized by diaphragm (Guroff & Udenfriend, 1960) and since its reincorporation into protein is inhibited by cycloheximide, the rate of release of tyrosine is considered to be a valid measurement of protein degradation in muscle (Fulks *et al.*, 1975). Media from incubations were taken and 100% (w/v) trichloroacetic acid was added to a final concentration of 5% (w/v). Samples were centrifuged in a bench centrifuge (3000 rev./min for 5 min) and samples were taken for the assay of tyrosine by the method of Waalkes & Udenfriend (1957). Rates of tyrosine release were linear with time over the 2h incubation period. To measure tissue tyrosine concentrations, muscles were frozen in liquid N_2 after incubation, crushed and ground in ground-glass homogenizers cooled in liquid N_2 and then were dispersed in water (2 ml). Trichloroacetic acid (100%, w/v) was added to 5% (w/v) and tyrosine was assayed as described above. A standard curve for tyrosine was carried out each time.

Measurement of tissue Ca^{2+} concentrations

After incubation, quarter diaphragms were blotted on filter paper and solubilized by heating at 110°C for 1h in 0.5 ml of Analar conc. HNO_3 in glass tubes washed in conc. HNO_3. The contents of the tube were evaporated to dryness *in vacuo* over solid NaOH and P_2O_5. The residues were dissolved in 1 ml of $LaCl_3$ solution [for atomic absorption spectroscopy, 1% (w/v) La^{3+}]/0.5 M-HCl (Analar). After centrifugation for 5 min at 3000 rev./min in a bench centrifuge, the Ca^{2+} concentrations of extracts (suitably diluted in $LaCl_3$/HCl) were determined in a Pye–Unicam SP.90A series 2 atomic absorption spectrophotometer.

Assay of phosphorylase

Phosphorylase was assayed essentially as described by Hedrick & Fischer (1965). Frozen muscle tissue was crushed and ground in a ground-glass homogenizer cooled in liquid N_2 and extracted in 50 mM-sodium glycero-2-phosphate/2 mM-EDTA, pH 7.0. The homogenates were centrifuged at 50000*g* for 10 min at 4°C and samples of the supernatant (50 μl) were assayed for phosphorylase

activity at 30°C in 50μl of assay medium (32mM-glucose 1-phosphate/2% oyster glycogen, pH 7.0) in the presence or absence of 2mM-AMP. After an appropriate time, stopping reagent [2ml of a solution containing 2.77g of $(NH_4)_6MO_7O_{24}$/litre and 14.4ml of conc. H_2SO_4/litre] was added. Then, 100μl of a solution containing 149g of sodium metabisulphite/litre, 5g of sodium sulphite/litre and 2.5g of 4-amino-3-hydroxynaphthalene-1-sulphonic acid/litre was added. The A_{700} was measured and the results are expressed an an activity ratio, i.e. activity in the absence of AMP/activity in the presence of AMP. Suitable blanks were subtracted.

Assay of lactate dehydrogenase

Following incubation of quarter-diaphragms, the media and quarter-diaphragms were frozen in liquid N_2. Diaphragms were homogenized (1g/19ml of buffer) in 50mM-potassium phosphate, pH 7.0, at 0°C in ground-glass homogenizers (see above). Suitably diluted tissue extracts and media were assayed for lactate dehydrogenase activity in a Gilford recording spectrophotometer (model 240) at 340nm in a total volume of 2ml containing 50mM-potassium phosphate (pH 7.0)/0.3mM-pyruvate/0.14mM-NADH.

Preparation of Ca^{2+}-activated proteinase from rat heart and diaphragm

The method was essentially as described for bovine heart by Waxman & Krebs (1978). Rat hearts (stored at −70°C) were homogenized in 4vol. of 5mM-Tris/HCl (pH7.5)/4mM-EDTA/50mM-NaCl/3mM-2-mercaptoethanol at 0°C by using a Polytron homogenizer (Kinematica G.m.b.H., Lucerne, Switzerland). The homogenate was centrifuged at 25000*g* for 45min. The supernatant was adjusted to pH 7.5 with 1M-KOH and diluted with 4 vol. of water at 0°C. The diluted supernatant was applied to a column of DEAE-cellulose DE-11 equilibrated with 5mM-Tris/HCl (pH 7.5)/10mM-NaCl / 0.1mM - EDTA / 3mM - 2 - mercaptoethanol (about 5ml of diluted supernatant/ml column bed vol.). The column was washed with 5–10vol. of equilibration buffer and a linear gradient of NaCl (10–500mM in equilibration buffer, total volume of ten column bed vols.). Fractions were collected and assayed for Ca^{2+}-dependent proteinase activity (see below). Those fractions containing activity (eluting at about 270mM-NaCl) were pooled and solid $(NH_4)_2SO_4$ was added to 60% saturation. The precipitated protein was collected by centrifugation at 38000*g* for 30min. The precipitate was taken up in a small volume (1–2ml) of 50mM-Tris/HCl (pH 7.5)/1mM-EDTA/2mM-dithiothreitol and was dialysed for 16h against 1000vol. of the same buffer at 4°C. The preparation was stored at −20°C. Usually 50–100g of rat hearts were used for each preparation of enzyme.

To demonstrate the presence of Ca^{2+}-activated proteinase in the rat diaphragm, the above method was followed up to the DEAE-cellulose column chromatography step. The Na^+ concentration was measured as described by Sugden *et al.* (1979*a*).

Assay of Ca^{2+}-activated proteinase

The enzyme was assayed by the release of trichloroacetic acid-soluble peptides and amino acids from α-casein (Sigma). The assay contained in 0.24ml: 50mM-Tris/HCl (pH7.5)/3mM-2-mercaptoethanol/5mg of α-casein/ml and a suitable amount of Ca^{2+}-activated proteinase [diluted in 50mM-Tris/HCl (pH7.5)/1mM-EDTA]. Assays were for 5–15min at 30°C. They were run in the presence of 5mM-$CaCl_2$ or 1mM-EDTA. The assay was terminated by the addition of 10% (w/v) trichloroacetic acid (0.24ml) and tubes were spun at 3000rev./min at 4°C in a bench centrifuge. Supernatant (0.4ml) was taken and assayed essentially as described by Lowry *et al.* (1951) as modified by Barrett (1972).

Collection of blood samples

A rat was killed by a blow on the head and cervical dislocation. The heart was exposed and a blood sample was collected by puncture of the right ventricle with a syringe. About 5ml was obtained from a 300–350g rat.

Other methods

Calmodulin-deficient cyclic nucleotide phosphodiesterase was prepared by the method of Klee & Krinks (1978) and assayed by the method of Sugden *et al.* (1979*b*). For metabolite assays, tissues were frozen in liquid N_2 after incubation. They were ground in ground-glass homogenizers under liquid N_2 and extracted with $HClO_4$ (conc. $HClO_4/H_2O$, 1:19, v/v). Protein was removed by centrifugation and the supernatants were neutralized to pH7–8 (Universal indicator) with 5M-KOH. Supernatants were assayed for metabolites. ATP was determined by the method of Lamprecht & Trautschold (1974), phosphocreatine by the method of Lamprecht *et al.* (1974) and ADP and AMP by the method of Jaworek *et al.* (1974).

Results

Effects of ionophore A23187, Ca^{2+} and EGTA on the rate of protein degradation in rat epitrochlearis and diaphragm muscle

Results are shown in Table 1. In both muscles, there was an increase in the rate of tyrosine release in the presence of 2.54mM-$CaCl_2$ (the concentration of $CaCl_2$ in Krebs–Henseleit bicarbonate

Table 1. *Effects of EGTA, Ca^{2+} and ionophore A23187 on the release of tyrosine from the diaphragm and epithrochlearis muscles*

Muscles were taken and incubated as described in the Experimental section. Quarter-diaphragms were used. Incubations were in 3ml of freshly prepared Krebs–Henseleit (1932) bicarbonate solution. The additions were at the following concentrations: EGTA, 0.5mM; $CaCl_2$, 2.54mM; ionophore A23187, 10μg/ml; the branched-chain amino acids L-leucine, L-isoleucine, L-valine at 1mM each, insulin, 0.1i.u./ml. The additions were present in both the preincubation (30min) and the test incubation (2h). Results are presented as means ± S.E.M. with the number of separate experiments in parentheses. The statistical significance of the results was determined by a paired *t* test in which quarter-diaphragms were compared with controls from the same animal and the epitrochlearis was compared with a contralateral control from the same animal. Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$.

Muscle preparation	Incubation	Tyrosine release into medium (pmol/h per mg wet. wt. of tissue)	Increase over control (%)
Epitrochlearis	Control (+EGTA)	207 ± 6	—
	+ $CaCl_2$	267 ± 13 (7)***	29 ± 4
Epitrochlearis	Control (+$CaCl_2$)	250 ± 19	
	+ A23187 + $CaCl_2$	324 ± 29 (7)**	30 ± 7
Diaphragm	Control (+EGTA)	193 ± 6	
	+ $CaCl_2$	291 ± 20 (9)***	51 ± 6
Diaphragm	Control (+$CaCl_2$)	261 ± 46	
	+ A23187 + $CaCl_2$	419 ± 30 (6)**	61 ± 13
Diaphragm	Control + insulin and branched-chain amino acids (+$CaCl_2$)	244 ± 21	
	+ A23187 + insulin and branched-chain amino acids + $CaCl_2$	404 ± 27 (8)***	66 ± 10
Diaphragm	Control (+EGTA)	162 ± 6	
	+ EGTA + A23187	157 ± 4 (6)	−3 ± 4

Table 2. *Effects of EGTA, Ca^{2+} and ionophore A23187 on tyrosine concentrations in the diaphragm muscle*

Quarter-diaphragms were incubated as described in the Experimental section and in the legend to Table 1. Additions were as described in the legend to Table 1. Results are presented as means ± S.E.M. with the number of observations in parentheses. The zero time point is taken after the initial 30min preincubation.

Addition to incubation	Time (min) ...	Tyrosine content (pmol/mg wet wt. of diaphragm) 0	120	Decrease
$CaCl_2$		95 ± 3 (6)	86 ± 2 (6)	9 ± 4
EGTA		83 ± 4 (3)	62 ± 8 (3)	21 ± 9
A23187 + $CaCl_2$		102 ± 4 (3)	94 ± 1 (3)	8 ± 4

solution) compared with controls where $CaCl_2$ was absent and 0.5mM-EGTA was present. The rate of tyrosine release could be further increased by inclusion of ionophore A23187 (10μg/ml) in the presence of 2.54mM-$CaCl_2$. The effects of ionophore A23187 were dependent upon the presence of Ca^{2+}. There was no significant difference in the rate of tyrosine release from quarter diaphragms in incubations omitting Ca^{2+} but containing EGTA compared with those omitting Ca^{2+} and containing EGTA and ionophore A23187 (Table 1). Furthermore, the possibility that differences in the rate of tyrosine release were caused by differences in the concentration of Mg^{2+} in incubations containing EGTA can be excluded because it was calculated that, at the concentration of EGTA used in experiments, the decrease in free Mg^{2+} concentration in the incubation medium was only about 3% (compared with that in the absence of EGTA).

Effects of the various agents on tissue total tyrosine concentrations were examined to exclude the possibility of effects of ionophore A23187 or EGTA on tissue tyrosine depletion (Table 2). In general, the greater the rate of tyrosine release over the course of the 2h incubation, the greater was the tissue tyrosine concentration. Tissue tyrosine concentrations (Table 2) did not decrease sufficiently over the incubation period to account for the observed changes in the rate of tyrosine release (Table 1). In incubations containing $CaCl_2$, and $CaCl_2$ plus ionophore A23187, decreases in tissue tyrosine concentrations were similar, although the overall rate of tyrosine release was greater in the

latter condition (Table 1). The largest decrease in tissue tyrosine concentration (in incubations plus EGTA) was observed under experimental conditions in which the rate of tyrosine release into the medium was the lowest observed of all experimental conditions tested. These experiments show that the action of the agents was not mediated solely by alterations in the diaphragm tyrosine content. These results suggest that, if there are effects of the agents on tyrosine transport, they are secondary to the effects on proteolysis. The results do not definitely exclude the possibility that ionophore A23187 or EGTA may cause alterations in tyrosine transport. These results strongly suggest that muscle tissue protein degradation *in vitro* can be increased by increasing the external Ca^{2+} concentrations. The results with ionophore A23187 suggest that facilitation of Ca^{2+} entry into muscle cells increases the rate of protein degradation.

From the amino acid composition of diaphragm (Odessey *et al.*, 1974) and the protein content of the diaphragm (0.2 mg/mg wet wt.), the proportion of muscle tyrosine mobilized during the experimental incubations can be calculated. The diaphragm tyrosine content is 52.7 nmol/mg wet wt. Thus the fractional loss of tyrosine calculated from Table 1 ranges between 0.37–0.80%/h.

However there are other possible interpretations of the role of Ca^{2+} in muscle protein degradation. The possibility that increased protein degradation was caused by an increase in proteolytic activity in contaminating blood in the presence of Ca^{2+} was excluded by incubating rat blood, as described in the Experimental section, in the presence of 2.54 mM-$CaCl_2$ or 0.5 mM-EGTA. No increase in tyrosine release was seen either with time or in the presence of Ca^{2+}. The concentration of tyrosine in the blood was about 0.027 mM. Furthermore no Ca^{2+}-dependent proteolytic activity was eluted from DEAE-cellulose columns at 0.27 M-NaCl when blood was used as a starting material in experiments analogous to the purification of the Ca^{2+}-activated proteinase from rat heart (see the Experimental section).

Ca^{2+} is important in exocytosis from mast cells [for a review, see Douglas (1974)]. Since these cells contain proteolytic activities, it seemed possible that Ca^{2+} could act to increase tyrosine release via inducing the degranulation of mast cells. To test this, experiments were performed with rat quarter-diaphragms (see the Experimental section) in the absence and presence of the mast cell degranulator compound 48/80 (condensation product of *p*-methoxy-*N*-methylphenethylamine with formaldehyde; see Rohlich *et al.*, 1971). No statistically significant differences were seen between incubations in the absence or presence of compound 48/80 (50 μg/ml) either in the presence of $CaCl_2$ (2.54 mM) or of EGTA (0.5 mM). It is therefore suggested that the effects of Ca^{2+} in increasing tyrosine release are not mediated by mast cells.

Effects of ionophore A23187, Ca^{2+} and EGTA upon the loss of lactate dehydrogenase from the diaphragm

Media from incubation of quarter-diaphragms with the above agents were assayed for the cytoplasmic enzyme lactate dehydrogenase as an indication of the integrity of the cell membrane and cell death (Figs. 1*a* and 1*b*). Over the course of a 2 h incubation, quarter-diaphragms incubated in the presence of Ca^{2+} lost only 3.5% of their lactate dehydrogenase to the medium. The rate of loss of lactate dehydrogenase from quarter-diaphragms incubated with EGTA or ionophore A23187 was 2–4 times greater than in incubations plus Ca^{2+}. After 2 h of incubation, diaphragms incubated with EGTA had lost 8.7% of their lactate dehydrogenase, whereas those incubated with ionophore A23187 plus Ca^{2+} lost 13.6% of their lactate dehydrogenase. There was no statistically significant difference between the release of lactate dehydrogenase in incubations plus EGTA compared to the incubations plus ionophore and Ca^{2+}. The loss of lactate dehydrogenase from incubations plus EGTA or from incubations plus ionophore A23187 and Ca^{2+} was significantly greater than from incubations plus Ca^{2+}. It therefore appears unlikely that the differences observed in tyrosine release in incubations (Table 1) can result from differences in membrane integrity in the various preparations, since the rate of tyrosine release from diaphragms incubated with ionophore A23187 and Ca^{2+} is significantly greater than from diaphragms incubated with EGTA (Table 1), although the rate of lactate dehydrogenase release from these preparations is not significantly different.

Effects of inhibitors of energy metabolism on protein degradation in the diaphragm

The results are shown in Table 3. The three inhibitors of energy metabolism tested (dinitrophenol, CN^- and iodoacetamide) caused significant decreases in the rate of protein degradation. The rates of tyrosine release into the media, and the tissue concentrations of tyrosine, are decreased in the presence of inhibitors. The results obtained with iodoacetamide are in conflict with the results of Chang & Goldberg (1978) who observed a 90% increase in the rate of tyrosine release in the presence of 0.2 mM-iodoacetate. The reasons for this difference are unknown.

Effects of ionophore A23187 and EGTA on Ca^{2+} uptake by the quarter diaphragm

To understand the effects of Ca^{2+}, the total Ca

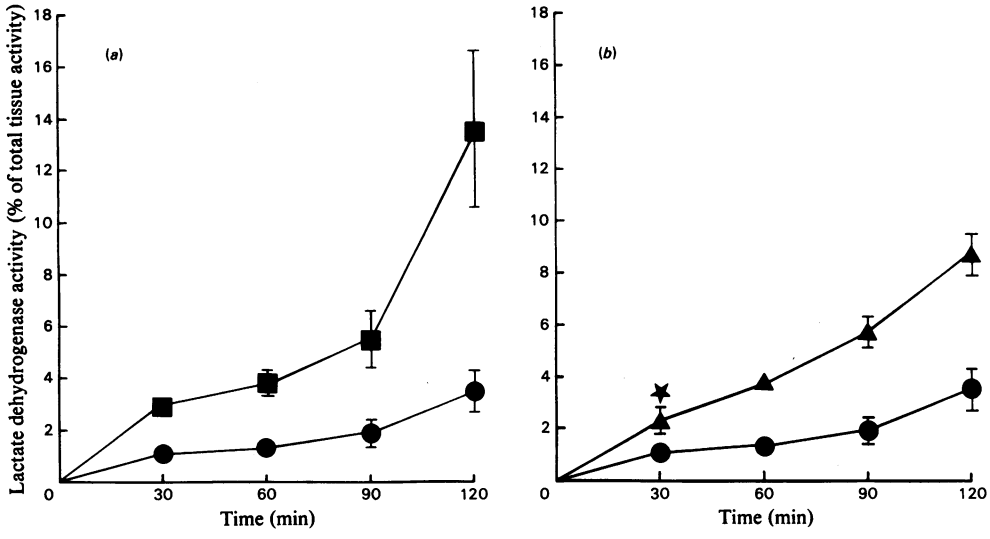

Fig. 1. *Effects of ionophore A23187 and EGTA on the release of lactate dehydrogenase from the quarter-diaphragm into the medium*

Incubations were carried out as described in the Experimental section and in the legend to Table 1. The following additions were made: (*a*) ●, 2.54 mM-$CaCl_2$; ■, 2.54 mM-$CaCl_2$ and ionophore A23187 (10 μg/ml); (*b*) ●, 2.54 mM-$CaCl_2$; ▲, 0.5 mM-EGTA. Zero-time is taken as after the 30 min preincubation. The release of lactate dehydrogenase is expressed as a percentage of the total tissue lactate dehydrogenase. Results are presented as means ± S.E.M. for three observations. With the exception of the point marked with a star, all results are statistically significantly different from the incubation with 2.54 mM-$CaCl_2$ at at least $P < 0.05$ by Student's *t*-test.

Table 3. *Effects of 2,4-dinitrophenol, CN^- and iodoacetamide on the release of tyrosine from the diaphragm*

Muscles were incubated in the absence of Ca^{2+} (in the presence of 0.5 mM-EGTA) as described in the Experimental section and in the legend to Table 1. The additions were at the following concentrations; 2,4-dinitrophenol, 0.2 mM; KCN, 2 mM; iodoacetamide, 0.5 mM. Results are presented as means ± S.E.M. with the number of observations in parentheses. Statistical significance was determined as described in Table 1: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(*a*) Release of tyrosine into the incubation medium

Inhibitor	Tyrosine release (pmol/h per mg wet wt. of tissue)	Inhibition (%)
2,4-Dinitrophenol		
No inhibitor	207 ± 20	—
+Inhibitor	112 ± 8 (10)***	46 ± 8
CN^-		
No inhibitor	173 ± 10	—
+Inhibitor	118 ± 7 (7)***	32 ± 5
Iodoacetamide		
No inhibitor	173 ± 10	—
+Inhibitor	110 ± 9 (7)***	36 ± 3

(*b*) Tyrosine concentrations in the diaphragm after 120 min of incubation

Inhibitor	Tyrosine concentration (pmol/mg wet wt. of tissue)	Decrease (%)
No inhibitor	94 ± 10 (4)	—
2,4-Dinitrophenol	60 ± 3 (4)*	37 ± 9
CN^-	61 ± 10 (4)**	35 ± 3
Iodoacetamide	60 ± 3 (4)*	36 ± 11

concentration in the quarter-diaphragm during incubation was measured (Fig. 2). In the presence of 2.54 mM-$CaCl_2$, there was a large and rapid increase in total tissue Ca. By using a dry wt. for the diaphragm of 0.213 ± 0.005 mg/mg wet wt. (mean ± S.E.M. for ten determinations) and an inulin space of 0.354 μl/mg wet wt. (Fulks *et al.*, 1975) it can be calculated that, were all the Ca free in the cell

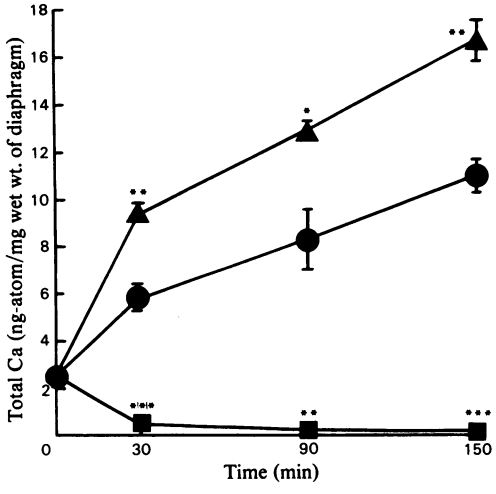

Fig. 2. *Effects of ionophore A23187 and EGTA on the quarter diaphragm total Ca concentration*
Incubations were carried out as described in the Experimental section and in the legend to Table 1. The following additions were made: ●, 2.54mM-$CaCl_2$; ▲, 2.54mM-$CaCl_2$ and ionophore A23187 (10μg/ml); ■, 0.5mM-EGTA. In this experiment, there was no preincubation. Statistically significant difference from the incubation with $CaCl_2$ was determined by Student's *t*-test. Results are presented as means ± S.E.M. for three to seven observations for each point; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

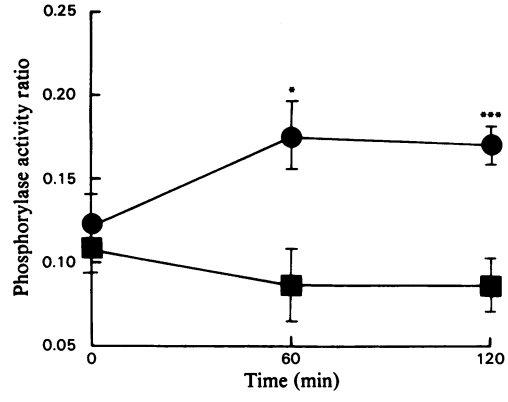

Fig. 3. *Effects of Ca^{2+} and EGTA on glycogen phosphorylase activity ratios in the diaphragm*
Incubations were carried out as described in the Experimental section and in the legend to Table 1. The following additions were made: ●, 2.54mM-$CaCl_2$; ▲, 0.5mM-EGTA. Zero time is taken as after the 30min preincubation. Results are presented as means ± S.E.M. for six to twelve observations at each point. Statistical significance was determined by Student's *t*-test: $^*P<0.05$, $^{***}P<0.001$.

water, the net Ca^{2+} concentration would be approx. 17mM after 150min of incubation. In the presence of ionophore A23187, Ca^{2+} uptake is further increased over the control (plus 2.54mM-$CaCl_2$) incubations. This demonstrates that ionophore A23187 increases the total Ca concentration in the diaphragm. In the absence of Ca^{2+} (plus 0.5mM-EGTA), the diaphragm is approx. 80% depleted of its total Ca within 30min.

There is a possibility, however, that Ca^{2+} is merely binding to extracellular structures rather than entering the cell. To examine this possibility, the activity of the cytoplasmic enzyme glycogen phosphorylase (EC 2.4.1.1) was examined. Phosphorylase kinase can be activated by Ca^{2+}, either by binding to its δ subunit (Brostrom *et al.*, 1971; Cohen *et al.*, 1978) or via a Ca^{2+}-activated limited proteolysis (Drummond & Duncan, 1968; Huston & Krebs, 1968). An increase in phosphorylase kinase activity will cause an increase in the phosphorylase activity ratio. An increase in the phosphorylase activity ratio in the presence of Ca^{2+} but in the absence of agents causing alterations in cyclic AMP concentrations is thus qualitatively indicative of an increase in cytosolic Ca^{2+} concentration, although it does not provide information about the mechanism of activation of phosphorylase kinase or about the extent of the increase in cytosolic Ca^{2+} concentration. As can be seen in Fig. 3 incubation of quarter diaphragms in the presence of 2.54mM-$CaCl_2$ causes a significant increase in the activity ratio of phosphorylase, possibly indicating an increase in cytosolic Ca^{2+} concentration during the test incubation. When the diaphragm was removed from the animal, the phosphorylase activity ratio was 0.233 ± 0.030 (mean ± S.E.M. for 12 observations). At the start of the test incubation, the activity ratio was 0.122 ± 0.018 in incubations plus Ca^{2+} and 0.107 ± 0.013 in incubations plus EGTA (mean ± S.E.M. for six observations). Thereafter, the activity ratio in the incubations plus Ca^{2+} increased (Fig. 3) whereas the activity ratio in the incubations plus EGTA decreased slightly. The decline in phosphorylase activity ratio during the preincubation is probably caused by the fact that the phosphorylase activity ratio is initially elevated because of the release of catecholamines engendered by the death of the animals causing an elevation in cyclic AMP concentrations. A preincubation is necessary to allow a decline in cyclic AMP concentration in the tissue and a reversal of phosphorylase activation. The changes in phosphorylase activity ratio may only be a crude qualitative indication of a rise in cytoplasmic Ca^{2+} concentration. There is no significant difference in

the phosphorylase activity ratios in the two incubations (plus Ca^{2+} ions and plus EGTA) after the preincubation although there is a significant difference in tissue total Ca^{2+} content (Fig. 2). This may reflect the time taken to cause a sufficient increase in cytoplasmic Ca^{2+} concentration. It is unlikely that Ca^{2+} could cause an increase in phosphorylase activity ratio via an increase in tissue cyclic AMP concentrations, since Ca^{2+} inhibits adenylate cyclase (Perkins, 1973) and activates cyclic nucleotide phosphodiesterase (Cheung, 1970).

Effects of Ca^{2+} concentration and ionophore A23187 on muscle contents of ATP, ADP, AMP and phosphocreatine

Because agents which interfere with energy metabolism (CN^- and 2,4-dinitrophenol) decrease the rate of tyrosine release by the quarter-diaphragm (Table 3), it was important to establish the effects of incubation in the presence and absence of Ca^{2+} and in the presence of A23187 on the concentrations of compounds involved in energy metabolism in the quarter diaphragm (Table 4). Both EGTA and A23187, at the concentrations used, caused significant decreases in ATP and phosphocreatine concentrations and in the ATP/ADP concentration ratio as compared with control incubations in the presence of 2.54 mM-$CaCl_2$. This result raises the possibility that the effects of Ca^{2+} upon proteolysis (Table 1) may occur via the same mechanism as the effects of 2,4-dinitrophenol and CN^-, i.e. decreases in ATP and phosphocreatine concentrations somehow inhibit proteolysis. Further work is necessary to examine this correlation. Although A23187 decreases ATP and phosphocreatine concentrations and the ATP/ADP concentration ratio, (Table 4), it increases the rate of proteolysis in the presence of 2.54 mM-$CaCl_2$ (Table 1) suggesting that this latter effect is caused by increasing intracellular Ca^{2+} concentrations. This is further supported by measurements of diaphragm total Ca. The effects of A23187 upon the concentrations of energy metabolites differ from those observed by Kameyama & Etlinger (1979) who deduced that there was no change in ATP concentrations on the basis of rates of protein synthesis. The direct measurements shown here should provide a better indication.

Although the results using the activity ratio of glycogen phosphorylase are possibly indicative of a rise in cytosolic Ca^{2+} concentrations in diaphragms incubated with $CaCl_2$, in view of the above results the decrease in the ATP concentration could cause a decrease in the glycogen phosphorylase activity ratio. However, by calculating the intracellular ATP concentration and by using a K_m of phosphorylase kinase for ATP of 0.24 mM (Krebs *et al.*, 1964), it

Table 4. *Effects of EGTA and ionophore A23187 on the tissue concentrations of phosphocreatine, ATP, ADP and AMP*

Muscles were incubated as described in the Experimental section and in the legend to Table 1. Additions were as described in Table 1. Results are presented as means ± S.E.M. with the number of observations in parentheses. Statistical significance of results compared with incubations in the presence of Ca^{2+} was determined by Student's *t* test: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Abbreviation: N.D., not detectable.

Muscle	Addition to incubation	Tissue content (nmol/mg wet wt. of tissue)				
		Phosphocreatine	ATP	ADP	AMP	ATP/ADP ratio
Diaphragm	$CaCl_2$	5.990 ± 0.546 (8)	3.240 ± 0.226 (8)	0.567 ± 0.022 (8)	0.048 ± 0.007 (8)	5.27 ± 0.32 (8)
	EGTA	2.820 ± 0.281 (8)***	1.990 ± 0.158 (8)***	0.497 ± 0.032 (8)	0.054 ± 0.009 (8)	4.00 ± 0.14 (8)**
	A23187 + $CaCl_2$	3.085 ± 0.253 (4)**	1.785 ± 0.065 (4)**	0.451 ± 0.026 (4)**	0.026 ± 0.008 (4)	4.00 ± 0.32 (4)*
Epitrochlearis	$CaCl_2$	6.580 ± 0.540 (9)	2.800 ± 0.246 (9)	0.645 ± 0.036 (6)	N.D.	4.68 ± 0.41 (6)

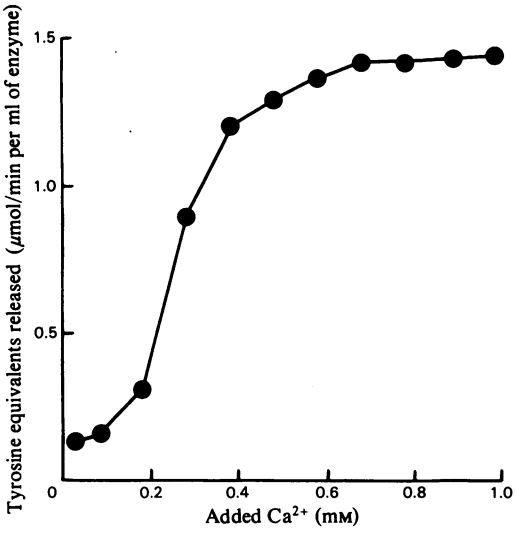

Fig. 4. *Effects of Ca^{2+} concentration on the activity of partially purified Ca^{2+}-activated proteinase from rat heart*

The Ca^{2+}-activated proteinase was partially purified from rat heart as described in the Experimental section. The assay was as described in the Experimental section at various concentrations of $CaCl_2$.

can be shown that the tissue concentrations of ATP in the incubations in the presence and absence of Ca^{2+} are sufficient to saturate the enzyme to the extent of 96.9 and 95.1%, respectively.

Effects of Ca^{2+} concentration on the activity of the Ca^{2+}-activated proteinase from the rat

A Ca^{2+}-activated proteinase is present in the rat diaphragm (results not shown). This enzyme is probably identical to that from pig muscle (Dayton *et al.*, 1976*a*,*b*) and bovine heart (Waxman & Krebs, 1978) in view of the similar behaviour during chromatography on DEAE-cellulose columns. The Ca^{2+} requirement of the rat heart enzyme was investigated (Fig. 4). The concentration of Ca^{2+} required for half-maximal activity was 0.25 mM. This requirement was not altered in the presence of calmodulin (1.6 μg/ml). There was no evidence that boiled fractions of Ca^{2+}-activated proteinase activity eluted from DEAE-cellulose would activate calmodulin-deficient Ca^{2+} dependent cyclic nucleotide phosphodiesterase (Klee & Krinks, 1978). The Ca^{2+}-activated proteinase was not inhibited by 67 μM-trifluoperazine [an inhibitor of calmodulin-dependent cyclic nucleotide phosphodiesterase and calmodulin-dependent glycogen synthetase phosphorylation; see Levin & Weiss (1977) and Srivastava *et al.* (1979)]. These results indicate that the Ca^{2+}-activated proteinase is not a calmodulin-dependent enzyme.

Discussion

The results presented in the present paper suggest that an increase in the intracellular concentration of Ca^{2+} can increase the rate of muscle protein degradation *in vitro*. This was shown for a relatively aerobic muscle (the diaphragm) and a relatively anaerobic skeletal muscle (the epitrochlearis). While these studies were in progress, Kameyama & Etlinger (1979) showed analogous results with an aerobic skeletal muscle, the soleus of the rat. The results in the present paper, however, question the importance of changes in intracellular Ca^{2+} concentrations in steady state protein turnover *in vivo*. Large amounts of Ca were taken up rapidly by the diaphragm incubated *in vitro* (Fig. 2) and this rapid uptake can be further increased by the inclusion of ionophore A23187 in the incubation medium. It seems to be generally accepted that the cytosolic concentration of Ca^{2+} in the muscle under resting conditions is about 10^{-8} M and this can increase to 10^{-6} M upon contraction. While it is still possible that the Ca^{2+} taken up by the incubated diaphragm is bound to extracellular structures, it would seem that the magnitude of the increase and the data on glycogen phosphorylase activity ratios (Fig. 3) renders this explanation unlikely. In experiments with ionophore A23187, the increase in proteolysis is probably dependent on the increase in tissue Ca^{2+} concentrations rather than on the 'energy status' of the tissue. This finding is not so apparent in incubations carried out in the presence and absence of $CaCl_2$, when there is a decrease in the concentrations of phosphocreatine and ATP in the absence of $CaCl_2$ (Table 4). Furthermore, the only Ca^{2+}-activated proteinase so far described in muscle tissue appears to have an apparent K_A for Ca^{2+} of approx. 0.25 mM, many times greater than the cytoplasmic Ca^{2+} concentration (see Fig. 4).

Although the putative importance of Ca^{2+} in steady-state intracellular protein turnover *in vivo* is diminished by the above findings, it is important to understand the process whereby a low (about 2 ng-atoms/mg wet wt.) tissue Ca content is normally maintained *in vivo*. This is clearly altered in the diaphragm incubated *in vitro*. There might possibly be a pathological role for Ca^{2+}in autolysis after cell death (both in the living animal and after death of the animal) and in the various myopathic diseases. Cellular necrosis can occur in the living animal. For example, a cellular necrosis occurs in the heart following non-fatal myocardial infarction. The dissolution of such tissue may involve increases in cytoplasmic Ca^{2+} concentration. These autolytic processes may be mediated by the Ca^{2+}-activated

proteinase described here. Evidence has also been presented that Ca^{2+} handling by dystrophic muscle is defective (Takagi *et al.*, 1973; Engel, 1977; Engel *et al.*, 1977; Samaha, 1977). This possibility was also discussed by Kameyama & Etlinger (1979). The diaphragm incubated *in vitro* may be a better model of a dystrophic muscle than of a normal muscle preparation *in vivo*.

A further finding is that agents that produce tissue anoxia (2,4-dinitrophenol and CN^-) decrease muscle tissue proteolysis. Other workers have made analogous observations in other tissues (Simpson, 1953; Steinberg & Vaughn, 1956; Brostrom & Jeffay, 1970; Hershko & Tomkins, 1971; Poole & Wibo, 1973; Chua *et al.*, 1979). Tissue proteolysis is decreased when energy metabolism is inhibited and presumably when there is a fall in ATP concentrations. It is therefore important to understand the basis of the 'energy requirement' of proteolysis. Factors such as an ATP-requiring lysosomal proton pump (see also Ballard, 1977) or an ATP-requiring proteinase (Etlinger & Goldberg, 1977) have been suggested to be implicated in this finding.

The characteristics of Ca^{2+} uptake by the isolated diaphragm lead one to question whether this tissue is a satisfactory model for the study of hormonal effects. It is known that the activities of certain enzymes of the cyclic AMP system and glycogen metabolism are affected by Ca^{2+} concentrations. Whether it is valid to examine hormonal affects on these enzymes when the diaphragm is taking up Ca^{2+} at such a rapid rate seems problematical.

I thank the Wellcome Trust for a Senior Research Fellowship and Miss Caroline E. Smith for technical assistance.

References

Ballard, F. J. (1977) *Essays Biochem.* **13**, 1–39

Barrett, A. J. (1972) in *Lysosomes* (Dingle, J. T., ed.), pp. 46–135, North-Holland, Amsterdam and London

Brostrom, C. O. & Jeffay, H. (1970) *J. Biol. Chem.* **245**, 4001–4008

Brostrom, C. O., Hunkeler, F. L. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1961–1967

Busch, W. A., Stromer, M. H., Goll, D. E. & Suzuki, A. (1972) *J. Cell Biol.* **52**, 367–381

Chang, T. W. & Goldberg, A. L. (1978) *J. Biol. Chem.* **253**, 3677–3684

Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **38**, 533–538

Chua, B., Kao, R. L., Rannels, D. E. & Morgan, H. E. (1979) *J. Biol. Chem.* **254**, 6617–6623

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. & Nairn, A. C. (1978) *FEBS Lett.* **92**, 287–293

Dayton, W. R., Goll, D. E., Zeece, M. G., Robson, R. M. & Reville, W. J. (1976*a*) *Biochemistry* **15**, 2150–2158

Dayton, W. R., Reville, W. J., Goll, D. E. & Stromer, M. H. (1976*b*) *Biochemistry*, **15**, 2159–2167

Douglas, W. W. (1974) *Biochem. Soc. Symp.* **39**, 1–28

Drummond, G. I. & Duncan, L. (1968) *J. Biol. Chem.* **243**, 5532–5538

Engel, W. K. (1977) in *Pathogenesis of Human Muscular Dystrophies* (Rowland, L. P., ed.), pp. 277–309, Excerpta Medica, Amsterdam

Engel, A. G., Mokhri, B., Jerusalem, F., Sakakibara, H. & Paulson, O. B. (1977) in *Pathogenesis of Human Muscular Dystrophies* (Rowland, L. P., ed.), pp. 310–324, Excerpta Medica, Amsterdam

Etlinger, J. D. & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 54–58

Fulks, R. M., Li, J. B. & Goldberg, A. L. (1975) *J. Biol. Chem.* **250**, 290–298

Goldberg, A. L. & St. John, A. C. (1976) *Annu. Rev. Biochem.* **45**, 747–803

Guroff, G. & Udenfriend, S. (1960) *J. Biol. Chem.* **235**, 3518–3522

Hedrick, J. L. & Fischer, E. H. (1965) *Biochemistry* **4**, 1337–1343

Hershko, A. & Tomkins, G. M. (1971) *J. Biol. Chem.* **246**, 710–714

Huston, R. B. & Krebs, E. G. (1968) *Biochemistry* **7**, 2116–2122

Jaworek, D., Gruber, W. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., vol. 4, pp. 2127–2131, Academic Press, New York and London

Kameyama, T. & Etlinger, J. D. (1979) *Nature (London)* **279**, 344–346

Klee, C. B. & Krinks, M. H. (1978) *Biochemistry* **17**, 120–126

Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L. & Fischer, E. H. (1964) *Biochemistry* **3**, 1022–1033

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Lamprecht, W. & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., vol. 4, pp. 2101–2110, Academic Press, New York and London

Lamprecht, W., Stein, P., Heinz, F. & Weisser, H. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. ed.), 2nd edn., vol. 4, pp. 1777–1781, Academic Press, New York and London

Levin, R. M. & Weiss, B. (1977) *Mol. Pharmacol.* **13**, 690–697

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Odessey, R., Khairallah, E. A. & Goldberg, A. L. (1974) *J. Biol. Chem.* **249**, 7623–7629

Okitani, A., Otsuka, Y., Sugitani, M. & Fujimaki, M. (1974) *Agric. Biol. Chem.* **38**, 573–579

Perkins, J. P. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 1–64

Poole, B. & Wibo, M. (1973) *J. Biol. Chem.* **248**, 6221–6226

Publicover, S. J., Duncan, C. J. & Smith, J. L. (1978) *J. Neuropathol. Exp. Neurol.* **37**, 544–557

Rannels, D. E., McKee, E. E. & Morgan, H. E. (1976) in *Biochemical Actions of Hormones* (Litwack, G., ed.), pp. 135–195, Academic Press, New York, San Francisco and London

Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977

Reijngoud, D. J. & Tager, J. M. (1973) *Biochim. Biophys. Acta* **297**, 174–178
Rohlich, P., Anderson, P. & Uvnas, B. (1971) *J. Cell Biol.* **51**, 465–483
Samaha, F. J. (1977) in *Pathogenesis of Human Muscular Dystrophies* (Rowland, L. P., ed.), pp. 633–639, Excerpta Medica, Amsterdam
Simpson, M. V. (1953) *J. Biol. Chem.* **201**, 143–154
Srivastava, A. K., Waisman, D. M., Brostrom, C. O. & Soderling, T. R. (1979) *J. Biol. Chem.* **254**, 583–586
Statham, H. E., Duncan, C. J. & Smith, J. L. (1976) *Cell Tissue Res.* **173**, 193–209
Steinberg, D. & Vaughn, M. (1956) *Arch. Biochem. Biophys.* **65**, 93–105
Sugden, M. C., Ashcroft, S. J. H. & Sugden, P. H. (1979*a*) *Biochem. J.* **180**, 219–229
Sugden, M. C., Christie, M. R. & Ashcroft, S. J. H. (1979*b*) *FEBS Lett.* **105**, 95–100
Takagi, A., Schotland, D. L. & Rowland, L. P. (1973) *Arch. Neurol.* **28**, 380–388
Waalkes, T. P. & Udenfriend, S. (1957) *J. Lab. Clin. Med.* **50**, 733–736
Waxman, L. & Krebs, E. G. (1978) *J. Biol. Chem.* **253**, 5888–5891