Role of Ca^{2+} for protein turnover in isolated rat hepatocytes

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1. Experiments with bivalent-cation chelators (EGTA and EDTA), a Ca^{2+} ionophore (A23187) and a Ca^{2+} -channel blocker (verapamil) indicate that Ca^{2+} is required for the lysosomal degradation of endogenous protein in hepatocytes. A distinction is made between lysosomal and non-lysosomal degradation by using the lysosomotropic agent methylamine. 2. As Ca^{2+} does not appear to be required for the lysosomal degradation of endocytosed asialo-fetuin, the Ca^{2+} -dependence for the degradation of endogenous protein is probably connected with the formation of autophagic vacuoles or the fusion of autophagic vacuoles with lysosomes. 3. EGTA and EDTA had a slight inhibitory effect on the non-lysosomal degradation. This effect could be due to the activity of non-lysosomal Ca^{2+} -dependent thiol proteinases. Together with previous experiments with thiol-proteinase inhibitors, the present experiments indicate that these proteinases have a very limited impact on the bulk protein degradation in the isolated hepatocytes.

Two bivalent cations, Ca^{2+} and Mg^{2+} , might be expected to be of importance for protein degradation, Mg^{2+} because it is needed for many cellular processes requiring ATP. All the lysosomal and most of the non-lysosomal protein degradation in hepatocytes has been shown to be energy-dependent (Seglen *et al.*, 1979). Ca^{2+} is necessary for the activation of certain proteinases, and might possibly be involved in processes connected with autophagy.

Most tissues seem to contain non-lysosomal Ca^{2+} -dependent thiol proteinases, referred to as calpains, as well as the specific calpain inhibitor protein calpastatin (Murachi *et al.*, 1980; Murachi, 1983). In rat liver at least two different Ca^{2+} -dependent proteinases have been reported from total liver mitochondria (Beer *et al.*, 1982; Rapoport *et al.*, 1982), and two from hepatocytic cytosol (DeMartino, 1981, 1982). Ca^{2+} has been shown to stimulate protein degradation in muscle (Kameyama & Etlinger, 1979; Sugden, 1980).

Regarding the role of Ca^{2+} in autophagy, it should be noted that Ca^{2+} is known to stimulate exocytosis in many different systems (Douglas, 1974), possibly by being required for the fusion of exocytic vesicles with the plasma membrane. In a cell-free system it has been shown that the fusion of secretory vesicles (with each other) depends on the Ca^{2+} -activated protein synexin (Creutz & Pollard, 1982). Similarly, Ca^{2+} may be needed for the fusion of autophagic vacuoles with lysosomes; or a Ca^{2+} requirement might be exerted via microtubules. Lysosomal degradation in hepatocytes appears to depend on

microtubules (Grinde & Seglen, 1981), and Ca^{2+} , via calmodulin-binding proteins, is involved in the control of aggregation of microtubules and microfilaments (Kakiuchi & Sobue, 1983).

It is well known that protein synthesis requires Mg^{2+} . In muscle preparations Ca^{2+} has been reported to stimulate protein synthesis as well as protein degradation (Kameyama & Etlinger, 1979).

The main purpose of the present paper is to evaluate the role of Ca^{2+} in the degradation of endogenous protein. Lysosomotropic weak bases, such as methylamine, appear to cause a relatively complete and selective inhibition of lysosomal protein degradation in hepatocytes (Seglen *et al.*, 1979, 1981; Grinde & Seglen, 1980; Amenta & Brocher, 1980; Grinde, 1982). By using methylamine it should therefore be possible to distinguish between the effect of Ca^{2+} on non-lysosomal degradation, possibly related to Ca^{2+} -dependent proteinases, and the effect of Ca^{2+} on lysosomal degradation, possibly related to effects on autophagy or fusion of vesicles.

Experimental

Cells

Isolated hepatocytes were prepared from the liver of 16h-starved male Wistar rats (220–260 g) by the collagenase-perfusion method (Seglen, 1976). The cells were incubated in suspension as previously described (Grinde & Seglen, 1980). The buffer employed, suspension buffer (Seglen, 1976), was

modified by removing bivalent cations and adding 15 mM-pyruvate and 10 mM-glucose. Viability was routinely tested, both before and after incubation with the various additions, and was found to be in the range 90–95% according to the Trypan Blue-exclusion test.

Degradation of endogenous protein

For the radioactive labelling of hepatocytic protein, a rat received an intravenous injection of 50 μ Ci of [^{14} C]valine 24 h before cell preparation. At the end of the experimental incubation, cells were precipitated by the addition of 0.1 ml of 10% (w/v) HClO_4 . Free (i.e. acid-soluble) and protein-incorporated (i.e. acid-precipitable) [^{14} C]valine were measured by liquid-scintillation counting (Grinde & Seglen, 1980). The net release of [^{14} C]valine during the incubation period was expressed as percentage of the initial protein-incorporated (i.e. acid-precipitable), radioactivity of the sample. Unlabelled valine (5 mM) was added to prevent reutilization of [^{14} C]valine, although reutilization, even without added valine, does not appear to be any problem in this system (Seglen *et al.*, 1979).

Degradation of endocytosed asialo-fetuin

The degradation of asialo-fetuin was measured as described by Grinde & Seglen (1981). Briefly, cells were incubated for 15 min with ^{125}I -labelled asialo-fetuin. Extracellular asialo-fetuin was then removed by washing the cells three times with ice-cold washing buffer (Seglen, 1976). After the subsequent experimental incubation, cells were precipitated with phosphotungstic acid, and radioactivity in the pellet and in the supernatant was measured by gamma counting. The net release of acid-soluble ^{125}I radioactivity was expressed as a percentage of the initial endocytosed protein-incorporated radioactivity of the sample.

Protein synthesis

Protein synthesis was measured as the incorporation of [^{14} C]valine under conditions of constant specific radioactivity (5 mM; 781 μ Ci/l), and the percentage synthesis, related to total protein measured as the wet weight of the cells, was calculated as previously described (Seglen, 1978).

Materials

^{125}I -labelled asialo-fetuin, prepared as described by Tolleshaug *et al.* (1977), was kindly given by Dr. H. Tolleshaug (Institute for Nutrition Research, Oslo, Norway). [^{14} C]Valine (Amersham CFB75; 275 Ci/mol, 50 mCi/litre, 0.18 mM) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; leupeptin was from The Peptide Institute, 476 Ina, Mino-shi, Osaka 562, Japan; methylamine was from Koch-Light Laboratories,

Colnbrook, Bucks., U.K.; ionophore A23187 was from Calbiochem, LaJolla, CA, U.S.A.; and verapamil (trade name isoptin) was from Knoll A.G., Ludwigshafen a. Rhein, West Germany. Other biochemicals were from Sigma. A23187 was dissolved in dimethyl sulphoxide. Bivalent cations were added as chloride salts, except $\text{Ba}(\text{OH})_2$. All additions were made iso-osmotic and pH-neutral.

Results

Effect of bivalent-cation chelations on the degradation of endogenous protein

The bivalent-cation chelators EGTA and EDTA inhibit the degradation of endogenous protein in isolated hepatocytes equally, causing up to 50% inhibition (Fig. 1). Methylamine appears to be a relatively complete and selective inhibitor of the lysosomal degradation of endogenous protein (Seglen *et al.*, 1979, 1981; Grinde & Seglen, 1980; Amenta & Brocher, 1980; Grinde, 1982). Accepting that methylamine has this effect, Fig. 1 shows that the two chelators block most of the lysosomal (i.e. methylamine-sensitive) degradation, and cause a slight inhibition of the non-lysosomal (i.e. methylamine-insensitive) degradation. Cells incubated with Ca^{2+} in the medium have a somewhat higher rate of degradation compared with cells incubated in a bivalent-cation-free medium, and a surplus of Ca^{2+} completely abolishes the effect of both chelators.

Both EGTA and EDTA have a strong preference for Ca^{2+} rather than Mg^{2+} . The fact that Ca^{2+} prevents their effect does not therefore indicate whether the inhibition caused by the chelators is due to a depletion of Ca^{2+} or Mg^{2+} or both. However, as shown in Table 1 (Expt. I) the addition of an excess of Mg^{2+} does not prevent the effect of EGTA, denoting that Ca^{2+} is necessary for protein degradation. Mg^{2+} causes some 20% stimulation of degradation, as compared with a bivalent-cation-free medium, in both the absence and the presence of EGTA, indicating that Mg^{2+} too is of importance.

Ca^{2+} -dependent proteinases tend to be inhibited by the thiol-proteinase inhibitor leupeptin (Sugita *et al.*, 1980; Beer *et al.*, 1982; DeMartino & Croall, 1982). If the effect of EGTA on non-lysosomal degradation is due to Ca^{2+} -dependent proteinases, then EGTA should not be expected to produce an effect additive to the combined effect of methylamine and leupeptin. As shown in Table 1 (Expt. II), EGTA does not appear to have such an additive effect. The effect of EGTA on non-lysosomal degradation was found to be reproducible (in three experiments), but the size of the effect makes an interpretation uncertain.

The chelators are not believed to enter the cells. They may, however, be able to influence the intracellular concentration of bivalent cations from

Fig. 1. *Effect of bivalent-cation chelators on the degradation of endogenous protein*

Hepatocytes were isolated from rats pre-labelled for 24 h *in vivo* with [¹⁴C]valine. The cells were incubated for 2 h in a bivalent-cation-free medium with various concentrations of EGTA (a) or EDTA (b). Either no other additions were present (○), or 10 mM-methylamine (●) or 2 mM-Ca²⁺ (Δ) was added. The net release of [¹⁴C]valine (acid-soluble radioactivity) was measured, and is expressed as % of the initial protein radioactivity. Each point represents the mean of two or three cell samples.

an extracellular position. There is a steady exchange of Ca²⁺ (and most likely of Mg²⁺ too) over the plasma membrane (Foden & Randle, 1978). Absorbing the extracellular bivalent cations may therefore lead to a net export of bivalent cations from the cells.

Time curves for the effect of EGTA shows that it takes 30–40 min from adding EGTA until maximal effect is achieved (Fig. 2). This lag is compatible with the explanation given above, i.e. that EGTA slowly depletes intracellular Ca²⁺ by stopping the influx. The effect of EGTA is much greater when the cells are preincubated for 20 min with EGTA. If cells preincubated with EGTA are incubated further without EGTA, then the rate of protein degradation is partly restored.

Effect of other modulators of Ca²⁺ concentration, and various bivalent cations, on the degradation of endogenous protein

When extracellular Ca²⁺ is absent, the Ca²⁺ ionophore A23187 (Reed & Lardy, 1972) causes a 40% inhibition of degradation (Fig. 3). Adding an increasing concentration of Ca²⁺ to the medium partly abolishes the effect of A23187. The ionophore was dissolved in dimethyl sulphoxide (final concn. 0.35%). The dimethyl sulphoxide was found not to influence protein degradation or synthesis (results not shown).

The Ca²⁺-channel-blocker verapamil (Kohlhardt *et al.*, 1972) and the inhibitor of calmodulin action,

Table 1. *Effect of EGTA on the degradation of endogenous protein*

Hepatocytes were isolated from rats pre-labelled for 24 h *in vivo* with [¹⁴C]valine. In Expt. I the cells were incubated in a bivalent-cation-free medium with or without EGTA (1 mM) and Mg²⁺ (2 mM). In Expt. II Ca²⁺ (1 mM) and Mg²⁺ (1 mM) were included in the medium, leupeptin was added at 1 mM, EGTA at 1.5 mM and methylamine at 10 mM. In both experiments the net release of [¹⁴C]valine (acid-insoluble radioactivity), over a 2 h incubation, was measured, and is expressed as % of initial protein radioactivity. Each value is the mean ± S.E.M. for three cell samples.

Addition	Protein degradation (%)	Inhibition (%)
Expt. I		
None	9.8 ± 0.30	0
Mg ²⁺	11.8 ± 0.11	-20
EGTA	5.7 ± 0.07	42
EGTA + Mg ²⁺	7.2 ± 0.05	25
Expt. II		
None	10.3 ± 0.10	0
EGTA	7.2 ± 0.08	30
Leupeptin	4.4 ± 0.08	57
Methylamine	3.7 ± 0.05	64
EGTA + leupeptin	4.2 ± 0.07	59
EGTA + methylamine	3.4 ± 0.03	67
Methylamine + leupeptin	2.6 ± 0.05	75
EGTA + methylamine + leupeptin	2.5 ± 0.06	76

Fig. 2. Time course and reversibility of the effect of EGTA on the degradation of endogenous protein

Hepatocytes were isolated from a rat pre-labelled for 24 h *in vivo* with [^{14} C]valine. The cells were preincubated for 20 min with (Δ , $\blacktriangle$) or without ($\circ$, $\bullet$) 1 mM-EGTA, washed three times in ice-cold washing buffer (Seglen, 1976), and finally incubated for up to 2 h in the absence ($\circ$, Δ) or presence ($\bullet$, $\blacktriangle$) of 1 mM-EGTA. No bivalent cations were present. The net release of [^{14} C]valine (acid-soluble radioactivity) was measured, and expressed as % of the initial protein radioactivity. Each point represents the mean of two cell samples.

trifluoperazine (Reinhart *et al.*, 1980), also inhibit protein degradation, their effect being on lysosomal (i.e. methylamine-sensitive) degradation (Table 2).

The effects of various bivalent cations on protein degradation were examined on cells pretreated with or without EGTA or EDTA (Table 3). Pretreatment with chelators was carried out to decrease the intracellular bivalent-cation concentrations, thereby making the cells more responsive to the addition of cations. Adding both Ca^{2+} and Mg^{2+} to cells pretreated with EGTA brings the rate of degradation back to that in untreated cells. When added alone, Ca^{2+} and Mg^{2+} are both stimulatory, although to a lesser extent than their combined effect, and with Mg^{2+} being more potent than Ca^{2+} .

The two experiments in Table 3 were performed on cells from different rats. The difference in rate of degradation between the controls (no addition) in the two experiments is due to variability between cell batches.

Three other bivalent cations were also tested, Mn^{2+} , being a relatively common bivalent cation,

Fig. 3. Effect of the ionophore A23187 on the degradation of endogenous protein

Hepatocytes were isolated from a rat pre-labelled for 24 h *in vivo* with [^{14} C]valine. The cells were incubated for 2 h with various concentrations of Ca^{2+} , in the absence ($\circ$), or presence (Δ) of A23187 (30 μM). Mg^{2+} (0.5 mM) and dimethyl sulphoxide (0.35%) were present in all samples. The net release of [^{14} C]valine (acid-soluble radioactivity) was measured, and expressed as % of the initial protein radioactivity. Each point represents the mean of two or three cell samples.

Co^{2+} , which may act as a Ca^{2+} antagonist, and Ba^{2+} , which may act as a Ca^{2+} agonist (Table 3, Expt. II). None of these ions were able to stimulate protein degradation, indicating that the effects of Ca^{2+} and Mg^{2+} are specific for those two ions. Instead a slight, although somewhat varying, inhibition was found repeatedly in three experiments. This inhibition might be due to antagonistic effects, but it could be also be caused by unspecific toxicity.

Effect of modulators of bivalent-cation concentrations on protein synthesis and on the degradation of endocytosed asialo-fetuin

The effectors investigated in connection with protein degradation were also tested on protein synthesis (Table 4). As shown, cells incubated with Ca^{2+} and/or Mg^{2+} exhibit a significantly increased protein synthesis compared with cells incubated in a bivalent-cation-free medium. EGTA causes a further drastic decrease in protein synthesis. The effect of EGTA is almost completely prevented by the addition of excess Ca^{2+} and Mg^{2+} . Ca^{2+} is much

Table 2. *Effect of trifluoperazine and verapamil on the degradation of endogenous protein*

Hepatocytes were isolated from a rat pre-labelled for 24 h *in vivo* with [¹⁴C]valine. The cells were incubated for 2 h in a medium containing Ca²⁺ and Mg²⁺ (1 mM each), with or without methylamine (12 mM), trifluoperazine and verapamil as noted. The net release of [¹⁴C]valine (acid-soluble radioactivity) was measured and is expressed as % of the initial protein radioactivity. Each value is the mean $\pm$ S.E.M. for three cell samples.

Addition	-Methylamine		+Methylamine	
	Protein degradation (%)	Inhibition (%)	Protein degradation (%)	Inhibition (%)
None	10.3 $\pm$ 0.10	0	3.3 $\pm$ 0.10	68
Trifluoperazine (0.01 mM)	10.5 $\pm$ 0.04	-2	3.3 $\pm$ 0.18	68
Trifluoperazine (0.1 mM)	8.7 $\pm$ 0.12	16	3.4 $\pm$ 0.06	67
Verapamil (0.2 mM)	7.5 $\pm$ 0.13	27	3.2 $\pm$ 0.03	69
Verapamil (0.4 mM)	4.4 $\pm$ 0.10	57	3.3 $\pm$ 0.01	68

Table 3. *Effect of various bivalent cations on the degradation of endogenous protein*

Hepatocytes were isolated from rats pre-labelled for 24 h *in vivo* with [¹⁴C]valine. The cells were pre-incubated for 20 min in a bivalent-cation-free medium with or without 1 mM-EGTA (Expt. I) or -EDTA (Expt. II), then washed twice with ice-cold medium, and incubated for 2 h with or without bivalent cations (1 mM) as noted. The net release of [¹⁴C]valine (acid-insoluble radioactivity) during the 2 h incubation was measured, and is expressed as % of initial protein radioactivity. Each value is the mean $\pm$ S.E.M. for three cell samples.

Addition	Pretreated without chelator		Pretreated with chelator	
	Protein degradation (%)	Stimulation (%)	Protein degradation (%)	Stimulation (%)
Expt. I				
None	8.9 $\pm$ 0.03	0	6.4 $\pm$ 0.01	0
Ca ²⁺ + Mg ²⁺	10.3 $\pm$ 0.10	16	9.6 $\pm$ 0.07	50
Ca ²⁺			7.0 $\pm$ 0.02	9
Mg ²⁺			8.8 $\pm$ 0.15	37
Expt. II				
None	7.4 $\pm$ 0.18	0	6.6 $\pm$ 0.01	0
Ca ²⁺	8.9 $\pm$ 0.14	21	7.7 $\pm$ 0.09	17
Mg ²⁺	9.3 $\pm$ 0.10	26	8.3 $\pm$ 0.11	26
Mn ²⁺	7.2 $\pm$ 0.06	-3	5.9 $\pm$ 0.02	-11
Co ²⁺	7.0 $\pm$ 0.16	-6	6.3 $\pm$ 0.06	-5
Ba ²⁺	7.2 $\pm$ 0.17	-2	5.8 $\pm$ 0.03	-12

more powerful than Mg²⁺ in decreasing the effect of EGTA, indicating that the cellular Ca²⁺ concentration does influence protein synthesis.

The ionophore A23187 inhibits protein synthesis by 55% when Ca²⁺ is absent. As with protein degradation, adding Ca²⁺ only partly restores protein synthesis. Trifluoperazine does not inhibit protein synthesis appreciably, whereas verapamil causes up to 50% inhibition.

Table 4. *Role of Ca²⁺ and Mg²⁺ in protein synthesis*

Hepatocytes were incubated for 1 h in a medium without bivalent cations, but with a complete amino acid mixture (Kovács *et al.*, 1981). Protein synthesis was measured as the incorporation of [¹⁴C]valine during the incubation period, and is expressed as % of the total protein. Each value is the mean $\pm$ S.E.M. for three cell samples. Cations and cation effectors were added in concentration as noted in the Table or, where not noted, in the following concentrations: Ca²⁺ (1 mM), Mg²⁺ (1 mM), EGTA (1 mM) and A23187 (30 μ M). The effect of the additions is given as % inhibition.

Addition	Protein synthesis (%)	Inhibition (%)
Ca ²⁺ + Mg ²⁺	0.87 $\pm$ 0.01	0
Ca ²⁺	0.82 $\pm$ 0.01	6
Mg ²⁺	0.84 $\pm$ 0.01	3
None	0.67 $\pm$ 0.01	23
EGTA	0.25 $\pm$ 0.01	71
EGTA + Ca ²⁺ (2 mM)	0.71 $\pm$ 0.02	18
EGTA + Ca ²⁺ (2 mM)	0.46 $\pm$ 0.01	47
EGTA + Ca ²⁺ (2 mM) + Mg ²⁺ (2 mM)	0.83 $\pm$ 0.01	5
A23187 + Mg ²⁺	0.39 $\pm$ 0.01	55
A23187 + Mg ²⁺ + Ca ²⁺	0.55 $\pm$ 0.01	37
Trifluoperazine (0.01 mM) + Ca ²⁺ + Mg ²⁺	0.90 $\pm$ 0.01	-3
Trifluoperazine (0.1 mM) + Ca ²⁺ + Mg ²⁺	0.82 $\pm$ 0.01	6
Verapamil (0.2 mM) + Ca ²⁺ + Mg ²⁺	0.69 $\pm$ 0.00	21
Verapamil (0.4 mM) + Ca ²⁺ + Mg ²⁺	0.44 $\pm$ 0.00	49

¹²⁵I-labelled asialo-fetuin is rapidly internalized (via receptor-mediated endocytosis) and degraded by isolated hepatocytes (Tolleshaug *et al.*, 1977). To test the role of Ca²⁺ in the degradation of this protein, the cells were incubated for 15 min in the

Fig. 4. Effect of EGTA on the degradation of endocytosed asialo-fetuin

Hepatocytes were allowed to endocytose ^{125}I -labelled asialo-fetuin for 15 min (in the presence of 1 mM of both Ca^{2+} and Mg^{2+}), then washed to remove extracellular asialo-fetuin, and incubated for up to 90 min in a bivalent-cation-free medium in the absence (O) or presence (●) of EGTA (1 mM). The net release of acid-soluble ^{125}I was measured, and expressed as % of the initial protein radioactivity. Each point represents the mean of three cell samples.

presence of ^{125}I -labelled asialo-fetuin. Then the cells were washed to remove extracellular ^{125}I -labelled asialo-fetuin and incubated further in the absence or presence of EGTA (Fig. 4); this showed that EGTA does not influence the degradation of the endocytosed protein.

Discussion

The main conclusion from the present experiments is that the degradation of endogenous protein in isolated rat hepatocytes depends on Ca^{2+} . The conclusion is based on the inhibitory effect of substances known to decrease intracellular Ca^{2+} concentration. These substances include the bivalent-cation chelators EGTA and EDTA, which might be expected to cause a net efflux of bivalent cations by halting their influx. EGTA is known to decrease Ca^{2+} concentration in diaphragm muscle (Sugden, 1980), and has been previously used to decrease Ca^{2+} concentration in isolated rat hepatocytes (Assimakopoulos-Jeannet *et al.*, 1977). The ionophore A23187 causes a net efflux of Ca^{2+} from hepatocytes when the cells are incubated in a Ca^{2+} -free medium (Foden & Randle, 1978). Verapamil is known to inhibit the Ca^{2+} influx in

muscle by blocking the Ca^{2+} channels (Kohlhardt *et al.*, 1972). In liver verapamil probably does not inhibit the influx of Ca^{2+} , but instead inhibits the mobilization of Ca^{2+} from intracellular pools (Kimura *et al.*, 1982).

Experiments with the lysosomotropic agent methylamine (Fig. 1 and Table 2) indicate that the Ca^{2+} requirement mainly concerns the lysosomal degradation. It is tempting to speculate that the step(s) requiring Ca^{2+} are connected with the formation of autophagic vacuoles or the fusion of these vacuoles with lysosomes. The finding (Fig. 4) that Ca^{2+} does not appear to be needed for the (lysosomal) degradation of the endocytosed protein asialo-fetuin supports the above speculation.

Previous experiments with hepatocytes have indicated that lysosomal degradation requires intact microtubules (Grinde & Seglen, 1981). Ca^{2+} , via calmodulin, is involved in the control of microtubule aggregation (Kakiuchi & Sobue, 1983). The inhibitor of calmodulin action, trifluoperazine, does have a small inhibitory effect on lysosomal protein degradation (Table 2). The effective concentration of trifluoperazine (0.1 mM) is, however, so high as to be indicative probably of detergent effects rather than an action on calmodulin. It is more likely that the Ca^{2+} requirement is connected with the autophagic process or with the fusion of vesicles.

In addition to blocking most of the lysosomal degradation, EGTA and EDTA seem to have a small effect on non-lysosomal (i.e. methylamine-insensitive) degradation (Fig. 1). It is tempting to connect this effect with the calpains (Ca^{2+} -dependent proteinases). In support of this connection, it was found that the response to EGTA is not additive to the combined effect of methylamine and leupeptin (Table 1). Leupeptin is a thiol-proteinase inhibitor known to inhibit calpains; in hepatocytes it blocks most of the lysosomal degradation and a small part of the non-lysosomal degradation (Grinde & Seglen, 1980). Experiments with thiol-proteinase inhibitors (Grinde & Seglen, 1980; Grinde, 1982) together with the present experiments indicate, however, that the calpains have a very limited impact on the bulk degradation of protein in isolated hepatocytes. In this connection it should be mentioned that rat liver has a very high concentration of the specific calpain inhibitor, calpastatin, whereas it is relatively low in calpains (Murachi, 1983).

When cells are first preincubated with EGTA, washed to remove the EGTA and the Ca^{2+} extracted from the cells, and incubated further, then the protein degradation recuperates (Fig. 2). Apparently incubation with EGTA does not lead to a complete depletion of all intracellular Ca^{2+} , but merely decreases the concentration of free Ca^{2+} in cytosol. After removal of EGTA, the Ca^{2+} concentration in the cytosol will probably increase, owing to the

release of Ca²⁺ from a non-cytosolic Ca²⁺ pool. Hepatocytes have a highly labile mitochondrial pool of Ca²⁺ (Murphy *et al.*, 1980).

The present data indicate that not just Ca²⁺, but also Mg²⁺, is important for protein degradation (Tables 1 and 3). This is not unexpected, since Mg²⁺ is required for many ATP-dependent processes, and most of the protein degradation of hepatocytes is energy-dependent (Seglen *et al.*, 1979). It is difficult to evaluate how important Mg²⁺ is, since there is no reliable means by which the intracellular Mg²⁺ concentration can be decreased without also decreasing the Ca²⁺ concentration.

Protein synthesis is known to utilize Mg²⁺, but, at least in cell-free systems, Mg²⁺ is not required. The data in Table 4 indicate that the 70% inhibition of protein synthesis caused by EGTA is mainly due to a Ca²⁺ depletion.

Since both protein synthesis and protein degradation are energy-requiring, the inhibition caused by EGTA could be secondary to a decrease in energy charge owing to the depletion of Ca²⁺. Assimacopoulos-Jeannet *et al.* (1977) found, however, that EGTA treatment of hepatocytes (1 mM-EGTA in a Ca²⁺-free buffer for 30 min) did not change ATP concentration of gluconeogenesis rates. When hepatocytes are incubated under decreasing oxygen concentration, thereby decreasing energy charge, it is found that a decrease sufficient to cause a 70% inhibition of protein synthesis does not influence protein degradation (Seglen *et al.*, 1979). Furthermore EGTA does not inhibit the, presumably energy-dependent, degradation of asialo-fetuin (Fig. 4). Although the effect of EGTA on protein synthesis might be (partly) due to a decrease in energy charge, it seems likely that the bulk effect on protein degradation is not.

Protein degradation requires protein synthesis (Goldberg & St. John, 1976). The effect of Ca²⁺ depletion on protein degradation might therefore be secondary to the inhibition of protein synthesis. However, EGTA causes a 70% inhibition of protein synthesis (Table 4) and a 55% inhibition of protein degradation (Fig. 1). In short-term (1–2 h) experiments comparable with those with EGTA, cycloheximide and puromycin inhibit protein degradation by only about 15% at concentrations that cause a 70% decrease in protein synthesis, or by 30% at concentrations that cause an almost complete block in protein synthesis (Kovács & Seglen, 1981). The effect of EGTA on protein degradation does therefore not appear to be secondary to an effect on protein synthesis.

In muscle the increased intracellular Ca²⁺ concentration, caused by including both the ionophore A23187 and Ca²⁺ in the medium, is reported to stimulate protein degradation (Kameyama & Etlinger, 1979; Sugden, 1980). Such a stimulation was

not found for the hepatocytes (Fig. 3). There are at least three possible explanations for this difference in response. First, according to Chen *et al.* (1978), A23187 does not cause a net influx of Ca²⁺ in hepatocytes. Foden & Randle (1978), however, conclude that A23187 leads to a net uptake of Ca²⁺. Second, protein degradation in the hepatocytes, under the present experimental conditions, is probably already maximally stimulated. The third explanation is that there are differences in the regulation of protein degradation between muscle and liver, e.g. Ca²⁺-dependent proteinases may undertake a larger portion of the protein degradation in muscle than in hepatocytes.

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