Biochem. J. (1983) **212**, 529–537
Printed in Great Britain

Differences in degradation processes for insulin and its receptor in cultured foetal hepatocytes

Eric PRINGAULT and Christiane PLAS
Laboratoire Interactions Cellulaires, U.E.R. Odontologie, Université Paris 7, 75005 Paris, France

(*Received 4 November 1982/Accepted 15 February 1983*)

Binding and degradation of ^{125}I-labelled insulin were studied in cultured foetal hepatocytes after exposure to the protein-synthesis inhibitors tunicamycin and cycloheximide. Tunicamycin (1 μg/ml) induced a steady decrease of insulin binding, which was decreased by 50% after 13 h. As the total number of binding sites per hepatocyte was 20000, the rate of the receptor degradation could not exceed 13 sites/min per hepatocyte. Cycloheximide (2.8 μg/ml) increased insulin binding by 30% within 6 h, an effect that persisted for up to 25 h. This drug had a specific inhibitory effect on the degradation of proteins prelabelled for 10 h with [^{14}C]glucosamine, without affecting the degradation of total proteins. Chronic exposure to 10 nM-insulin neither decreased insulin binding nor modified the effect of the drugs. The absence of down-regulation of insulin receptors cannot be attributed to rapid receptor biosynthesis in foetal hepatocytes. Cellular insulin degradation, which is exclusively receptor-mediated, was determined by two different parameters. First, the rate of release of degraded insulin into the medium was 600 molecules/min per hepatocyte with 1 nM labelled hormone, and increased (preincubation with cycloheximide) or decreased (tunicamycin) as a function of the amount of cell-bound insulin. Secondly, the percentage of cell-bound insulin degraded was not changed by the presence of protein-synthesis inhibitors (25–30%). The stability of insulin degradation suggested that this process was dependent on long-life proteinase systems. Such differences in degradation rates and cycloheximide sensitivity imply that hormone- and receptor-degradation processes utilize distinct pathways.

The physiological effect of a variety of macromolecular substances in eukaryotic cells is accompanied by a cycle of events which involves ligand binding to cell surface sites, aggregation of occupied receptors and transfer of ligand–receptor complexes to an internal compartment by endocytosis. The receptor can be degraded or recycled back to the plasma membrane, whereas the fate of the internalized ligand depends essentially on its nature (Goldstein *et al.*, 1979; Kaplan, 1981; Pearse & Bretscher, 1981). This scheme applies to several polypeptide hormones, particularly to insulin (Gorden *et al.*, 1980; Schlessinger, 1980; Goldfine, 1981; King & Cuatrecasas, 1981; Pastan & Willingham, 1981). Insulin has been shown to be degraded in the cell, and its degradation is coupled to the binding of the hormone (Terris & Steiner, 1975). Whether or not the fate of the receptor is linked to that of the ligand is debatable. Cultured foetal hepatocytes, where insulin degradation is known to be receptor-mediated (Plas & Desbuquois, 1982), enable comparative studies of the effects of protein-synthesis inhibitors on insulin binding and degradation. The present study shows that the degradation pathways of insulin and its receptor are distinguishable by their differences in degradation rates and sensitivity to cycloheximide. It suggests that degradation of the receptor is not a prerequisite for active receptor-mediated insulin degradation and that the cell therefore has the possibility of reutilizing the insulin receptor.

Materials and methods

Culture procedure

Primary cultures were obtained from 18-day rat foetuses (Sprague–Dawley) as described previously (Plas *et al.*, 1973). The culture medium (2 ml/dish) consisted of NCTC 109 medium (Evans *et al.*, 1964) supplemented with 10% (v/v) foetal-calf serum,

10 μM-cortisol and 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.3. It was supplemented daily with glucose (0.5 mg/dish). Experiments were performed after 2 or 3 days of culture in the presence of cortisol, when the glycogenic effect of insulin is fully expressed (Plas & Nunez, 1976).

Insulin-binding and -degradation studies

Measurements were performed as described previously (Desbuquois *et al.*, 1974; Plas & Desbuquois, 1982). Incubations of 1 nM-^{125}I-labelled insulin with hepatocytes grown in monolayers (about 1.2×10^6 cells/dish) were performed at 37°C and pH 7.3, in 2 ml of culture medium. On completion of the incubation, the medium was removed and the cells were washed with 4×2.5 ml of ice-cold Hanks solution. This washing step was performed within 12 s. The cell-associated radioactivity was then extracted for 30 min at 4°C by two successive additions of 1 ml of 8.5 M-acetic acid containing 0.35 mM-bacitracin. The recovery of the radioactivity in the extract was about 95%. The small amount of radioactivity remaining was solubilized with 1 ml of 0.5 M-NaOH for 30 min at 75°C. The sum of radioactivity measured in this solution and in the acid extract represents the 'total binding'. Corrections were made for the non-specific association of ^{125}I-labelled insulin to cells by performing parallel incubations in the presence of native insulin (3 μM). The acid-soluble labelled material recovered from the cells was allowed to dry at 23°C in a vacuum desiccator containing solid NaOH. The residue was dissolved in 1 ml of 0.1 M-HCl and the solution neutralized with 1 mM-Tris. The material was then tested for its ability to be precipitated by 10% (w/v) trichloroacetic acid. The soluble fraction represents the cell-bound insulin that was degraded. The effects of the protein-synthesis inhibitors on insulin binding and degradation were estimated as follows: after 2 days of culture, cells were incubated in the presence of either cycloheximide (2.8 μg/ml) or tunicamycin (1 μg/ml) or no inhibitor for various times. ^{125}I-labelled insulin was then added to the medium and cultures were incubated for 30 min. The amount of specifically bound insulin, the percentage of cell-bound insulin degraded and the amount of degraded insulin released in the medium were measured as described above.

Glycogenesis studies

Glycogen labelling in the presence of [^{14}C]-glucose was determined as described previously (Plas *et al.*, 1973). At day 3 of the culture, [^{14}C]glucose (1 μCi/mg) was introduced in the medium together with 10 nM-insulin or its solvent (10 μM-HCl). The radioactivity present in glycogen was measured after 2 h of incubation.

Protein-synthesis and -degradation studies

The rate of protein synthesis was estimated by the incorporation of [^{14}C]leucine (0.2 μCi/ml) or [^{3}H]glucosamine (1 μCi/ml). Protein-synthesis inhibitors cycloheximide (2.8 μg/ml) or tunicamycin (1 μg/ml) were added at various times before labelling. Control cultures were grown without inhibitor. At zero time, the tracer was introduced in the medium and the cultures were stopped after 2 h of incubation. The radioactivity incorporated into proteins and the protein concentration were determined by the procedure described by Kitagawa (1980).

Protein degradation was studied by measuring either cell protein breakdown or release of protein-degradation products in the medium. Hepatocytes were prelabelled for 10 h with [^{14}C]leucine (0.05 μCi/ml) or [^{3}H]glucosamine (0.25 μCi/ml). Cultures were washed twice with unlabelled culture medium and incubated for 10 min to allow the release of labelled precursors not incorporated into proteins (flushing). This medium was then replaced and drugs were added, or not, at this time (zero time of the experiment). Then, or after 6 h of incubation in unlabelled medium, cultures were stopped and trichloroacetic acid-precipitable radioactivity incorporated into proteins was determined (Kitagawa, 1980). Trichloroacetic acid-precipitable radioactivity was also measured in samples of the medium at various times.

Results

Effect of protein-synthesis inhibitors on insulin binding

The effect of cycloheximide (2.8 μg/ml) and tunicamycin (1 μg/ml) was tested from day 1 to day 3 of the culture. Insulin binding to hepatocytes was measured at various times of incubation after addition of the drug. The amount of specifically bound ^{125}I-labelled insulin was determined after 30 min at 37°C, i.e. under steady-state binding conditions. In control cultures grown in the absence of drugs, the only modification was an occasional slight decrease of specific insulin binding during the 50 h experimental period. After 5 h in the presence of cycloheximide, specific binding increased by 30% and remained steady for up to 25 h. Afterwards it declined, to reach 60% of the maximal value after a 50 h preincubation (Fig. 1). The increase in specific binding observed after a 8 h exposure to the drug was always present when measured at concentrations of ^{125}I-labelled insulin ranging from 0.1 to 5 nM (results not shown). The presence of tunicamycin induced a significant decrease in insulin binding after 5 h of incubation. The time-course curve was used to determine the time needed to decrease specific binding by half, which was 13 h.

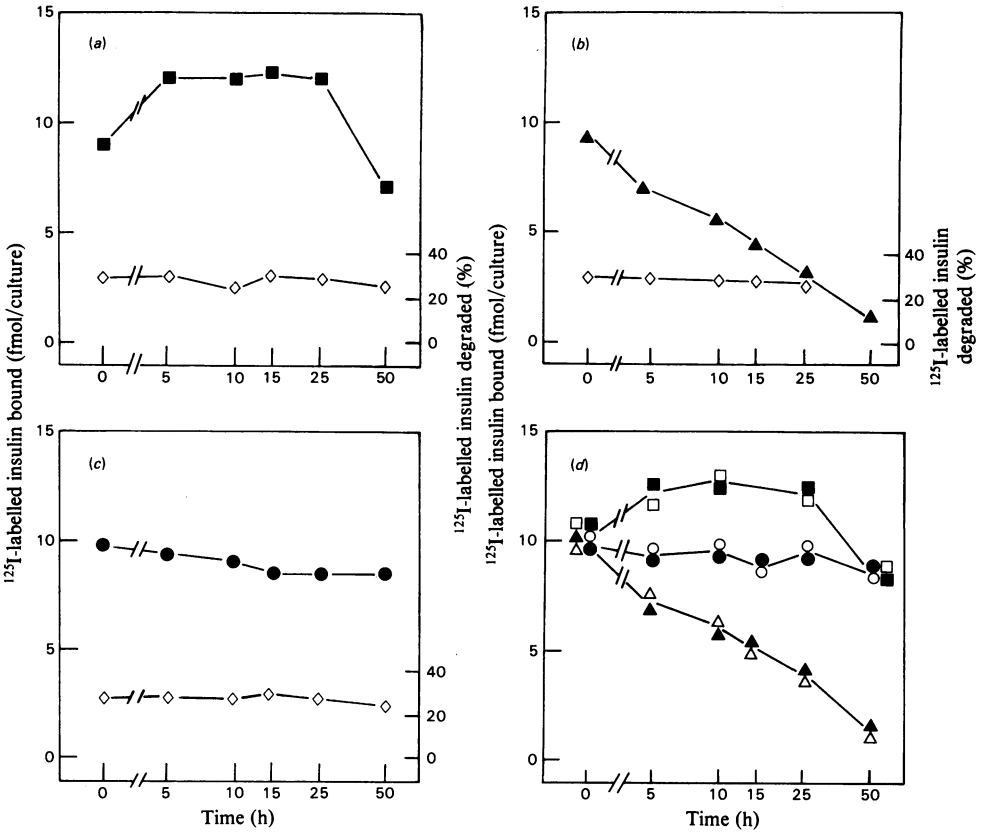

Fig. 1. *Effect of protein-synthesis inhibitors and chronic exposure to insulin on insulin binding and degradation*
After preincubation of hepatocytes with cycloheximide (2.8 μg/ml) (*a*), tunicamycin (1 μg/ml) (*b*) or without drug (*c*) for various times indicated on the abscissa, 1 nM-^{125}I-labelled insulin was allowed to bind to cells for 30 min at 37°C. The amount of specifically bound labelled insulin and the percentage of cell-associated degraded labelled insulin were determined as described in the Materials and methods section. In another experiment, this protocol was repeated, except that preincubation was performed in the presence of 10 nM native insulin (*d*). Then cultures were washed four times with insulin-free medium. In control cultures not preincubated with insulin, 10 nM native hormone was added just before the washing step, to eliminate any possible carry-over effect of insulin. The specific binding of cultures preincubated with cycloheximide (■), cycloheximide and 10 nM-insulin (□), tunicamycin (▲), tunicamycin and 10 nM-insulin (△), 10 nM-insulin (○) or solvent alone (●), and the percentage of cell-associated insulin degraded (◇), are shown. Three experiments gave the same results.

Shorter-time-interval binding kinetic studies were then performed. Cycloheximide increased specific insulin binding as early as in the first hours of incubation, whereas tunicamycin decreased insulin binding after a lag time of 4 h (Fig. 2). Thus the behaviour of the specifically bound insulin depended on the inhibitor used: the half-time for the disappearance of insulin binding was 13 h in the presence of tunicamycin, but could not be determined in the presence of cycloheximide.

Specific insulin binding was also measured after chronic exposure to native insulin in the presence or absence of inhibitors. The experiments described above were repeated with hepatocytes preincubated with each drug and 10 nM native insulin. At the end of the preincubation period, cultures were washed to eliminate free native insulin before determining insulin binding. A preincubation with native insulin did not alter insulin binding (Fig. 1). In the presence of cycloheximide, an initial increase in labelled insulin bound, followed by a decrease after 25 h, was also observed. With tunicamycin, the half-time for the disappearance of insulin binding remained close to 13 h.

Parallel cultures were used to measure the effect of these inhibitors on hepatocyte protein synthesis. The amount of [^{14}C]leucine or [^{3}H]glucosamine incorporated into cellular proteins after 2 h was

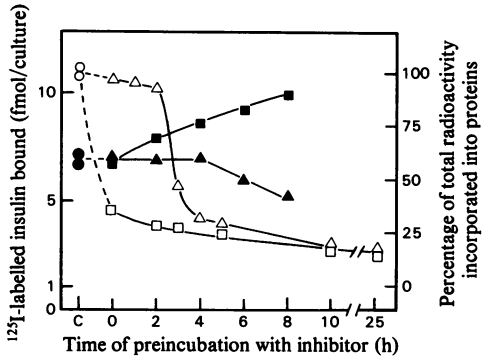

Fig. 2. *Effect of cycloheximide and tunicamycin on protein synthesis and insulin binding*

After preincubation with cycloheximide (2.8 μg/ml) or tunicamycin (1 μg/ml) for various times indicated on the abscissa, cultures received [^{14}C]leucine (0.2 μCi/ml of medium) or [^{3}H]glucosamine (1 μCi/ml of medium) respectively and were incubated for a further 2h period in the presence of the drug. Parallel cultures received 1 nM-^{125}I-labelled insulin, which was allowed to bind to cells for 30min at 37°C. Control cultures were incubated in the absence of drug. The radioactivity incorporated into proteins (□, △, ○) or the amount of specifically bound labelled insulin (■, ▲, ●) were determined as described in the Materials and methods section. □, ■, Cultures preincubated with cycloheximide; △, ▲, cultures preincubated with tunicamycin; ○, ●, control cultures without inhibitor. Three experiments gave the same results. Abbreviation: C, control.

Table 1. *Effect of protein-synthesis inhibitors on the glycogenic response to insulin*

Cell monolayers were preincubated with tunicamycin (1 μg/ml), cycloheximide (2.8 μg/ml) or without drug for various times. [^{14}C]Glucose was then added at a specific radioactivity of 1 μCi/mg in the medium, at the same time as 10 nM native insulin or its solvent (10 μM-HCl), and the hepatocytes were incubated for 2h. The radioactivity incorporated into glycogen was measured as described in the Materials and methods section. The stimulation index represents the ratio of [^{14}C]glucose incorporated (nmol/h per mg of protein) in cultures treated with insulin to that in control cultures. Results are presented as means ± S.D. for five experiments performed with different cell preparations.

Additions	Time of preincubation (h)	[^{14}C]Glucose incorporation into glycogen (nmol/h per mg of protein) Solvent	10 nM-insulin	Stimulation index
None (control)	—	8.2 ± 0.7	24.0 ± 0.5	2.93
Tunicamycin (1 μg/ml)	0	10.1 ± 2.3	36.2 ± 4.9	3.58
	10	3.7 ± 0.3	15.1 ± 1.7	4.08
	15	4.2 ± 0.2	15.3 ± 2.3	3.64
	25	5.2 ± 0.3	23.1 ± 0.2	4.44
None (control)	—	8.3 ± 0.6	18.7 ± 2.0	2.25
Cycloheximide (2.8 μg/ml)	0	11.0 ± 1.2	22.8 ± 3.1	2.08
	5	2.1 ± 0.1	2.5 ± 0.4	1.18
	10	1.7 ± 0.1	1.6 ± 0.2	0.95
	25	0.75 ± 0.05	0.56 ± 0.08	0.76

determined as a function of increasing preincubation times in the presence of cycloheximide (2.8 μg/ml) or tunicamycin (1 μg/ml). The addition of cycloheximide together with [^{14}C]leucine caused a 64% inhibition of protein labelling after 2h. This inhibition reached 75% after 5h, then remained close to about 80% until 25h of preincubation (Fig. 2). Tunicamycin induced no significant decrease in [^{3}H]glucosamine incorporation during the first 2h. A 50% inhibition was obtained during the 3–5h labelling period, and the maximal effect (80% inhibition) appeared after 10h of preincubation. A lag time was thus observed in the inhibitory effect of tunicamycin, but protein synthesis was efficiently inhibited by both drugs, at all the preincubation times used.

Effect of protein-synthesis inhibitors on the glycogenic effect of insulin

The glycogenesis stimulation by insulin was determined after various times of incubation in the presence of cycloheximide or tunicamycin. Incorporation of [^{14}C]glucose into glycogen was measured over a 2h incubation period in the presence or absence of 10 nM native insulin. This dose is known to produce maximal stimulation of glycogenesis in foetal hepatocytes (Plas *et al.*, 1979). In control cultures grown without drug, insulin clearly stimulated glycogenesis. When cycloheximide or tunicamycin was added together with the native hormone, the stimulatory effect of insulin was maintained (Table 1). Preincubation with cycloheximide rapidly decreased both basal and insulin-stimulated glycogenesis, so that after a 10h exposure to the drug the glycogenic effect of insulin disappeared. None of the preincubation time periods with tunicamycin, i.e. 10, 15 or 25h, significantly modified the glycogenic effect of the hormone. Thus pre-exposure to the two protein-synthesis inhibitors did not produce the same modifications in basal or insulin-stimulated glycogenesis.

Effect of protein-synthesis inhibitors on insulin degradation

The percentage of cell-associated insulin degraded was measured after exposure to cycloheximide (2.8 μg/ml) or tunicamycin (1 μg/ml). Cells were allowed to bind 1 nM-^{125}I-labelled insulin for 30min at 37°C, at different times of preincubation

with drugs. After extraction of cell-bound labelled insulin, the percentage of degraded hormone was determined as described in the Materials and methods section. In control cultures without inhibitors, this percentage was 28–32% for 50h of incubation. It was unchanged in the presence of cycloheximide or tunicamycin for up to 25h (Fig. 1). Chronic exposure to native insulin did not modify the percentage of cell-associated degradation, which remained constant (28–31%), and was again not changed in the presence of the drugs.

The ^{125}I-labelled insulin that was degraded by the cells and released into the culture medium was determined at various times of incubation in the presence of 1nM labelled hormone. The amount of trichloroacetic acid-soluble radioactivity in the medium increased linearly with time. This corresponded to a rate of 62.5fmol of labelled insulin degraded/h per culture in the absence of inhibitors. When cells were preincubated with cycloheximide and tunicamycin for 15h, this rate was increased by cycloheximide and decreased by tunicamycin (94.0 and 33.5fmol/h culture respectively). The contrary effects of the drugs on the rate of insulin degradation were less marked when the amount of degraded insulin in the medium was expressed as a fraction of the corresponding amount of cell-bound hormone (Fig. 3). Likewise, the insulin-degradation rate was proportional to the amount of cell-bound insulin when various concentrations of labelled hormone were used (results not shown). As it has been shown that the binding in foetal hepatocytes is saturable only above 10nM-insulin (Menuelle & Plas, 1981), the degradation rate determined for 1nM-insulin in the present study was not maximal.

The ability of the culture medium to degrade insulin was also tested. After preincubation of cultures with or without drugs, the medium was removed and incubated for 4h in the presence of 1nM-^{125}I-labelled insulin. Insulin degradation in the medium was not detectable even when the cells were preincubated with protein-synthesis inhibitors (Fig. 3). The culture medium did not reveal any insulin-proteolytic activity. The release of trichloroacetic acid-soluble radioactivity into the medium corresponded to the cell-mediated insulin-degradation rate, which for 1nM labelled insulin was 600 molecules/min per hepatocyte in the absence of drug.

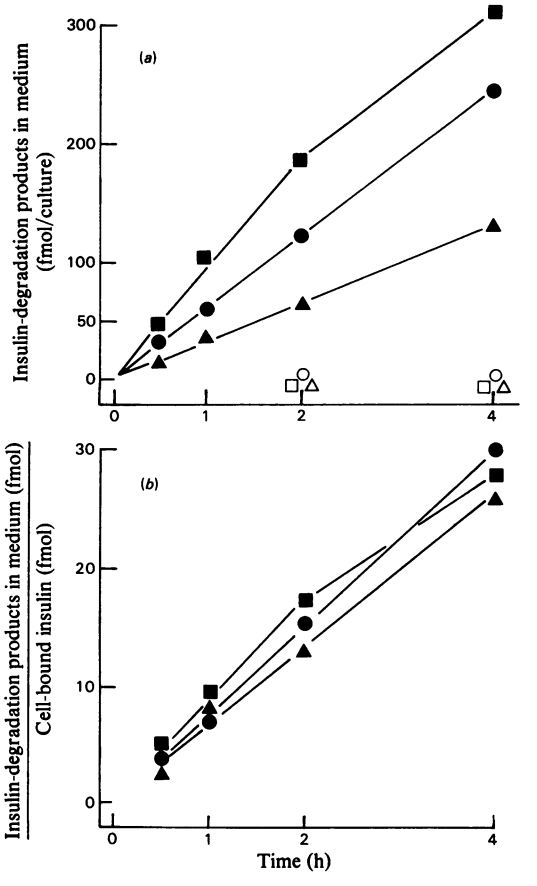

Fig. 3. *Effect of protein-synthesis inhibitors on the appearance of degraded insulin in the medium*

Cell monolayers were preincubated for 15h with either cycloheximide (2.8μg/ml) or tunicamycin (1μg/ml), or without drug. At the end of the preincubation period, 1nM-^{125}I-labelled insulin was added and the cells were incubated for various times indicated on the abscissa. Culture medium was then removed and the trichloroacetic acid-soluble radioactivity was determined. The amount of cell-associated labelled insulin was also measured. The appearance of insulin-degradation products in the medium is represented in (*a*), and these values are expressed as a fraction of the corresponding amounts of specifically cell-bound insulin in (*b*) (■, ▲, ●). In parallel cultures, medium was removed at the end of the preincubation period and the insulin degradation was measured in the absence of cells (□, △, ○). ■, □, Cultures preincubated with cycloheximide; ▲, △, cultures preincubated with tunicamycin; ●, ○, control cultures without inhibitor. Four experiments gave the same results.

Protein-degradation processes in foetal hepatocytes

The degradation of proteins prelabelled for 10h with [^{14}C]leucine or [^{3}H]glucosamine was studied. The decrease in radioactivity incorporated into proteins and the release of trichloroacetic acid-soluble radioactivity into the medium were measured over a 6h incubation period after removal of the tracer. Cycloheximide was added, or not, at the end of the prelabelling period. In cultures prelabelled with [^{14}C]leucine, 24% of the initially labelled proteins were degraded after 6h (Table 2). This

Table 2. *Effect of cycloheximide and native insulin on protein breakdown*

Cell monolayers were labelled with [^{14}C]leucine (0.05 μCi/ml) or [^{3}H]glucosamine (0.25 μCi/ml). Cultures were then washed twice and flushed for 10 min with unlabelled medium. This flushing medium was replaced by the unlabelled medium containing cycloheximide (2.8 μg/ml), or 10 nM-insulin, or cycloheximide (2.8 μg/ml) plus 10 nM-insulin, or solvent alone (zero time of the experiment). The radioactivity incorporated into proteins was measured at zero time and after 6 h of incubation under these conditions. Results are presented as means ± S.D. for five experiments performed with different cell preparations.

Additions at zero time	[^{14}C]Leucine incorporation into proteins (c.p.m./culture)			[^{3}H]Glucosamine incorporation into proteins (c.p.m./culture)		
	At zero time	At 6 h	(% degraded)	At zero time	At 6 h	(% degraded)
None (control)	49440 ± 620	37430 ± 990	24.3	35010 ± 800	20160 ± 1210	42.4
10 nM-Insulin	50670 ± 1210	39380 ± 1540	22.3	32840 ± 1390	18730 ± 560	43.0
Cycloheximide (2.8 μg/ml)	50140 ± 1735	39750 ± 1745	20.7	34500 ± 320	26990 ± 230	21.8
Cycloheximide (2.8 μg/ml) + 10 nM-insulin	46830 ± 1350	36400 ± 1480	22.3	34220 ± 700	26730 ± 470	21.9

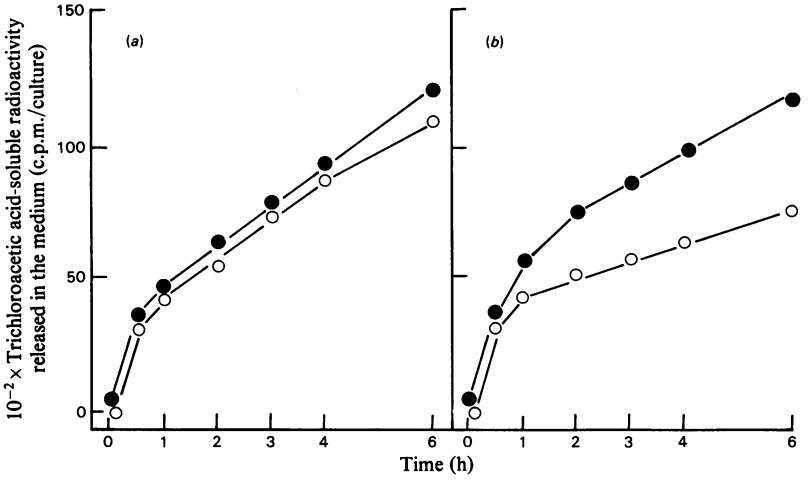

Fig. 4. *Effect of cycloheximide on the release of protein-degradation products into the medium*

Cell monolayers were prelabelled for 10 h with [^{14}C]leucine (0.05 μCi/ml) or [^{3}H]glucosamine (0.25 μCi/ml). Cultures were then washed twice and flushed for 10 min with unlabelled medium. This flushing medium was replaced by the unlabelled medium containing cycloheximide (2.8 μg/ml) or not (zero time of the experiment). Samples of the medium were taken at various times and trichloroacetic acid-soluble radioactivity was determined. The radioactivity so released in the presence of cycloheximide (○) or in its absence (●) is represented after prelabelling in (*a*) with [^{14}C]leucine and in (*b*) with [^{3}H]glucosamine. Four experiments gave the same results.

percentage was 42% in cultures prelabelled with [^{3}H]glucosamine. Thus the turnover of glycoproteins was faster than that of total proteins. Cycloheximide induced no significant decrease in total protein degradation, but significantly decreased the percentage of glycoprotein degraded (from 42 to 22%). When 10 nM-insulin was added at the beginning of the chase period, similar results were obtained.

The release of trichloroacetic acid-soluble radioactivity in the medium was directly proportional to a loss of radioactivity in proteins. An initial rapid release followed by a slower rate was observed for both radioactive precursors (Fig. 4). Cycloheximide did not modify this pattern in cells prelabelled with [^{14}C]leucine. As expected, this drug slowed the rate of release of degraded products from cells preincubated with [^{3}H]glucosamine. Under such experi-

mental conditions, cycloheximide had a specific inhibitory effect on glycoprotein degradation.

Discussion

Cultured foetal hepatocytes are suitable for correlative studies of insulin binding, insulin degradation and subsequent stimulation of glycogenesis (Menuelle & Plas, 1981; Plas & Desbuquois, 1982). Insulin degradation is receptor-mediated in cultured foetal hepatocytes (Plas & Desbuquois, 1982) as well as in isolated adult hepatocytes (Terris & Steiner, 1975). The close parallel observed between insulin degradation and insulin binding suggests a mandatory involvement of the insulin receptor in the degradation of the hormone. The present study used appropriate protein-synthesis inhibitors to distinguish receptor site degradation from degradation of the hormone within the cell.

Tunicamycin, an inhibitor of protein *N*-glycosylation (Takatsuki *et al.*, 1975; Duksin & Bornstein, 1977), induced a linear decrease in the specific binding of insulin: the half-time for the disappearance of binding was 13h. As the tunicamycin-induced inhibition of [^{3}H]glucosamine incorporation into proteins showed a lag time of 3h, this half-time is probably overestimated. These results suggest that protein *N*-glycosylation is required to produce functional insulin receptors. In 3T3-L1 adipocytes, the use of a heavy-isotope density-shift technique gave a half-life of 7.5h for degradation of the insulin receptor, compared with a value of 9h as determined by the tunicamycin-induced loss of the insulin-binding capacity (Rosen *et al.*, 1979; Reed *et al.*, 1981). Cycloheximide has been shown to lengthen the half-life of insulin receptor degradation in these cells from 7.5h to 25h, thereby suggesting that the continued synthesis of a short-lived protein is required for insulin-receptor turnover (Reed *et al.*, 1981). In the present study, the half-time for the disappearance of insulin binding in the presence of cycloheximide was also lengthened (to 50h), and an early unexpected increase in binding (maximal after 5h) was observed for all insulin concentrations in the range 0.1–5nM. These results suggest an effect of cycloheximide on the rate of receptor degradation in cultured foetal hepatocytes. However, direct measurement of the turnover rate of the receptor will be necessary to confirm this interpretation.

Cycloheximide did not alter the degradation rate of total proteins in cultured foetal hepatocytes, yet it specifically inhibited the degradation of proteins prelabelled for 10h with [^{3}H]glucosamine. As this inhibition was already obvious after 2h, the proteolytic system responsible for the degradation of glycoproteins responded rapidly to cycloheximide. This might be due to an inhibitory effect of the drug on the synthesis of a short-lived protein involved in the proteolytic process, but other possibilities, such as the release of some preformed proteinase from the cell (Hille *et al.*, 1970), cannot be excluded. The structure of the insulin receptor includes a carbohydrate moiety, and its glycoprotein nature is well documented (Jacobs *et al.*, 1980; Hedo *et al.*, 1981). The insulin-receptor degradation participates in the general pattern of membrane glycoprotein turnover, which could depend on a proteolytic system sensitive to cycloheximide during the early exposure to the drug.

The degradation rate of insulin, as measured by the release of the degraded products into the medium, corresponded to about 600 molecules/min per hepatocyte for 1nM-insulin. It was increased by 50%, or decreased by 45%, after 15h exposure to cycloheximide or tunicamycin respectively. These variations seem to be related to the modifications in insulin binding, rather than to a direct effect of the drugs on insulin degradation, since they were no longer observed when insulin degradation was expressed as a fraction of cell-bound hormone. Furthermore, the percentage of cell-associated insulin degraded was unchanged in the presence of the drugs. These results are consistent with the linear dependence which holds between the insulin-degradation rate and the concentration of insulin bound at steady state (Terris & Steiner, 1975, 1976). They confirm the receptor-mediated characteristic of insulin degradation in foetal hepatocytes (Plas & Desbuquois, 1982). Besides, as insulin degradation is unaffected by protein-synthesis inhibitors, it must be under the dependence of long-life proteolytic systems. Several proteinases have been proposed for cellular insulin degradation, e.g. glutathione–insulin transhydrogenase (Ansorge *et al.*, 1973; Varandani *et al.*, 1975) and neutral glutathione-independent insulinase (Burghen *et al.*, 1972; Duckworth *et al.*, 1979; Goldstein & Livingston, 1981). The most likely candidate in foetal hepatocytes should be sought among those proteinases that are not sensitive to cycloheximide and tunicamycin.

A clear glycogenic effect of insulin persisted after a 25h incubation in the presence of tunicamycin, even though the insulin binding was decreased by 80%. In cultured foetal hepatocytes (Menuelle & Plas, 1981), as in isolated soleus muscle (Le Marchand-Brustel *et al.*, 1978), insulin induces a half-maximal stimulation of glycogenesis when only a small fraction of the total receptor sites are occupied. Tunicamycin might therefore interfere with the synthesis of spare receptors. When cycloheximide was added together with insulin, the early glycogenic effect of insulin was maintained. This suggests that the insulin effect does not require protein synthesis *de novo*. However, a more prolonged incubation in the presence of the drug, which depressed both basal and insulin-stimulated glyco-

genesis, revealed that cycloheximide rapidly interferes with some step along the glycogen metabolic pathways. A specific inhibition of the glycogenic effect of insulin has been obtained in the presence of increasing chloroquine concentrations which in parallel inhibit cellular insulin degradation (Plas & Desbuquois, 1982). This suggested that receptor-mediated insulin degradation, or some associated pathway, is functionally related to the insulin glycogenic effect in cultured foetal hepatocytes. Cycloheximide could be a useful tool with which to investigate this aspect, but under the conditions used in the present study its inhibitory effect does not appear to be specific to insulin-mediated events.

Chronic exposure of cultured foetal hepatocytes to native hormone does not induce a decrease in insulin binding (Menuelle & Plas, 1981; the present paper). The insulin-induced down-regulation of insulin receptors, first described in IM9 lymphocytes (Gavin *et al.*, 1974), has also been reported in cultured adult rat and chick hepatocytes (Blackard *et al.*, 1978; Caro & Amatruda, 1980; Krupp & Lane, 1981). It has been attributed to an insulin-induced increase in the receptor-degradation rate in IM9 lymphocytes (Kosmakos & Roth, 1980; Kasuga *et al.*, 1981). Another mechanism has been implied in chick hepatocytes, namely the translocation of receptors from the cell surface to an intracellular compartment or to a sequestration of receptors in a state which is inaccessible to insulin (Krupp & Lane, 1981). In foetal hepatocytes, the estimated half-life of insulin receptors was comparable with that reported in other systems, but chronic exposure to insulin did not reveal any down-regulation, even when protein synthesis was inhibited by cycloheximide or tunicamycin. Thus the absence of down-regulation cannot be explained by an exceptionally rapid rate of receptor synthesis (possibly insulin-induced), which is related to the foetal stage of the cells. These results suggest the existence of a pathway which is distinct from receptor biosynthesis *de novo* and which permits the maintenance of a constant pool of functional receptors in the plasma membrane.

Morphological evidence of internalization of insulin in the cell, after its binding to plasma-membrane receptors, has been obtained in isolated hepatocytes (Carpentier *et al.*, 1979*a*) and intact liver (Bergeron *et al.*, 1979; Carpentier *et al.*, 1979*b*), and the hormone has been shown to be associated with lysosomes, or closely related structures. Other studies suggest that the insulin receptor is internalized or translocated into the cell after its interaction with the hormone (Desbuquois *et al.*, 1979; Hofmann *et al.*, 1981; Desbuquois *et al.*, 1982; Felhmann *et al.*, 1982). Receptor-mediated degradation of insulin is an active process in cultured foetal hepatocytes, where it represents 600 insulin molecules/min per hepatocyte for 1 nM-insulin. Maximal insulin binding, measured at a steady state at 37°C in the presence of 30 nM-insulin, corresponds to 20000 sites/hepatocyte (Menuelle & Plas, 1981). As the half-life of the insulin receptor can be expected to be equal to 13 h in the presence of tunicamycin, the rate of receptor degradation cannot exceed 13 sites degraded/min per hepatocyte. The rate of insulin degradation was therefore much higher than that for receptor degradation, in the same way as in chick hepatocytes (Krupp & Lane, 1982). This suggests that receptor site degradation is not required for the degradation of an insulin molecule that has interacted with its receptor, and thus implies the possible reutilization of the receptor. Furthermore, in cultured foetal hepatocytes, the proteolytic systems implicated in degradation of insulin and receptors differed in their sensitivity to cycloheximide. The degradation pathways of insulin and its receptor thus seem to be distinct. It is possible that the handling of insulin and its receptor depends on different enzymes, which may belong to a given subcellular compartment or be linked to a given single process, e.g. endocytosis.

This research was supported by the Centre National de la Recherche Scientifique (grants ATP-1981 Endocrinologie and Contract Programme-1982 Nutrition Fondamentale) and a generous gift from Organon Laboratories. This study is part of the Doctorate Thesis that E. P. will be submitting to the University of Paris 7, France.

References

Ansorge, S., Bohley, P., Kirschke, H., Langner, J., Wiederanders, B. & Hanson, H. (1973) *Eur. J. Biochem.* **32**, 27–35

Bergeron, J. J. M., Sikstrom, R., Hand, A. R. & Posner, B. I. (1979) *J. Cell Biol.* **80**, 427–443

Blackard, W. G., Guzelian, P. S. & Small, M. E. (1978) *Endocrinology* **103**, 548–553

Burghen, G. A., Kitabchi, A. E. & Brush, J. S. (1972) *Endocrinology* **91**, 633–642

Caro, J. F. & Amatruda, J. M. (1980) *J. Biol. Chem.* **255**, 10052–10055

Carpentier, J. L., Gorden, P., Barazzone, P., Freychet, P., Le Cam, A. & Orci, L. (1979*a*) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2803–2807

Carpentier, J. L., Gorden, P., Freychet, P., Le Cam, A. & Orci, L. (1979*b*) *J. Clin. Invest.* **63**, 1249–1261

Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120

Desbuquois, B., Willeput, J. & Huet de Froberville, A. (1979) *FEBS Lett.* **106**, 338–344

Desbuquois, B., Lopez, S. & Burlet, H. (1982) *J. Biol. Chem.* **257**, 10852–10860

Duckworth, W. C., Stentz, F. B., Heinemann, M. & Kitabchi, A. E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 635–639

Duksin, D. & Bornstein, P. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3433–3437
Evans, V. J., Bryant, J. C., Kerr, H. A. & Schilling, E. L. (1964) *Exp. Cell Res.* **36**, 439–474
Fehlmann, M., Carpentier, J. L., Le Cam, A., Thamm, P., Saunders, D., Braudenburg, D., Orci, L. & Freychet, P. (1982) *J. Cell Biol.* **93**, 82–87
Gavin, J. R., III, Roth, J., Neville, D. M., Jr., De Meyts, P. & Buell, D. N. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 84–88
Goldfine, I. D. (1981) *Biochim. Biophys. Acta* **650**, 53–67
Goldstein, B. J. & Livingston, J. N. (1981) *Endocrinology* **108**, 953–961
Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1979) *Nature (London)* **279**, 679–685
Gorden, Ph., Carpentier, J. L., Freychet, P. & Orci, L. (1980) *Diabetologia* **18**, 263–274
Hedo, J. A., Kasuga, M., Van Obberghen, E., Roth, J. & Kahn, C. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4791–4795
Hille, M. B., Barrett, A. J., Dingle, J. T. & Fell, H. B. (1970) *Exp. Cell Res.* **61**, 470–472
Hofmann, C., Ji, T. H., Miller, B. & Steiner, D. F. (1981) *J. Supramol. Struct.* **15**, 1–13
Jacobs, S., Hazum, E. & Cuatrecasas, P. (1980) *J. Biol. Chem.* **255**, 6937–6940
Kaplan, J. (1981) *Science* **212**, 14–20
Kasuga, M., Kahn, C. R., Hedo, J. A., Van Obberghen, E. & Yamada, K. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6917–6921
King, A. C. & Cuatrecasas, P. (1981) *N. Engl. J. Med.* **305**, 77–88
Kitagawa, T. (1980) *J. Cell. Physiol.* **102**, 37–43
Kosmakos, F. C. & Roth, J. (1980) *J. Biol. Chem.* **255**, 9860–9869
Krupp, M. & Lane, M. D. (1981) *J. Biol. Chem.* **256**, 1689–1694
Krupp, M. N. & Lane, M. D. (1982) *J. Biol. Chem.* **257**, 1372–1377
Le Marchand-Brustel, Y., Jeanrenaud, B. & Freychet, P. (1978) *Am. J. Physiol.* **234**, E348–E358
Menuelle, P. & Plas, C. (1981) *Diabetologia* **20**, 647–653
Pastan. I. H. & Willingham, M. C. (1981) *Annu. Rev. Physiol.* **43**, 239–250
Pearse, B. M. F. & Bretscher, M. S. (1981) *Annu. Rev. Biochem.* **50**, 85–101
Plas, C. & Desbuquois, B. (1982) *Biochem. J.* **202**, 333–341
Plas, C. & Nunez, J. (1976) *J. Biol. Chem.* **251**, 1431–1437
Plas, C., Chapeville, F. & Jacquot, R. (1973) *Dev. Biol.* **32**, 82–91
Plas, C., Menuelle, P., Moncany, M. L. J. & Fulchignoni-Lataud, M. C. (1979) *Diabetes* **28**, 705–712
Reed, B. C., Ronnett, G. V. & Lane, M. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2908–2912
Rosen, O. M., Chia, G. H., Fung, C. & Rubin, C. S. (1979) *J. Cell. Physiol.* **99**, 37–42
Schlessinger, J. (1980) *Trends Biochem. Sci.* **5**, 210–214
Takatsuki, A. K., Kohno, K. & Tamura, G. (1975) *Agric. Biol. Chem.* **39**, 2089–2091
Terris, S. & Steiner, D. F. (1975) *J. Biol. Chem.* **250**, 8389–8398
Terris, S. & Steiner, D. F. (1976) *J. Clin. Invest.* **57**, 885–896
Varandani, P. T., Nafz, M. A. & Chandler, M. L. (1975) *Biochemistry* **14**, 2115–2120