Cell-surface insulin receptor cycling and its implication in the glycogenic response in cultured foetal hepatocytes

Pascal SOUBIGOU, Eric PRINGAULT* and Christiane PLAS

Laboratoire Interactions Cellulaires, U.E.R. Odontologie, Université Paris 7, 2 place Jussieu, 75005 Paris, France

The insulin-receptor cycle was investigated in cultured foetal rat hepatocytes by determining the variations in insulin-binding sites at the cell surface after short exposure to the hormone. Binding of ^{125}I -insulin was measured at 4 °C after dissociation of prebound native insulin. Two protocols were used: exchange binding assay and binding after acid treatment; both gave the same results. Cell-surface ^{125}I -insulin-receptor binding decreased sharply (by 40%) during the first 5 min of 10 nM-insulin exposure ($t_{\frac{1}{2}} = 2$ min) and remained practically constant thereafter; subsequent removal of the hormone restored the initial binding within 10 min. This fall-rise sequence corresponded to variations in the number of insulin receptors at the cell surface, with no detectable change in receptor affinity. The reversible translocation of insulin receptors from the cell surface to a compartment not accessible to insulin at 4 °C was hormone-concentration- and temperature-dependent. SDS/polyacrylamide-gel electrophoresis after cross-linking of bound ^{125}I -insulin to cell-surface proteins with disuccinimidyl suberate showed that these variations were not associated with changes in M_r of binding components, in particular for the major labelled band of M_r 130 000. The insulin-receptor cycle could be repeated after intermittent exposure to insulin. Continuous or intermittent exposure to the hormone gave a similar glycogenic response, contrary to the partial effect of a unique short (5–20 min) exposure. A relationship could be established between the repetitive character of the rapid insulin-receptor cycle and the maximal expression of the biological effect in cultured foetal hepatocytes.

INTRODUCTION

A wide variety of ligands, including low-density lipoprotein, transferrin, galactose- or mannose-terminal glycoproteins, α_2 -macroglobulin and IgG, are brought into cells by receptor-mediated endocytosis. After dissociation of the internalized receptor–ligand complex in a pre-lysosomal acidic compartment, the ligand or part of it is despatched to its intracellular destination, while the receptor may be recycled back to the cell surface (for reviews see Kaplan, 1981; Anderson *et al.*, 1982; Ashwell & Harford, 1982; Ciechanover *et al.*, 1983; Tycko *et al.*, 1984; Schneider *et al.*, 1985). For hormones such as insulin, the internalization of the ligand–receptor complex via endocytosis as well as the subsequent cellular degradation of insulin are well documented (Terris & Steiner, 1975; Gorden *et al.*, 1980; Olefsky *et al.*, 1982; Posner *et al.*, 1982). Large differences have been found between the survival half-time of the receptor and insulin itself in cultured chick hepatocytes, suggesting the sparing of the receptor and its reutilization (Krupp & Lane, 1982). Insulin-receptor recycling has been also predicted from the effect of lysosomotropic agents on insulin-induced receptor loss in isolated rat adipocytes (Marshall *et al.*, 1981). A progressive recycling of the internalized photoaffinity-labelled insulin receptors back to the cell surface has been demonstrated in isolated rat hepatocytes (Fehlmann *et al.*, 1982b). On the other hand, the insulin-induced translocation of cell-surface insulin receptors to a proteinase-resistant

intracellular compartment has been shown to be rapidly reversed in 3T3- C_2 fibroblasts (Knutson *et al.*, 1983) and in isolated rat adipocytes (Arsenis *et al.*, 1985; Marshall, 1985) after removal of insulin.

Cellular insulin degradation in cultured foetal rat hepatocytes is predominantly mediated by the endocytosis of the insulin–receptor complex (Plas & Desbuquois, 1982). As its maximal rate (6000 molecules/min per cell) largely exceeds the rate of the insulin-receptor degradation (estimated at 13 sites/min per cell), the insulin receptor would appear to be efficiently reutilized (Pringault & Plas, 1983; Pringault *et al.*, 1985). These cells, moreover, unlike cultured adult hepatocytes (Blackard *et al.*, 1978), show no evidence for a decrease in insulin-receptor cell concentration after chronic exposure to the hormone (receptor down-regulation) (Menuelle & Plas, 1981). These findings suggest that insulin receptors are rapidly internalized and recycled back to the cell surface in cultured foetal hepatocytes, which appear to be particularly suitable for the investigation of the insulin–receptor cycle. The present paper describes the variations in insulin receptor sites present on the cell surface at any given moment after transitory exposure to insulin. The large amplitude of the fall-rise sequence in cell-surface receptor numbers permitted the receptor-cycling process to be characterized. It was found to be rapid, repetitive, and dose- and temperature-dependent. The relationship between the cycling process and the glycogenic response to insulin was investigated by taking advantage of the kinetic aspect of the two processes.

* Present address: Unité de Biologie des Membranes, Département de Biologie Moléculaire, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France.

MATERIALS AND METHODS

Materials

Medium NCTC 135 was obtained from Gibco and foetal calf serum from Sorga (batch 95). Pig mono-component insulin was purchased from Novo Laboratories, Na^{125}I was from Amersham Corp., and insulin was iodinated at 130–170 Ci/g by a modification (Desbuquois *et al.*, 1974) of the chloramine-T method. Cortisol 21-succinate (sodium salt) was from Sigma.

Culture procedure

Primary cultures of hepatocytes were obtained from 18-day rat (Sprague-Dawley) fetuses as described previously (Plas *et al.*, 1973). The culture medium consisted of NCTC 109 medium (Evans *et al.*, 1964) supplemented with 10% (v/v) foetal-calf serum, 10 μM -cortisol and 10 mM-Hepes, pH 7.3. Experiments were performed after 2 days of culture in the presence of cortisol, when the glycogenic effect of insulin is fully expressed (Plas & Nunez, 1976).

Cell-surface insulin binding

Incubations of ^{125}I -insulin (1–30 nM) with foetal hepatocytes grown in monolayers (about 1.3×10^6 cells/6 cm-diameter dish) were performed for 4 h at 4 °C in 1 ml of 'binding medium'. This medium, which consists of glucose-free NCTC 109 medium + 1 mM-KCN, pH 7.8, and the low temperature used, were chosen to avoid any receptor redistribution. At the end of the incubation, the medium was removed and the cells were washed with 4×2.5 ml of ice-cold Hanks solution. Cell-surface-associated radioactivity was then extracted and measured as previously described (Plas & Desbuquois, 1982). Corrections were made for the non-specific association of ^{125}I -insulin with cells by performing parallel incubations in the presence of 10 μM native insulin. Insulin specific binding was expressed as fmol of ^{125}I -insulin bound per culture. For the concentration of 10 nM- ^{125}I -insulin generally used, the non-specific binding represented 12% of the total binding.

Cell-surface binding after a brief exposure to native insulin was determined in cells which were first exposed to 10 nM-insulin in culture medium at 37 °C for various times (up to 30 min in most experiments). Insulin was removed or not after 10 min. Culture medium was removed at the end of the incubation, and the cells were cooled to 4 °C, and washed once with cold culture medium containing 10 nM native insulin to eliminate any possible effect of insulin carry-over. In all subsequent operations, the temperature was carefully maintained at 4 °C. Two different protocols were used to quantify cell-surface insulin binding after dissociation of prebound native insulin: (1) cultures were washed once with binding medium containing 10 nM- ^{125}I -insulin and incubated for 4 h in the same medium (exchange binding assay); (2) cultures were washed four times with Hanks solution, incubated for 5 min in barbital/sodium acetate buffer, pH 3.0, to remove cell-surface-bound insulin (Olefsky & Kao, 1982), then washed twice with binding medium to restore pH, and incubated for 4 h in binding medium containing 10 nM- ^{125}I -insulin (binding after acid treatment). The amount of cell-surface specifically bound ^{125}I -insulin was determined as described above.

Analysis of ^{125}I -insulin covalently linked to cell-surface receptors

^{125}I -insulin bound to cells was covalently cross-linked to receptors by using the bifunctional chemical reagent disuccinimidyl suberate as previously described by Pilch & Czech (1980). First, cultured foetal hepatocytes were allowed to bind ^{125}I -insulin for 4 h at 4 °C. After four washings in ice-cold Hanks solution, cells were incubated for 15 min at 4 °C in binding medium containing 2 mM-disuccinimidyl suberate and 5% (v/v) dimethyl sulphoxide (stock solution of 0.2 M-disuccinimidyl suberate was freshly prepared in dimethyl sulphoxide). The reaction was quenched by replacement of the medium by ice-cold Hanks solution. Cell material was solubilized in Laemmli buffer under reducing conditions and subjected to electrophoresis on SDS/polyacrylamide gels as described by Laemmli (1970), followed by autoradiography. The relative intensities of the bands were determined by scanning the autoradiographs with a Joyce-Loebl microdensitometer and by direct counting of radioactivity of the bands cut out from the dried gels.

Glycogenesis

Glycogen labelling in the presence of [^{14}C]glucose was determined as previously described (Plas *et al.*, 1973).

Definitions

Each protocol involved at least three experiments performed on different cell preparations. Each symbol on the graphs corresponds to a different culture. The results are expressed per culture. The cell population of a culture dish is of the order of 1.3×10^6 hepatocytes, which corresponds to 500 μg of protein and to 3.6 mg wet wt. of liver.

RESULTS

Variations in cell-surface ^{125}I -insulin binding after brief exposure to insulin

Foetal hepatocytes were first exposed to 10 nM native insulin for various times (up to 40 min) in culture medium at 37 °C; then cell-surface specific insulin binding was measured after incubation at 4 °C for 4 h in the presence of 10 nM- ^{125}I -insulin as described in the Materials and methods section. Cell-surface insulin binding decreased sharply during the first 5 min of insulin exposure and thereafter remained practically constant ($t_{1/2} = 2$ min) (Fig. 1). A new steady state of insulin binding was thereby achieved, which represented about 60% of the value when no insulin was present. The initial rate of the decrease in cell-surface binding represented about 12% of the initial binding/min, which corresponded to an estimated rate of 12000 insulin-binding sites/min per cell. When 10 nM native insulin was removed after a 10 min (or a 30 min) exposure to the hormone and the cells were maintained in insulin-free culture medium at 37 °C, cell-surface insulin binding gradually returned to a value close (about 95%) to the initial value (Fig. 1). Maximal recovery of binding occurred within 10 min, with a partial (about half-maximal) effect after 5 min.

Similar insulin-induced variations in cell-surface insulin binding were obtained when ^{125}I -insulin was

Fig. 1. Variations in cell-surface ^{125}I -insulin binding after short exposure to the hormone

Foetal hepatocytes were first incubated in the presence of 10 nM native insulin in culture medium at 37 °C for various times up to 40 min, insulin being removed or not after 10 or 30 min. Then the cells were transferred at 4 °C and cell-surface insulin binding was measured after a 4 h incubation in 'binding medium' containing 10 nM- ^{125}I -insulin, by using exchange binding assay (●, ○) or binding after acid treatment (▲, △) as described in the Materials and methods section. The cell-surface insulin binding in cultures incubated with insulin (●, ▲), or with insulin and then after removal of the hormone (○, △) is shown.

allowed to bind to cells at 4 °C under two different conditions, i.e. exchange binding assay and binding after acid treatment, as described in the Materials and methods section. The amount of cell-surface insulin bound was reproducibly identical by both methods (Fig. 1). According to Haigler *et al.* (1980) and Olefsky & Kao (1982), the acid extraction technique permits the removal of surface-bound ligand, leaving only intracellular cell-associated hormone. In cultured foetal hepatocytes, this treatment allowed dissociation of more than 85% of cell-surface associated ^{125}I -insulin, when binding was performed at a hormone concentration of 10 nM under steady-state conditions at 4 °C, i.e. after 3 h. When cells were incubated with ^{125}I -insulin at the same concentration but at 37 °C for 10 min, the extent of dissociation was decreased to 35%; the remaining cell-associated radioactivity represented internalized hormone. The same proportion (35%) of insulin prebound at 37 °C was released during a 2–3 h chase incubation at 4 °C in the presence of 10 nM native hormone, thereby indicating that dissociation of cell-surface-bound insulin effectively occurred under such conditions. A 4 h incubation time was chosen for both exchange binding assay and binding after acid treatment to ascertain the results, a time when cell permeabilization had not yet happened, as shown by the Trypan Blue dye-exclusion test.

Changes in cell-surface insulin binding were also measured as a function of ligand concentration. For ^{125}I -insulin concentrations in the range 1–30 nM, cell-surface insulin binding was always decreased by 35% after a 10 min exposure to 10 nM-insulin, and was then restored to 90% of the initial value after removal of the hormone (Fig. 2). Scatchard plots of the binding data were curvilinear and approximately parallel. Although it

Fig. 2. Cell-surface ^{125}I -insulin binding as a function of the hormone concentration after short exposure to native insulin

Foetal hepatocytes were incubated with 10 nM-insulin or hormone solvent for up to 20 min at 37 °C, insulin being removed or not after 10 min. Then cell-surface insulin binding was measured for 4 h at 4 °C in the presence of increasing concentrations of ^{125}I -insulin after liberation of sites from native insulin by acid treatment as described in the Materials and methods section. The cell-surface insulin binding in cultures incubated for 10 min with insulin (●), with insulin and then for a further 10 min after removal of the hormone (○), or with hormone solvent alone (■), is shown. Inset: Scatchard plot of the binding data.

was difficult to determine accurately the total number of sites, because of this curvilinearity, these plots revealed a reversible decrease in cell-surface insulin receptor amount, with no change in the apparent affinity of the receptors. The decrease represented receptor translocation from the cell surface to a compartment which is not accessible to insulin at 4 °C, and the recovery reflects receptor translocation from a compartment not accessible to insulin at 4 °C towards the cell surface. In the present study, 'internalized' and 'recovered' receptors refer respectively to those concerned with these two converse processes.

The decrease in cell-surface insulin binding was measured after 10 min incubation of cells at 37 °C in the presence of various concentrations of native insulin. The effect progressively developed with the dose of insulin used; maximal effect was obtained with 30 nM-insulin (54% decrease) and half-maximal effect with about 3 nM (25%). A 12% decrease was already seen with 1 nM. The dose of 10 nM-insulin, which produces a maximal glycogenic response (Plas *et al.*, 1979), was used to investigate the effect of temperature on the cycling process of the insulin receptors. The decrease in cell-surface binding induced by a 10 min exposure to 10 nM-insulin at various temperatures was not detectable at 4 °C, but was present at 16 °C (28%). The pronounced effect at 22 °C was similar to that observed at 37 °C (40%) (Fig. 3a). After previous internalization of insulin receptors at 37 °C, the recovery of cell-surface binding

during the subsequent 10 min incubation in the absence of the hormone was not detectable at 4 °C. It was partial at 16 °C and nearly total at 22 °C and 37 °C (Fig. 3b).

Fig. 3. Temperature-dependence of the insulin-receptor cycling

(a) Foetal hepatocytes were first incubated in the presence of 10 nM native insulin or hormone solvent for 10 min in culture medium at various temperatures. (b) Foetal hepatocytes were first incubated in the presence of 10 nM native insulin or hormone solvent for 10 min in culture medium at 37 °C, then insulin was removed or not, and the cells were incubated for a further 10 min at various temperatures. For (a) and (b), at the end of the incubation, cell-surface ^{125}I -insulin specific binding was measured by the exchange binding assay as described in the Materials and methods section. The cell-surface insulin binding in cultures incubated with insulin (●), with insulin and then after removal of the hormone (○), or with hormone solvent alone (■), is shown.

Characterization of cell-surface receptors cross-linked covalently to ^{125}I -insulin

After covalent cross-linking of bound ^{125}I -insulin to cell-surface proteins by using disuccinimidyl suberate (Pilch & Czech, 1980), the labelled material was subjected to electrophoresis, followed by autoradiography. The major ^{125}I -insulin-labelled polypeptide from cells not previously exposed to native insulin at 37 °C, which entered the gel, had M_r 130 000 (Fig. 4). Two minor components with mobilities corresponding to M_r 300 000 (appearing as a doublet) and M_r 90 000 were also present. An additional band, of M_r 115 000, was also reproducibly labelled. The five binding components were insulin-specific, since their labelling could be prevented by a simultaneous incubation with excess native insulin.

The cell-surface radioactive material was then analysed during variations in receptor amounts after exposure to 10 nM-insulin and subsequent removal of the hormone. This approach does not permit a quantitative determination of the amount of receptors, because, as previously described (Ronnett *et al.*, 1983), only 10% of specifically bound insulin cross-linked covalently to cell-surface proteins. This poor efficiency of cross-linking was nonetheless reproducible, and the densitometry pattern of autoradiographs reflected the variations in cell-surface binding (Fig. 4). Moreover, the five labelled components were always present during the binding decrease and recovery processes. The relative proportion of these bands was maintained; it averaged, for the M_r -300 000–305 000, M_r -130 000, M_r -115 000 and M_r -90 000 components, 5, 60, 25 and 10% respectively of the total ^{125}I -insulin labelling cross-linked to cell-surface binding components, as determined by scanning the radioactivity and by the direct counting of excised bands.

The major protein band, of M_r 130 000, corresponds to the main insulin-binding subunit of the insulin

Fig. 4. Characterization of cell-surface insulin receptor cross-linked covalently to ^{125}I -insulin after short exposure to insulin

Foetal hepatocytes were first incubated in the presence of 10 nM native insulin or hormone solvent in culture medium at 37 °C for various times up to 30 min, insulin being removed or not after 10 min. Then cells were transferred to 'binding medium' containing 10 nM- ^{125}I -insulin for 4 h at 4 °C, and ^{125}I -insulin bound to cells was covalently cross-linked to receptors by using disuccinimidyl suberate as described in the Materials and methods section. Labelled material was extracted with Laemmli (1970) buffer under reducing conditions and subjected to SDS/polyacrylamide-gel electrophoresis, followed by autoradiography. Autoradiograms from cross-linking of bound ^{125}I -insulin in cells incubated for the times indicated with 10 nM native insulin (a), or in insulin-free medium after previous exposure to 10 nM-insulin for 10 min (b), are shown. Lane (x) derives from cells where an excess of unlabelled insulin was present during the incubation with ^{125}I -insulin. The M_r values were determined by comparison with the following standards: myosin, 205 000; β -galactosidase, 116 000; phosphorylase b, 97 400; bovine serum albumin, 66 000; ovalbumin, 45 000; carbonic anhydrase, 29 000.

receptor, which is referred to as the α -subunit of the receptor in other cell systems (Pilch & Czech, 1980). A band similar to the M_r 115000 component, with a M_r slightly lower than that of the α -subunit, has been described in isolated adult hepatocytes by using photoaffinity labelling of the receptor (Fehlmann *et al.*, 1982b). This additional component was found to derive from the M_r -130000 component by proteolytic conversion (Fehlmann *et al.*, 1982a,b; Donner & Yonkers, 1983; Haynes & Yip, 1985).

Repetitive character of the insulin-receptor cycle and the glycogenic response to the hormone

When cells were exposed to insulin intermittently, by using three successive short exposures to 10 nM-insulin at 10 min intervals separated by incubations in the absence of the hormone, the fall-rise sequence of variations in cell-surface binding was reproduced three times (Fig. 5). The slight attenuation of the amplitude of the variations could be due to experimental difficulties linked to rapid transitions in hormone concentration in the medium and temperature.

When foetal hepatocytes were incubated in the continued presence of 10 nM-insulin, the insulin stimulation of [14 C]glucose incorporation into glycogen progressively developed with time to become 4-fold after 70 min (Fig. 6). Intermittent short exposure to insulin, with four successive 10 min exposures at 10 min intervals, evoked a nearly maximal insulin effect on glycogenesis (3.2-fold). Transitory exposure to 10 nM-insulin, from 2 to 20 min, only produced partial stimulation of glycogenesis: the stimulation was very poor for 2 min (1.4-fold), increased to 2.0-fold for 5 min and remained constant between 5 and 20 min. A second transitory exposure to 10 nM-insulin, 30 min later for example, gave some restoration of the insulin glycogenic effect (2.5-fold).

Fig. 5. Effect of intermittent insulin exposure on cell-surface 125 I-insulin binding

Cells were first exposed to 10 nM-insulin by using three successive short exposures at 10 min intervals separated by incubations in the absence of the hormone. Then cell-surface 125 I-insulin specific binding was measured by the exchange binding assay as described in the Materials and methods section. Data are presented as means $\pm$ s.d. for four experiments performed with different cell preparations.

Fig. 6. Effect of brief exposure to insulin on insulin-stimulated glycogenesis

At day 2 of the culture (zero time of the experiment), the medium was replaced by fresh NCTC 109 medium which was supplemented with 10 nM-insulin or hormone solvent together with [14 C]glucose (1 μ Ci/mg). Cultures were then incubated for up to 70 min with insulin present under different conditions, [14 C]glucose being maintained in the medium. The radioactivity present in glycogen was measured at the times indicated. Results are shown for cultures exposed continuously to 10 nM-insulin (●), intermittently by four successive short insulin exposures at 10 min intervals separated by incubations in the absence of the hormone (○), or two successive exposures from 0 to 10 min, and from 30 to 40 min (■), brief exposure to 10 nM-insulin from time zero to 2–20 min followed by removal of hormone (▲), or in the presence of hormone solvent alone (△).

DISCUSSION

The route of the insulin receptor inside the cell has been studied directly by using the insulin-receptor photoaffinity labelling, which leaves the possibility that the covalent insulin-receptor complexes are not processed by the cell in the same way as the receptors that have bound the freely dissociable hormone (Fehlmann *et al.*, 1982b; Chvatchko *et al.*, 1984). Another method has been developed to quantify the total and the internal insulin-receptor pools, which combines cell-surface trypsin treatment at low temperature with receptor solubilization by Triton X-100 (Olefsky *et al.*, 1982; Knutson *et al.*, 1983). It allows examination of the return to the cell surface of proteinase-resistant insulin receptors, previously internalized in the presence of the hormone (Knutson *et al.*, 1983; Arsenis *et al.*, 1985; Marshall, 1985). The present study investigated the variations in insulin-receptor sites on the cell surface after transitory exposure to insulin. This approach, which revealed a rapid and reversible depletion of cell-surface insulin-binding sites after a short exposure to the hormone, was facilitated by the large amplitude of the variations. It permitted study, in intact cells, of the kinetics of the recovery process, its regulation by temperature and the mode of exposure to the hormone. The two different methods used to determine cell-surface insulin-binding sites after exposure to native hormone,

i.e. exchange binding assay and binding after acid treatment, gave the same results.

Cell-surface insulin-binding sites decreased to 60% of the initial value after 5 min exposure to 10 nM-insulin at 37 °C and were restored (up to 95%) 5–10 min after the removal of the hormone. These variations corresponded to variations in the number of insulin receptors, with no change in their apparent affinity and no modification in the M_r of the binding components. In particular, no difference was detected in the relative proportion of M_r -130000 and M_r -115000 components, which correspond respectively to the α -subunit of the hepatocyte insulin receptor (Pilch & Czech, 1980) and to a proteolytically derived fragment from this subunit (Fehlmann *et al.*, 1982a,b; Donner & Yonkers, 1983; Haynes & Yip, 1985). This suggests, as previously described in isolated adipocytes (Arsenis *et al.*, 1985), that the structure of the recycled insulin receptor is intact. When studied by the photoaffinity labelling of insulin receptors in isolated adult hepatocytes, a component corresponding to M_r 115000 was also found to be internalized together with the M_r -130000 component, but was not recovered at the cell surface with it during the subsequent progressive recycling (within 4 h) of insulin receptors (Fehlmann *et al.*, 1982b).

Receptor internalization and recovery processes were found to be rapid and dose- and temperature-dependent in cultured foetal hepatocytes. A significant translocation was already observed with 0.3 nM-insulin, a concentration which gave a half-maximal glycogenic response in this cell system (Plas *et al.*, 1979). Both processes were clearly decreased at a temperature of 16 °C. These results could be compared as far as the internalization process with similar ones obtained in adult hepatocytes. Physiological increases in insulin concentrations have been shown to enhance the proportion of occupied insulin receptor sites which became internalized (acid-resistant) (Caro *et al.*, 1982; Draznin *et al.*, 1984). Kinetic studies of the intracellular localization of 125 I-insulin by autoradiography have revealed an internalization of insulin to a limited region of the peripheral cytoplasm, which occurred more slowly at 20 °C than at 37 °C (Carpentier *et al.*, 1979). Moreover, a single injection of insulin into rats induced a rapid translocation of liver insulin receptors from the plasma membrane to the Golgi-like enriched fractions (Posner *et al.*, 1980; Desbuquois *et al.*, 1982); the insulin-binding activity was decreased by approx. 50% in the plasma membrane, with a half-maximal value after 4 min and a steady state after 8 min (Desbuquois *et al.*, 1982). On the other hand, a rapid rate of insulin-receptor cycling has been predicted from studies on the effects of monensin on the time course of the uptake of insulin in isolated adult hepatocytes (Whittaker *et al.*, 1986).

The 40% decrease in cell-surface receptors after 5 min of insulin exposure implied a new distribution of insulin receptors in cultured foetal hepatocytes. This pronounced modification is in favour of a ligand-dependent alteration of receptor cycling, which could be due to accelerated receptor internalization and/or decreased externalization. Some insulin-receptor cycling could also occur regardless of the presence or absence of insulin, as in Fao hepatoma cells (Chvatchko *et al.*, 1984). A further question which arises is the source of the surface receptors that are replenished after removal of hormone. In isolated adipocytes few, if any, intracellular receptors exist before

insulin exposure, and insulin appears to trigger off receptor internalization essentially through a ligand-dependent process (Marshall, 1985). Also the cell-surface receptors rapidly recovered after removal of hormone necessarily originate from the receptor pool previously internalized (Arsenis *et al.*, 1985; Marshall, 1985). In the adult rat liver, it has been estimated from cell-fractionation studies that at least 35% of the insulin-receptor sites are localized in different structural compartments inside the cell (Posner *et al.*, 1980; Desbuquois *et al.*, 1982). The poor yield of solubilized foetal hepatocyte insulin receptors after Triton X-100 treatment did not allow us to quantify the cellular distribution of insulin receptors. Whether insulin receptor recovery in the present cell system occurs at the expense of the just internalized pool and/or of another internal one is not yet known.

The stimulation of glycogenesis caused by a transitory exposure to insulin, which allowed a complete cycle of the insulin receptor in cultured foetal hepatocytes, was poor, but could be improved by a second brief exposure to insulin. Furthermore, intermittent short exposures to insulin at 10 min intervals gave a nearly maximal response. This suggests that one cycle of the insulin receptors is not sufficient to develop the characteristic insulin glycogenic response, which is fully expressed when foetal hepatocytes are subjected to a prolonged or repeated exposure to the hormone. A 15 min exposure to insulin was also unable to produce a maximal activation of glycogenesis in isolated adult hepatocytes (Frank *et al.*, 1981). The recycled insulin receptors are likely to be functional, as previously shown in isolated adipocytes (Arsenis *et al.*, 1985). Cultured foetal hepatocytes are characterized by a rapid insulin-receptor cycling process, which is moreover repetitive, since it was possible to reproduce it at least three times successively. The fact that the receptor-endocytosis-mediated insulin degradation was maintained at a constant rate for several hours (Pringault & Plas, 1983) suggests that the cycling of the insulin receptors occurs very efficiently during this time. Such efficiency is probably related to a specific ability to maintain the maximal expression of the glycogenic effect of insulin for 3 h in the foetal hepatocyte system.

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REFERENCES

- Anderson, R. G. W., Brown, M. S., Beisiegel, U. & Goldstein, J. L. (1982) *J. Cell Biol.* **93**, 523–531
- Arsenis, G., Hayes, G. R. & Livingston, J. N. (1985) *J. Biol. Chem.* **260**, 2202–2207
- Ashwell, G. & Harford, J. (1982) *Annu. Rev. Biochem.* **51**, 531–554
- Blackard, W. G., Guzelian, P. S. & Small, M. E. (1978) *Endocrinology* (Baltimore) **103**, 548–553
- Caro, J. F., Muller, G. & Glennon, J. A. (1982) *J. Biol. Chem.* **257**, 8459–8466
- Carpentier, J.-L., Gorden, P., Freychet, P., Le Cam, A. & Orci, L. (1979) *Diabetologia* **17**, 379–384
- Chvatchko, Y., Van Obberghen, E. & Fehlmann, M. (1984) *Biochem. J.* **222**, 111–117

- Ciechanover, A., Schwartz, A. L. & Lodish, H. F. (1983) *J. Cell. Biochem.* **23**, 107–130
- Desbuquois, B., Krug, F. & Cuatrecasas, P. (1974) *Biochim. Biophys. Acta* **343**, 101–120
- Desbuquois, B., Lopez, S. & Burlet, H. (1982) *J. Biol. Chem.* **257**, 10852–10860
- Donner, D. B. & Yonkers, K. (1983) *J. Biol. Chem.* **258**, 9413–9418
- Draznin, B., Trowbridge, M. & Ferguson, L. (1984) *Biochem. J.* **218**, 307–312
- 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., Brandenburg, D., Orci, L. & Freychet, P. (1982a) *J. Cell Biol.* **93**, 82–87
- Fehlmann, M., Carpentier, J.-L., Van Obberghen, E., Freychet, P., Thamm, P., Saunders, D., Brandenburg, D. & Orci, L. (1982b) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5921–5925
- Frank, H. J. L., Davidson, M. B. & Serbin, P. A. (1981) *Metab. Clin. Exp.* **30**, 1159–1164
- Gorden, P., Carpentier, J.-L., Freychet, P. & Orci, L. (1980) *Diabetologia* **18**, 263–274
- Haigler, H. T., Maxfield, F. R., Willingham, M. C. & Pastan, I. (1980) *J. Biol. Chem.* **255**, 1239–1241
- Haynes, F. J. & Yip, C. C. (1985) *Diabetologia* **28**, 786–792
- Kaplan, J. (1981) *Science* **212**, 14–20
- Knutson, V. P., Ronnett, G. V. & Lane, M. D. (1983) *J. Biol. Chem.* **258**, 12139–12142
- Krupp, M. N. & Lane, M. D. (1982) *J. Biol. Chem.* **257**, 1372–1377
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Marshall, S. (1985) *J. Biol. Chem.* **260**, 4136–4144
- Marshall, S., Green, A. & Olefsky, J. M. (1981) *J. Biol. Chem.* **256**, 11464–11470
- Menuelle, P. & Plas, C. (1981) *Diabetologia* **20**, 647–653
- Olefsky, J. M. & Kao, M. (1982) *J. Biol. Chem.* **257**, 8667–8673
- Olefsky, J. M., Marshall, S., Berhanu, P., Sackow, M., Heidenreich, K. & Green, A. (1982) *Metab. Clin. Exp.* **31**, 670–690
- Pilch, P. F. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 1722–1731
- 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
- Posner, B. L., Patel, B., Verma, A. K. & Bergeron, J. J. M. (1980) *J. Biol. Chem.* **255**, 735–741
- Posner, B. I., Khan, M. N. & Bergeron, J. J. M. (1982) *Endocr. Rev.* **3**, 280–298
- Pringault, E. & Plas, C. (1983) *Biochem. J.* **212**, 529–537
- Pringault, E., Plas, C., Desbuquois, B. & Clauser, H. (1985) *Biol. Cell.* **52**, 13–22
- Ronnett, G. V., Tennekoon, G., Knutson, V. P. & Lane, M. D. (1983) *J. Biol. Chem.* **258**, 283–290
- Schneider, Y.-J., Octave, J.-N. & Trouet, A. (1985) *Curr. Top. Membr. Transp.* **24**, 413–458
- Terris, S. & Steiner, D. F. (1975) *J. Biol. Chem.* **250**, 8389–8398
- Tycko, B., DiPaola, M., Yamashiro, D. J., Fluss, S. & Maxfield, F. R. (1984) *Ann. N.Y. Acad. Sci.* **421**, 424–433
- Whittaker, J., Hammond, V. A., Taylor, R. & Alberti, K. G. M. M. (1986) *Biochem. J.* **234**, 463–468

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