Insulin receptor desensitization correlates with attenuation of tyrosine kinase activity, but not of receptor endocytosis

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A model of insulin-receptor down-regulation and desensitization has been developed and described. In this model, both insulin-receptor down-regulation and functional desensitization are induced in the human HepG2 cell line by a 16 h exposure of the cells to 0.1 μ M-insulin. Insulin-receptor affinity is unchanged, but receptor number is decreased by 50%, as determined both by 125 I-insulin binding and by protein immunoblotting with an antibody to the β -subunit of the receptor. This down-regulation is accompanied by a disproportionate loss of insulin-stimulated glycogen synthesis, yielding a population of cell-surface insulin receptors which bind insulin normally but which are unable to mediate insulin-stimulated glycogen synthesis within the cell. Upon binding of insulin, the desensitized receptors are internalized rapidly, with characteristics indistinguishable from those of control cells. In contrast, this desensitization is accompanied by a loss of the insulin-sensitive tyrosine kinase activity of insulin receptors isolated from these cells. Receptors isolated from control cells show a 5–25-fold enhancement of autophosphorylation of the β -subunit by insulin; this insulin-responsive autophosphorylation is severely attenuated after desensitization to a maximum of 0–2-fold stimulation by insulin. Likewise, the receptor-mediated phosphorylation of exogenous angiotensin II, which is stimulated 2–10-fold by insulin in receptors from control cells, is completely unresponsive to insulin in desensitized cells. These data provide evidence that the insulin-receptor tyrosine kinase activity correlates with insulin stimulation of an intracellular metabolic event. The data suggest that receptor endocytosis is not sufficient to mediate insulin's effects, and thereby argue for a role of the receptor tyrosine kinase activity in the mediation of insulin action.

INTRODUCTION

The binding of insulin to its receptor mediates a variety of intracellular metabolic and growth-promoting functions, and the molecular mechanism of insulin action remains the subject of active study. Several proximal physiological responses of the insulin receptor to insulin binding have been identified, which may be involved in the mediation of insulin action within the cell. The elucidation of the primary and quaternary structure of the transmembrane insulin receptor has focused attention on the interaction between the disulphide-linked α - and β -subunits of the receptor as a potential primary site of signal transduction (Massague *et al.*, 1980; Hedo *et al.*, 1981; Siegel *et al.*, 1981). Binding of insulin to the extracellular α -subunit (M_r 135000) of the receptor has been shown to activate a tyrosine kinase activity of the intracellularly exposed β -subunit (M_r 95000), leading to the autophosphorylation of the β -subunit (Kasuga *et al.*, 1982; Machicao *et al.*, 1982; Petruzzelli *et al.*, 1982). Receptor autophosphorylation *in vitro* occurs solely on tyrosine residues and is sufficient to stimulate the *trans*-phosphorylation of exogenous substrates.

In intact cells, the tyrosine autophosphorylation and stimulation of the kinase activity is rapidly followed by extensive phosphorylation on serine and threonine residues of the insulin receptor, suggesting the specific activation of other intracellular kinases (Kasuga *et al.*, 1982; Pang *et al.*, 1985). The rapidity and specificity of the insulin-stimulated receptor autophosphorylation and tyrosine kinase activity, as well as the identification of

several potential intracellular substrates for this enzyme (Rosen *et al.*, 1981; White *et al.*, 1985), have prompted the suggestion that these phosphorylation reactions form the basis for the mediation of insulin action within the cell. However, the causal relationship of the receptor tyrosine kinase activity to insulin function has been difficult to demonstrate directly.

A further consequence of the interaction of insulin with its receptor on intact cells involves the sequestration of the occupied receptor away from the surface. Upon binding of insulin to its receptor, the receptor–hormone complex is rapidly internalized within the cell (Fehlmann *et al.*, 1982; Green & Olefsky, 1982; Knutson *et al.*, 1983). Although temporally related to the most rapid of insulin's effector functions such as glucose transport, and preceding other insulin-stimulated events, the function of insulin-receptor internalization has not yet been delineated. The internalization process may serve merely as a clearance mechanism for transporting bound insulin inside the cell, where it can be degraded, freeing the receptor for recycling. On the other hand, the presence of insulin receptors on the nucleus has raised the possibility of a function for the internalized insulin itself (Goldfine *et al.*, 1977).

To approach the question of the functional relevance of the insulin-stimulated receptor sequestration and/or tyrosine kinase activity, we have utilized insulin-induced receptor desensitization to produce a population of insulin receptors which bind insulin normally but are unable to stimulate glycogen synthesis. An examination of the insulin-receptor tyrosine kinase activity of these

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receptors shows that the desensitization of insulin function is accompanied by a complete attenuation of insulin-stimulated receptor autophosphorylation and of tyrosine kinase activity toward exogenous substrates. These data establish a strong correlation between the tyrosine kinase activity of the insulin receptor and insulin-stimulated glycogen synthesis within the cell. In contrast, the insulin-mediated sequestration of the cell-surface receptors appears to be unaltered by desensitization, indicating that receptor endocytosis is not, of itself, sufficient to mediate insulin action.

METHODS

Cell culture

HepG2 cells, obtained from Dr. B. Knowles, Wistar Institute, were grown as monolayer cultures in MEM (Modified Eagle's Medium) as described by Knowles *et al.* (1980). For binding studies, cells were grown in 24-well plates and used at a stage of early confluence, 3–4 days after plating (approx. 10^6 cells per well). For glycogen-synthesis experiments, cells were subcultured in Dulbecco's MEM, containing 10% (v/v) fetal-calf serum, 2 mM-L-glutamine, 100 units of penicillin/ml and 100 μ g of streptomycin/ml.

125 I-insulin binding

Insulin binding was performed on cell monolayers in 13 mm-diam. wells in 0.25 ml of MEM with 20 mM-Hepes/0.1% bovine serum albumin, pH 7.4, in the presence of 0.12 nM- 125 I-insulin (New England Nuclear). Non-specific binding, determined with the addition of 1 μ M unlabelled insulin (Elanco), was less than 5% of the total bound. After binding, cells were quickly washed three times in cold Hanks buffer (Hanks balanced salt solution containing 20 mM-Hepes and 0.1% albumin, pH 7.4), removed from the dish with 1 M-NaOH, and counted for radioactivity in a Beckman γ -counter. For Scatchard analysis, binding was performed at 4 °C overnight, and data were analysed by using the LIGAND computer program (Munsen & Rodbard, 1980). Solubilized-insulin-receptor binding was performed in 0.1% Triton X-100, with Sephadex G-75 gel filtration to separate bound 125 I-insulin from free. Non-specific binding, determined with the addition of 1 μ M-insulin, was 10–20% of the total bound in this assay.

For internalization of the receptor, cell monolayers were incubated with 125 I-insulin at 37 °C for various times as indicated in the Figure legends. At the end of the incubation, cells were washed three times with cold Hanks buffer, and the cell-surface receptors were inactivated by trypsin (0.5 mg/ml for 30 min at 25 °C) (Marshall *et al.*, 1981; Green & Olefsky, 1982; Knutson *et al.*, 1983; Peterson *et al.*, 1983; Reed *et al.*, 1984). After the addition of soya-bean trypsin inhibitor (1.5 mg/ml), the cells were removed from the plate, washed twice with Hanks buffer, and the 125 I present in the cell pellet was counted to quantify internalized receptor. Total cellular receptor was measured in parallel plates by counting cell-associated radioactivity directly after removal from the plate with no further washing.

Down-regulation of insulin receptors

For down-regulation of cell surface insulin receptors, cells were cultured in the presence of 0.1 μ M-insulin for

18 h. Bound insulin was removed by washing monolayers three times quickly in Hanks buffer, then washing (for 3 min each) at room temperature in Hanks buffer, pH 6.8–7.0, and then twice in Hanks buffer, pH 7.4, before measurement of insulin receptors by 125 I-insulin binding.

Incorporation of [14 C]glucose into glycogen

Cell monolayers were incubated with 25 μ M-[U- 14 C]-glucose in DMEM with 20 mM-Hepes, pH 7.4, with or without insulin for 90 min at 37 °C. After washing of the cells with cold Hanks buffer, 1 ml of 5 mM-KOH was added to the wells and samples were processed for the determination of [14 C]glycogen by the method of Hofmann *et al.* (1980). Cells in which insulin receptors had been previously down-regulated were washed three times in Dulbecco's MEM to remove the added insulin before the assay was begun.

Partial purification of insulin receptors

Detergent extracts enriched in insulin receptors were prepared by a modification of the method of Siegel *et al.* (1981). Briefly, HepG2 cells were grown to confluence in T-75 cm² flasks, washed several times with ice-cold phosphate-buffered saline (Gibco), pH 7.4, and monolayers were scraped and cells pelleted by centrifugation (1000 *g* for 5 min). Cell pellets were resuspended in solubilization buffer containing 50 mM-Hepes, pH 7.6, 150 mM-NaCl, 10 mM-MgSO₄, 1% Triton X-100, 1 μ g of leupeptin/ml and 1 mg of bacitracin/ml, and placed on a rotary wheel for 2 h at 4 °C. After centrifugation at 12000 *g* for 15 min, supernatants were applied to 1.5 ml wheat-germ-agglutinin-Sepharose columns and washed at 4 °C with 75 ml of solubilization buffer containing 0.1% Triton X-100. The glycoprotein fraction was eluted from the column with the same buffer containing 0.3 M-N-acetylglucosamine. Preparations were eluted at 4 °C and used immediately without freezing. Protein was determined by the method of Lowry *et al.* (1951).

Protein immunoblotting

Both whole cells (10^5 – 10^6 cells) and partially purified insulin-receptor preparations (0.5–10 fmol of insulin-binding activity) from control and desensitized cell preparations were subjected to SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Antigenic proteins were detected after transfer to nitrocellulose by the immunoblotting procedure of Towbin *et al.* (1979), as modified by Johnson & Elder (1983). The antibody used was raised in rabbits to the synthetic peptide EEHIPYTHMNGGKK, corresponding to residues 1317–1330 of the human insulin receptor (Ullrich *et al.*, 1985). This antibody was found to react with the β -subunit of the human insulin receptor, both by immunoprecipitation and by Western immunoblotting (results not shown). The antibody was used at a 1:100 dilution, followed by 125 I-Protein A (10^6 c.p.m./25 ml) for detection by autoradiography. Autoradiograms were quantified by scanning densitometry on Optronics high-speed scanner system.

Autophosphorylation assay and immunoprecipitation of the insulin receptor

Assays for receptor autophosphorylation were carried out with preparations of partially purified insulin receptor, containing 3–5 fmol of insulin-binding activity.

The receptor preparation was incubated for 15 min at 23 °C with various concentrations of insulin in a final volume of 50 µl, containing 0.02% Triton X-100, 20 mM-Hepes buffer, pH 7.4, with 5% (v/v) glycerol, 30 mM-NaCl, 2 mM-MnCl₂ and 12 mM-MgSO₄. [γ -³²P]ATP (50 µM; 50 c.p.m./fmol; Amersham) was used to initiate the phosphorylation reaction at 23 °C (Petrucelli *et al.*, 1982). The reaction was terminated after 30 min by dilution with a stopping buffer containing 50 mM-Hepes, pH 7.4, 150 mM-NaCl, 12 mM-MgSO₄, 0.1% Triton X-100, 5 mM-EDTA and 10 mM-ATP. Murine anti-(insulin receptor) antibody, obtained from Dr. S. Jacobs (Burroughs-Wellcome), was added at a final dilution of 1:300, and the mixture was incubated overnight at 4 °C. Then 50 µl of goat anti-(mouse IgG) conjugated to agarose (Sigma) was added to each tube. After a 2 h further incubation at 4 °C, the samples were centrifuged for 4 min at 12000 rev./min at 4 °C, and the agarose pellets were repeatedly washed with the stopping buffer. The pellets were then heated at 100 °C for 3 min in SDS/polyacrylamide-gel-electrophoresis sample buffer and the proteins analysed by SDS/polyacrylamide-gel electrophoresis. ³²P-labelled protein was detected by autoradiography and quantified by scanning densitometry.

Phosphorylation of exogenous angiotensin II

The partially purified insulin receptor (2.5–5 fmol of insulin-binding activity) was first incubated with insulin and [γ -³²P]ATP as for the autophosphorylation assay, except that 10 mM-dithiothreitol, 0.5 mM-sodium orthovanadate and 60 mM-*p*-nitrophenyl phosphate were included in the reaction. After the 15 min incubation with [γ -³²P]ATP, 2 mM-angiotensin II (Boehringer-Mannheim) was added and the incubation continued for a further 15 min before the protein was removed by trichloroacetic acid precipitation and the ³²P-labelled angiotensin II measured by binding to P-81 cellulose paper (Whatman) (Casnellie *et al.*, 1982). The paper was washed for 4 × 4 min in 75 mM-H₃PO₄, dried briefly in acetone, and the bound ³²P-labelled angiotensin II quantified by scintillation counting.

RESULTS

Desensitization and down-regulation of insulin receptors

As previously reported for HepG2 cells (Podskalny *et al.*, 1985; Strader *et al.*, 1985), as well as for many other cell systems (Krupp & Lane, 1981; Marshall *et al.*, 1981),

Fig. 1. Insulin binding to untreated and down-regulated HepG2 cells

Cells were incubated in the absence (Δ) or presence (×) of 0.1 µM-insulin for 18 h at 37 °C. After surface-bound insulin was washed away, the binding of ¹²⁵I-insulin was measured at 4 °C for 24 h. Data shown are representative of three experiments.

prolonged exposure of cells to insulin results in insulin-receptor down-regulation and desensitization. As shown in Fig. 1, this is expressed in HepG2 cells as a 50% loss in the number of cell-surface receptors, with no change in the affinity of the remaining receptors for insulin. This process was time- and dose-dependent, reaching a maximal decrease of 50% after 18 h of exposure to 0.1 µM-insulin. This 50% decrease in ¹²⁵I-insulin binding was also observed after detergent extraction of the receptors from the cell and partial purification by wheat-germ-agglutinin–Sephadex affinity chromatography, as shown in Table 1, suggesting that the down-regulated receptors had been degraded in this cell line. The 50% loss of total cellular insulin receptors was also confirmed by quantitative protein immunoblotting of whole cells against an antibody specific for the β-subunit of the insulin receptor (Table 1).

Down-regulated insulin receptors were functionally uncoupled from insulin stimulation of glycogen synthesis, as shown in Fig. 2. In contrast with control HepG2 cells, where insulin stimulated the synthesis of [¹⁴C]glycogen in a dose-dependent fashion with an EC₅₀ (concn. giving 50% of maximum stimulation) of 0.5 nM, there was a

Table 1. Characteristics of insulin receptors after down-regulation

Insulin binding to intact cells was performed on monolayer cultures as described in the Methods section. Results are means ± S.D. of three experiments, each done in triplicate. Binding to wheat-germ-agglutinin–Sephadex-purified receptor was measured by Sephadex G-75 chromatography (see the Methods section), and values are means ± S.E.M. of three experiments, each done in duplicate. Immunoblotting results are from densitometric scans of autoradiograms and are expressed as arbitrary units, as the mean of two experiments.

	Intact cells		Purified receptor	
	¹²⁵ I-insulin bound (fmol/10 ⁶ cells)	Immunoblotting (units/10 ⁶ cells)	¹²⁵ I-insulin bound (fmol/mg of protein)	Immunoblotting (units/µg of protein)
Control	1.14 ± 0.16	9.1	398 ± 66	3.4
Desensitized	0.50 ± 0.06	5.1	200 ± 32	1.7

Fig. 2. Insulin stimulation of glycogen synthesis

Control (●) or down-regulated (○) HepG2 monolayers were washed, incubated with insulin and [^{14}C]glucose for 90 min at 37 °C, and the accumulation of [^{14}C]glycogen within the cells was measured as described in the Methods section. The data shown are the means $\pm$ S.E.M. of triplicate determinations, averaged from three separate experiments. Basal values were 3.1 and 3.4 nmol of glycogen/min per 10^6 cells for control cells and for desensitized cells respectively. Maximal stimulation by insulin was 2.3-fold, corresponding to 7.2 nmol of [^{14}C]glycogen formed/min per 10^6 cells.

marked desensitization of insulin-stimulated glycogen synthesis after a 18 h exposure to insulin, with the EC_{50} of this process in down-regulated cells less sensitive to insulin by three orders of magnitude ($0.5 \mu\text{M}$). Thus prolonged exposure to insulin produces on the surface of HepG2 cells a population of insulin receptors which bind insulin, but do not mediate insulin stimulation of glycogen synthesis.

Insulin-receptor endocytosis

Upon binding to its receptor, insulin was rapidly sequestered away from the surface of the cell. This process was measured by the inaccessibility of the internalized receptors to inactivation by exogenously applied trypsin (Fig. 3) (Marshall *et al.*, 1981; Green & Olefsky, 1982; Peterson *et al.*, 1983; Knutson *et al.*, 1983; Reed *et al.*, 1984). The trypsin-treatment conditions were adjusted such that approx. 95% of the cell-surface bound insulin was removed when the insulin binding was first performed at 4 °C, a non-permissive temperature for internalization. Both the binding of insulin to its receptor and its translocation from the cell surface to a trypsin-resistant intracellular compartment were found to be rapid at 37 °C. As shown in Fig. 3(a), insulin binding to its receptor at this temperature was saturable and occurred with a $t_{1/2}$ of 6 min. Once bound to the cell-surface receptor, the insulin was internalized by the cell even more rapidly, with a $t_{1/2}$ of 2 min (Fig. 3b).

To examine the effects of receptor desensitization on internalization, the receptor-mediated endocytosis of insulin in control cells was compared with this process in cells in which the insulin receptors were maximally down-regulated by prolonged exposure to insulin. Prior down-regulation of insulin receptors did not affect the

Fig. 3. Binding and internalization of ^{125}I -insulin by HepG2 cells

(a) Binding of ^{125}I -insulin to HepG2 cells at 37 °C, showing total binding (●) and trypsin-resistant binding (○). The trypsin-resistant ^{125}I -insulin binding represents internalization, which is plotted as a function of time in (b). Percentage internalization was calculated as [^{125}I -insulin bound after trypsin]/[total ^{125}I -insulin bound]. Maximal binding in the experiment shown was 1.1 fmol/ 10^6 cells, of which 0.5 fmol/ 10^6 cells was trypsin-resistant at the maximum. The data shown are the means $\pm$ S.E.M. of triplicate determinations and are representative of five similar experiments.

kinetics of binding of insulin to the remaining cell-surface receptors. This is shown in Fig. 4(a), where the time course of ^{125}I -insulin binding to control and down-regulated cells was the same ($t_{1/2} = 6$ min), even though the total amount of ^{125}I -insulin bound to the down-regulated cells was attenuated by 44% as compared with the control cells. Fig. 4(b) demonstrates that down-regulation of insulin receptors had no effect on insulin-mediated sequestration of the receptor away from the cell surface (as measured by resistance to removal by trypsin), and shows the time courses of internalization of ^{125}I -insulin by control and down-regulated cells. The two curves are virtually superimposable, with 40% of the total bound ^{125}I -insulin internalized within 6 min ($t_{1/2} = 2$ min) in both cases. Thus neither the initial rate nor the final extent of receptor-mediated insulin endocytosis was affected by prior down-regulation of the receptors. These data indicate that, although the down-regulation process results in a decrease in the number of cell-surface insulin receptors, the remaining plasma-membrane receptors bind and internalize insulin under conditions which are indistinguishable from those of untreated cells.

Fig. 4. Time course of ^{125}I -insulin internalization of HepG2 cells as a function of receptor down-regulation

(a) Time-dependence of total ^{125}I -insulin binding at 37 °C for untreated (●) and insulin-down-regulated (○) cells. (b) Time course of internalization of ^{125}I -insulin by untreated cells (●) and down-regulated cells (○). Internalization was calculated as described in the legend to Fig. 3. Data are the means $\pm$ S.E.M. of triplicate determinations, representative of three experiments.

Autophosphorylation of insulin receptors

We next examined the insulin-stimulated autophosphorylation of insulin receptors isolated from control and down-regulated cells to determine whether changes in receptor autophosphorylation would accompany the observed desensitization of insulin-mediated glycogen synthesis in these cells. The insulin-stimulated autophosphorylation of partially purified receptors from control and desensitized HepG2 cells is shown in Fig. 5. As a control for these experiments, a 'washed' cell preparation was included (middle panel, Fig. 5) in which cells were incubated with insulin for 20 min rather than the 16 h used for desensitization; this was long enough for insulin binding and receptor phosphorylation to occur in the intact cell, but not long enough for the desensitization process to begin. The receptors isolated from control HepG2 cells show a strong insulin-stimulated phosphorylation of the β -subunit of the insulin receptor

Fig. 5. Autophosphorylation of the β -subunit of the insulin receptor

Insulin receptors were solubilized and partially purified by wheat-germ-agglutinin-Sepharose chromatography from control (left panel), 'washed' (middle panel) or desensitized (right panel) cells. The 'washed' preparation was from cells which had been treated with insulin exactly as the desensitized cells, except that the incubation with insulin was only for 20 min, as detailed in the Results section. These three receptor preparations were then incubated without or with 10 nM-insulin in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, immunoprecipitated with an anti-(insulin receptor) antibody, and electrophoresed as described in the Methods section. The arrow points to the position of the β -subunit of the insulin receptor.

above a low basal value. In contrast, receptors from desensitized cells show little or no insulin-stimulation of ^{32}P incorporation into the β -subunit ($n = 21$). The lack of insulin-responsive receptor autophosphorylation in desensitized cells was accompanied in many experiments by an increase in the basal phosphorylation; the magnitude of this increase ranged from 20 to 80% of the maximal extent of stimulation, and varied from one experiment to the next. This increased basal receptor phosphorylation was also observed in receptors from the 'washed' cell controls. However, these 'washed' cell receptors, which were not desensitized, could be further phosphorylated in response to insulin, whereas the phosphorylation of receptors isolated from desensitized cells was not insulin-sensitive. When gels such as those shown in Fig. 5 were hydrolysed under basic conditions to dephosphorylate phosphoserine and phosphothreonine residues before autoradiography, most, but not all, of the increase in basal autophosphorylation of the receptors from desensitized and 'washed' cells was reversed. The relative insulin-sensitivity was not affected by base hydrolysis, however, with the control and 'washed'-cell control preparations exhibiting insulin-stimulated receptor autophosphorylation, whereas the desensitized receptors showed no response to insulin (results not shown).

A quantitative evaluation of the changes in insulin-mediated receptor autophosphorylation with desensitization was obtained by scanning densitometry of autoradiograms. As shown in Fig. 6, insulin stimulated the phosphorylation of the β -subunit of its receptor in a dose-dependent fashion in receptors isolated from control cells. The EC_{50} of this autophosphorylation was approx. 4 nM, with a maximal stimulation of 5–25-fold.

Fig. 6. Densitometry of autophosphorylated insulin receptors

Insulin-receptor preparations from control (●) and desensitized (○) HepG2 cells were allowed to autophosphorylate in the presence of insulin and [γ - ^{32}P]ATP, and the ^{32}P -labelled β -subunit was identified by gel electrophoresis and quantified by scanning densitometry as detailed in the Methods section and the legend to Fig. 2. The data shown are means $\pm$ S.D. of three experiments. The phosphorylation of the control samples ranged from a basal value of 18 arbitrary units to a maximum of 317 (17.6-fold stimulation). For the desensitized preparations, the basal value was 72 arbitrary units, with a stimulation to 124 by $1 \mu\text{M}$ -insulin (1.7-fold).

In contrast, there was essentially no insulin stimulation of autophosphorylation of receptors from desensitized cells, even at concentrations of stimulating insulin as high as $1 \mu\text{M}$.

Phosphorylation of exogenous substrates

This decrease in insulin-receptor autophosphorylation with desensitization could reflect either an attenuation of the ability of the receptor to serve as a substrate for phosphorylation or a defect in the kinase activity of the receptor itself. To approach this question, we also examined the tyrosine kinase activity of the control and desensitized insulin receptors against an exogenous substrate, angiotensin II. As shown in Fig. 7, the receptors isolated from control cells phosphorylate angiotensin II in response to insulin in a dose-dependent fashion, with an EC_{50} of approx. 5 nM and a maximum stimulation of 2–10-fold. In contrast, receptors isolated from desensitized cells completely lack this insulin-sensitive kinase activity, showing no stimulation of angiotensin II phosphorylation by insulin at any concentration. Concomitant with this lack of insulin sensitivity was evidenced a substantial increase in the basal tyrosine kinase activity to a value representing 40–100% of the maximally stimulated activity. Thus desensitization of insulin-stimulated glycogen synthesis is accompanied by an increase in the basal activity of the tyrosine kinase and a severe attenuation of insulin-sensitive tyrosine kinase activity.

Fig. 7. Insulin-stimulated phosphorylation of exogenous angiotensin II

Insulin-receptor preparations from control (●) and desensitized (○) HepG2 cells were incubated with insulin, [γ - ^{32}P]ATP and angiotensin II, and the incorporation of ^{32}P into angiotensin II was measured as described in the Methods section. The data shown are representative of three similar experiments. With the control preparation, the basal angiotensin II phosphorylation was 2.7 pmol of ^{32}P /mg of protein, stimulated 9-fold to 24.3 pmol /mg protein with $1 \mu\text{M}$ -insulin. With the desensitized preparation, the basal phosphorylation was 11.6 pmol of ^{32}P /mg of protein, with no further stimulation by insulin.

DISCUSSION

Prolonged incubation of HepG2 cells with insulin leads to the down-regulation of insulin receptors, accompanied by a desensitization of insulin function, as measured by glycogen synthesis. This attenuation of insulin function, which has also been observed for a variety of insulin-stimulated events in other cell systems (Heaton & Gelehrler, 1981; Amatruda *et al.*, 1982; Crettaz & Kahn, 1984), is too great to be explained by the observed 50% decrease in insulin binding, and therefore must reflect a post-binding defect in the desensitized state. Thus, after insulin-mediated receptor down-regulation and desensitization, the HepG2 cell surface is populated by approximately half the original number of insulin receptors, which bind insulin normally but are unable to mediate insulin action within the cell. In the present study we have used these desensitized cells as a source of 'non-functional' insulin receptors with which to explore the involvement of various receptor-mediated events in insulin action.

One striking response of cellular insulin receptors to insulin is the rapid sequestration of the receptor away from the cell surface. We have measured the initial sequestration of the insulin-receptor complex away from the HepG2 cell surface by the accessibility of bound ^{125}I -insulin to removal by trypsin. Trypsin treatment of intact cells has been shown in a number of systems to degrade insulin-binding sites exposed on the cell surface,

without affecting receptors which have been internalized; the number of endocytosed receptors which can be recovered intracellularly by subcellular fractionation correlates well with the number of receptors resistant to degradation by trypsin (Marshall *et al.*, 1981; Green & Olefsky, 1982; Knutson *et al.*, 1983; Peterson *et al.*, 1983; Reed *et al.*, 1984). In the present study, the degree of exposure to trypsin was optimized to be the minimum amount necessary to remove the cell-surface receptors when the binding was first done at 4 °C to prevent endocytosis; the fact that fully 95% of this surface binding was removed by trypsin indicates that appreciable receptor endocytosis did not occur during the incubation of the cells with trypsin at 25 °C. This is borne out by the data shown in Fig. 3, where differences in the extent of internalization between 1 and 5 min of exposure of the cells to insulin can be distinguished: these differences between short time points would be masked if further sequestration of receptors away from the cell surface had occurred during the trypsin-treatment process. We have previously shown that the internalization of insulin by the HepG2 cell is 10-fold faster than the rate of hormone degradation and dissociation ($t_{1/2}$ of dissociation = 20 min) (Blake & Strader, 1986). Thus appreciable processing and release of internalized insulin also would not occur during the time of exposure of the cells to trypsin.

An examination of ^{125}I -insulin sequestration away from the surface of both control and desensitized cells showed no effect of receptor desensitization on internalization. Both the initial rate and the final extent of internalization of desensitized receptors were indistinguishable from those of normal receptors in HepG2 cells. Although it is possible that alterations in other aspects of receptor endocytosis may occur with tachyphylaxis, these data indicate that changes in the rate of insulin-induced receptor internalization are not a necessary consequence of insulin-receptor down-regulation. These results are consistent with the notion that, although internalization may well be a necessary step in insulin action, insulin-receptor internalization is not sufficient for insulin responsiveness in HepG2 cells.

Another rapid response of the insulin receptor which has been implicated in insulin action is its tyrosine kinase activity. An examination of insulin receptors from HepG2 cells after tachyphylaxis reveals a loss of insulin-sensitive kinase activity of receptors from desensitized cells, reflected both in the insulin-stimulated autophosphorylation of the β -subunit of the insulin receptor and in the phosphorylation of an exogenous peptide substrate.

The lack of insulin-sensitive tyrosine kinase activity in desensitized receptors was accompanied by an increase in the basal activity, as discussed above in Figs 5–7. Thus the lack of insulin-responsiveness of the kinase activity could, in theory, be due either to persistent activation or to persistent inactivation of the enzyme after desensitization. As shown by the data in Figs. 6 and 7, the basal kinase activity of the desensitized receptor is lower than the maximally stimulated activity in the control cells, and the activity cannot be stimulated to this control value by insulin. In contrast, a briefer exposure of the cells to insulin, sufficient for saturation of the binding sites, but not sufficient for desensitization to occur, results in a similar increase in the basal kinase activity, with no

concomitant loss of insulin sensitivity (Fig. 5). Taken together, these data suggest that the loss of insulin-sensitivity of the tyrosine kinase activity of desensitized receptors is at least partially due to an inactivation of this function. We cannot rule out the possibility that there is an accompanying irreversible activation of some of the receptors in these cells and that desensitization has affected the cellular mechanism of cycling receptors from an active to an inactive state. It is apparent, however, that the insulin-sensitivity of the receptor tyrosine kinase activity has been lost upon desensitization, in concert with the loss of insulin-sensitive glycogen synthesis, and with no alteration in insulin binding or of internalization of the remaining cell-surface receptors.

It is interesting that this attenuation of both the *trans*- and auto-phosphorylation activities of the insulin receptor from desensitized cells could be measured in isolated, partially purified, receptor preparations, suggesting some covalent modification of the insulin receptor in the desensitized state. One possible site of such a modification would be the phosphorylation of serine or threonine residues on the receptor, events which are known to occur in intact cells in response to insulin binding. In fact, it has been shown that desensitization of the β -adrenergic receptor (Sibley *et al.*, 1984) and of the tyrosine kinase activity of the epidermal-growth-factor receptor (Cochet *et al.*, 1984) can be correlated with receptor phosphorylation on serine and threonine residues, respectively. By analogy, it is interesting to speculate that some of the increase in the basal extent of serine or threonine autophosphorylation observed here in the desensitized insulin receptors was responsible for the failure of these receptors to exhibit insulin-sensitive tyrosine kinase activity. A study by Stadtmauer & Rosen (1986) has shown that increasing intracellular cyclic AMP in IM-9 lymphocytes promotes the phosphorylation of the β -subunit of the insulin receptor on serine and threonine residues, which correlates with an attenuation of insulin-stimulated receptor autophosphorylation and kinase activity, suggesting that insulin-receptor phosphorylation may in fact serve as a mechanism for the modulation of insulin-responsive tyrosine kinase activity.

Arsenis & Livingston (1986) have also reported a decrease in the insulin-stimulated tyrosine kinase activity in desensitized adipocytes, attributable primarily to an increase in basal activity; possible alterations in receptor autophosphorylation were not explored. However, in the adipocyte system, the dose-response of the kinase activity to insulin was unchanged with desensitization. This is in contrast with our current findings in the HepG2 cell, where there was no responsiveness of this parameter to insulin at any concentration. The desensitization of insulin action in the adipocyte was much less severe than that observed in the HepG2 cell, making the data more difficult to interpret. This difference between the adipocyte and the HepG2 cell may be related to the presence of 'spare receptors' in the adipocyte, where the stimulation of only a small percentage of the receptors can result in full activation of insulin function, perhaps making complete desensitization of both metabolic events and kinase activity difficult to achieve. The correlation in the HepG2 cell of insulin-responsiveness with receptor tyrosine kinase activity argues strongly for the involvement of this kinase activity in the mediation of insulin function within the cell.

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