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# Insulin stimulates tyrosine phosphorylation of its receptor β-subunit in intact rat hepatocytes

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We studied the phosphorylation of the β subunit of the insulin receptor in intact freshly isolated rat hepatocytes, labelled with [$^{32}$P]$P_i$. Insulin receptors partially purified by wheat-germ agglutinin chromatography were immunoprecipitated with either antibodies to insulin receptor or antibodies to phosphotyrosine. Receptors derived from cells incubated in the absence of insulin contained only phosphoserine. Addition of insulin to hepatocytes led to a dose-dependent increase in receptor β-subunit phosphorylation, with half-maximal stimulation being observed at 2 nM-insulin. Incubation of cells with 100 nM-insulin showed that, within 1 min of exposure to the hormone, maximal receptor phosphorylation occurred, which was followed by a slight decrease and then a plateau. This insulin-induced stimulation of its receptor phosphorylation was largely accounted for by phosphorylation on tyrosine residues. Sequential immunoprecipitation of receptor with anti-phosphotyrosine antibodies and with anti-receptor antibodies, and phosphoamino acid analysis of the immunoprecipitated receptors, revealed that receptors that failed to undergo tyrosine phosphorylation were phosphorylated on serine residues. The demonstration of a functional hormone-sensitive insulin-receptor kinase in normal cells strongly supports a role for this receptor enzymic activity in mediating biological effects of insulin.

## INTRODUCTION

The diverse biological responses of cells to insulin are initiated by the binding of the hormone to its specific cell-surface receptors. This binding event activates one or more signalling mechanisms. Evidence has been gathered during the last few years that signals generated in response to insulin involve phosphorylation and dephosphorylation of cellular proteins (Avruch *et al.*, 1976; Denton *et al.*, 1981; Cohen, 1982). Those reactions are likely to play a central role in mediating insulin action, since the insulin receptor is an insulin-stimulated protein tyrosine kinase (for review see Gammeltoft & Van Obberghen, 1986). In broken cell systems, the phosphorylation of the purified insulin receptor occurs primarily on tyrosine residues (Kasuga *et al.*, 1983; Gazzano *et al.*, 1983; Petruzzelli *et al.*, 1984; Yu & Czech, 1984). In contrast, in intact cells, Kasuga *et al.* (1982) and Gazzano *et al.* (1983) found that the addition of insulin led to a preferential increase in insulin-receptor β-subunit phosphoserine content. However, in a study using another cultured cell-line, Fao hepatoma cells, Pang *et al.* (1985) and White *et al.* (1985) showed predominance of tyrosine phosphorylation of insulin receptor during the initial cellular response to insulin.

To appreciate the role of the insulin-receptor tyrosine kinase activity in normal (i.e. not transformed and not cultured) cells, we have investigated insulin-receptor phosphorylation in intact freshly isolated rat hepatocytes, by using antibodies which immunoprecipitate phosphotyrosine-containing proteins, combined with an improved procedure to minimize dephosphorylation during extraction and purification of insulin receptors. We demonstrate the following: (i) insulin-receptor phosphorylation is stimulated by insulin at concentrations within the physiological range; (ii) insulin-receptor phosphorylation occurs predominantly on tyrosine residues during the initial response to insulin.

## MATERIALS AND METHODS

### Materials

Triton X-100, *N*-acetylglucosamine, histone H2B, phosphoserine, phosphothreonine, phosphotyrosine and bacitracin were purchased from Sigma (St. Louis, MO, U.S.A.). Wheat-germ-agglutinin–agarose came from Miles Laboratories. [γ-$^{32}$P]ATP (triethylammonium salt, aqueous solution; 3000 Ci/mmol) was from Amersham International; pig insulin was a gift from Novo Research Institute (Copenhagen, Denmark). Protein A (Pansorbin) was purchased from Calbiochem, and phenylmethanesulphonyl fluoride from Serva. All reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Serum from patient B7, containing autoantibodies to insulin receptors (Kahn *et al.*, 1981), was a gift from Dr. P. Gorden (N.I.H., Bethesda, MD, U.S.A.). Anti-phosphotyrosine antibodies were prepared as described by Pang *et al.* (1985).

### Phosphorylation of insulin receptors in intact hepatocytes

Hepatocytes were isolated by collagenase digestion of livers from male Wistar rats (150–200 g) as described by Le Cam & Freychet (1977). Immediately after isolation, hepatocytes were washed twice in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 1% bovine serum albumin. Hepatocytes ($5 \times 10^6$ cells/ml) were incubated for 2 h at 37 °C in the same buffer lacking unlabelled

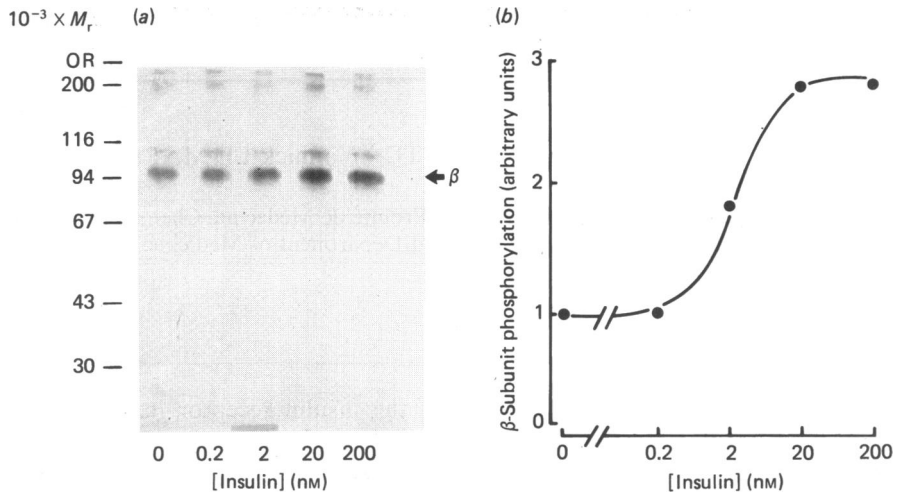


**Fig. 1. Insulin dose–response of receptor β-subunit phosphorylation**

Freshly isolated rat hepatocytes were labelled with [$^{32}$P]$P_i$ for 2 h at 37 °C, and then exposed for an additional 10 min at 37 °C to the indicated insulin concentrations. The cells were solubilized and glycoproteins were partially purified by wheat-germ-agglutinin–agarose. For each time point, an identical amount of glycoproteins was exposed to anti-insulin receptor antibodies at a final concentration of 50 μg of IgG/ml. Finally, the immunoprecipitated phosphoproteins were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions. (*a*) Autoradiogram of the gel; (*b*) densitometric analysis of the labelling of the insulin-receptor β-subunit as a function of the insulin concentration. OR, origin.

phosphate and supplemented with 10 mM-Hepes and [$^{32}$P]$P_i$ (250 μCi/ml). Cells were then exposed to insulin at different concentrations and for various incubation times as indicated in the Figure legends.

### Partial purification of insulin receptors

Incubation medium was rapidly discarded after a brief centrifugation (20 s, 2000 *g*) and the cells were immediately frozen in liquid $N_2$. Hepatocytes were thawed in 6 ml of solubilization buffer containing Hepes (50 mM, pH 7.4), sodium pyrophosphate (15 mM), NaF (100 mM), EDTA (10 mM), sodium orthovanadate (4 mM), Triton X-100 (1%), trypsin inhibitor (100 units/ml), bacitracin (2 mg/ml) and phenylmethanesulphonyl fluoride (1 mM). Cells were solubilized for 60 min at 4 °C. The insulin receptors were partially purified by affinity chromatography on wheat-germ-agglutinin–agarose (Van Obberghen *et al.*, 1981).

### Immunoprecipitation of insulin receptors with anti-receptor antibodies or with anti-phosphotyrosine antibody

Partially purified insulin receptors were exposed for 15 h at 4 °C to anti-receptor antibodies from patient B7 or to anti-phosphotyrosine antibodies at final concentrations of 50 or 20 μg of IgG/ml respectively. This concentration of anti-receptor antibodies immunoprecipitated 100% of the labelled receptors, since no receptors were detected after a second immunoprecipitation of the supernatant (results not shown). In each condition, the same amount of glycoproteins, as determined by the Bradford (1976) method, was subjected to immunoprecipitation. Thereafter the precipitation was achieved by addition of protein A (2 h at 4 °C). After centrifugation (5 min, 10000 *g*), the pellets were washed twice with Hepes buffer (50 mM, pH 7.6) containing NaCl (150 mM) and Triton X-100 (0.1%). The precipitated proteins were eluted by addition of 3% SDS/5% β-mercaptoethanol (final concns.). The samples were boiled and then analysed by SDS/polyacrylamide-gel electrophoresis as previously described (Laemmli, 1970). The gels were autoradiographed on 3M Trimax film. The radioactivity in the receptor β-subunit was measured by Čerenkov counting of the corresponding gel fragment or by densitometric scanning analysis (under conditions of linearity).

### Phosphoamino acid analysis

The region of the gel corresponding to the insulin-receptor β-subunit was excised, and then the proteins were eluted by incubating the gel pieces at 37 °C in 0.1% SDS solution containing $NH_4HCO_3$ (50 mM, pH 8) and precipitated with trichloroacetic acid (10%) after addition of 20 μg of IgG as carrier. Precipitates were hydrolysed in 6M-HCl at 110 °C for 90 min (Cooper *et al.*, 1983). Labelled phosphoamino acids were separated by thin-layer electrophoresis at pH 3.5 and located by autoradiography. Phosphoamino acid markers (phosphoserine, phosphothreonine, phosphotyrosine; 3 μg each) were added to each sample and located by spraying the plates with ninhydrin solution and heating.

## RESULTS

### Dose-dependence of insulin-stimulated insulin-receptor β-subunit phosphorylation in rat hepatocytes

Intact freshly isolated rat hepatocytes were labelled with [$^{32}$P]$P_i$ and incubated with various insulin concentrations. Insulin receptors were partially purified by affinity chromatography on wheat-germ-agglutinin–agarose and immunoprecipitated with antibodies to insulin receptors. As previously demonstrated (Van Obberghen *et al.*, 1983), as compared with control serum, anti-receptor antibodies immunoprecipitated one additional phosphoprotein, which migrated as a $M_r$-95000 protein under reducing conditions and which corresponds

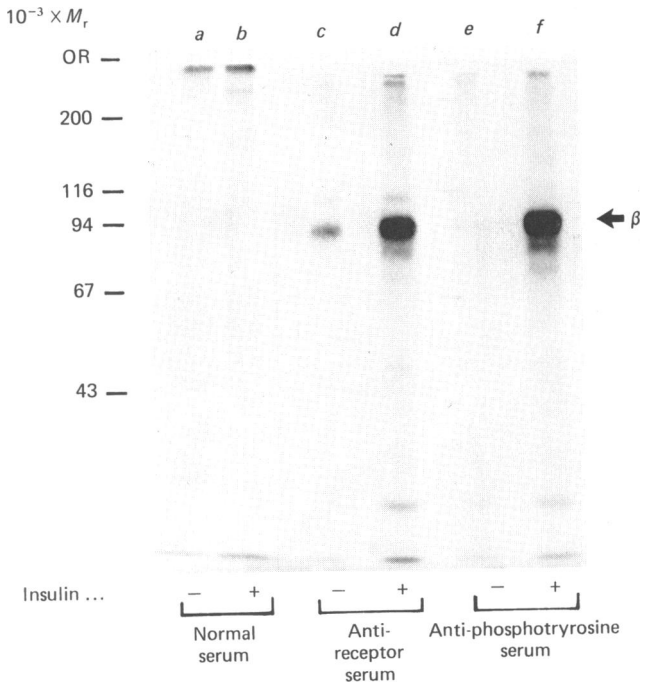


**Fig. 2. Immunoprecipitation of insulin receptors by anti-phosphotyrosine antibodies**

Freshly isolated hepatocytes were labelled for 2 h at 37 °C with [$^{32}$P]$P_i$, and then incubated for 3 min without or with insulin (100 nM). The cells were solubilized, and partially purified insulin receptors were prepared by wheat-germ-lectin chromatography. These receptors were incubated with control serum (50 $\mu$g of IgG/ml; lanes *a* and *b*), anti-receptor serum B7 (50 $\mu$g of IgG/ml; lanes *c* and *d*), and anti-phosphotyrosine antibody (20 $\mu$g of IgG/ml; lanes *e* and *f*). The immune precipitates were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography. OR, origin; arrow indicates position of $\beta$-subunit.

to the receptor $\beta$-subunit (Fig. 1*a*). This $M_r$-95000 phosphoprotein was observed with cells incubated in the absence of insulin, indicating occurrence of basal receptor phosphorylation. Insulin induced a dose-dependent increase in its receptor $\beta$-subunit phosphorylation. Thus, quantitative densitometric analysis of receptor $\beta$-subunit labelling revealed that the maximally stimulatory action of insulin on its receptor subunit phosphorylation was found at 20 nM and amounted to a 3-fold stimulation. Half-maximal stimulation was observed with 2 nM-insulin.

### Insulin's stimulatory effect on its receptor $\beta$-subunit phosphorylation is due to phosphorylation on tyrosine residues

Wheat-germ extracts obtained from hepatocytes were incubated for 3 min without or with insulin (100 nM), and then were exposed to control serum (Fig. 2, lanes *a* and *b*), anti-receptor antibodies (Fig. 2, lanes *c* and *d*) and anti-phosphotyrosine antibodies (Fig. 2, lanes *e* and *f*). With anti-receptor antibodies it was found that in rat hepatocytes the insulin-receptor $\beta$-subunit was phosphorylated in the absence of insulin, and that the hormone induced a several-fold increase in its receptor $\beta$-subunit phosphorylation. In contrast, with anti-phosphotyrosine antibodies, no phosphoprotein was detectable in an extract derived from cells incubated in the absence of insulin. However, incubation of hepatocytes with insulin promoted the occurrence of a $M_r$-95000 phosphoprotein, which was precipitated with anti-phosphotyrosine antibodies. The electrophoretic mobility of this phosphoprotein, as well as the fact that it is generated on addition of insulin, strongly suggest that it corresponds to the insulin-receptor $\beta$-subunit.

### Insulin rapidly stimulates tyrosine phosphorylation of its receptor $\beta$-subunit

Hepatocytes labelled with [$^{32}$P]$P_i$ were incubated with 100 nM-insulin for 0, 1, 2.5 and 7 min. Wheat-germ-lectin-purified glycoproteins were then subjected to immunoprecipitation by anti-phosphotyrosine antibodies (Fig. 3*a*) or by anti-receptor antibodies (Fig. 3*b*). When the immunoprecipitation was performed with anti-receptor antibodies, a basal phosphorylation of the receptor $\beta$-subunit was observed, and within 1 min insulin increased this phosphorylation. In hepatocytes not exposed to insulin, no phosphoprotein could be immunoprecipitated with anti-phosphotyrosine antibodies. However, addition of insulin led to a rapid appearance of the receptor $\beta$-subunit precipitable by anti-phosphotyrosine antibodies. Quantitative analysis revealed a similar pattern with both the anti-receptor antibodies and the anti-phosphotyrosine antibodies (Fig. 4). Thus the amount of receptor $\beta$-subunit precipitable by either anti-receptor antibodies or anti-phosphotyrosine antibodies reached a maximum within 1 min; this was followed by a rapid decline and an apparent plateau. This similar time course found with both antibodies suggests that the insulin receptors newly phosphorylated on insulin addition contain at least one phosphotyrosine residue. However, for each time point after the addition of insulin, the amount of receptors precipitated by anti-receptor antibodies tended to be higher when compared with the amount precipitated by anti-phosphotyrosine antibodies. This would indicate that this receptor population was not phosphorylated on tyrosine residues. Indeed, those receptors could be precipitated when the supernatant obtained after precipitation with anti-phosphotyrosine antibody was subjected to a second precipitation with anti-receptor antibodies (Fig. 3*c*). Furthermore, the extent of labelling of those insulin receptors remained virtually constant during the incubation with insulin (Fig. 3*c*).

### Phosphoamino acid analysis of insulin receptors precipitated by anti-receptor antibodies

Receptors immunoprecipitated by anti-receptor antibodies were excised from the polyacrylamide gel shown in Fig. 3, and analysed for their phosphoamino acids content (Fig. 5). In the absence of insulin, the insulin-receptor $\beta$-subunit was phosphorylated only on serine residues. After 1 min exposure to the hormone, a large amount of phosphotyrosine appeared, with a moderate stimulation of serine phosphorylation (Fig. 5*a*). Further, after 2.5 min incubation with insulin, the amount of phosphotyrosine decreased more than the amount of phosphoserine. After addition of insulin, phosphorylation was always more pronounced on tyrosine residues than on serine residues. Further, phosphoamino acid analysis of insulin receptors remain-

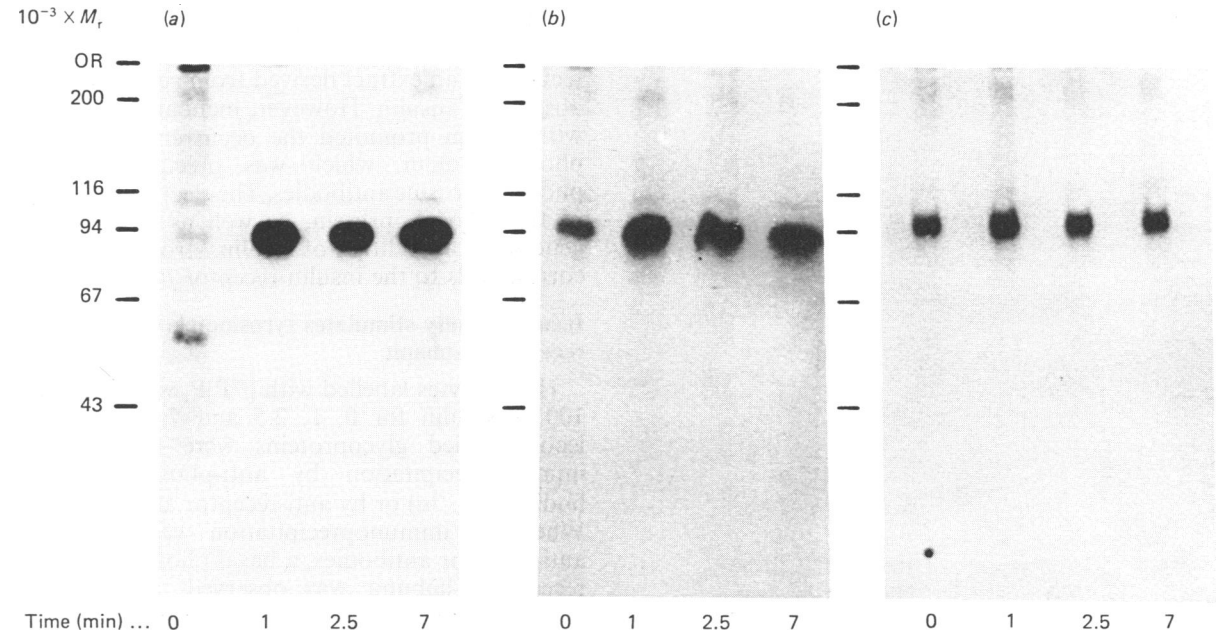


**Fig. 3. Time course of insulin-stimulated phosphorylation of its receptor β-subunit**

Hepatocytes labelled with [$^{32}$P]$P_i$ were exposed to insulin (100 nM) for 0, 1, 2.5 and 7 min. Thereafter, insulin receptors partially purified by wheat-germ-agglutinin chromatography were subjected to immunoprecipitation either with anti-phosphotyrosine serum (20 μg of IgG/ml; *a*) or with serum containing antibodies to insulin receptor (serum from patient B7; 50 μg of IgG/ml; *b*). In another experiment, the supernatants obtained after immunoprecipitation with anti-phosphotyrosine antibodies were subjected to a second immunoprecipitation with anti-receptor antibodies, as shown in (*c*). The immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography. OR, origin.

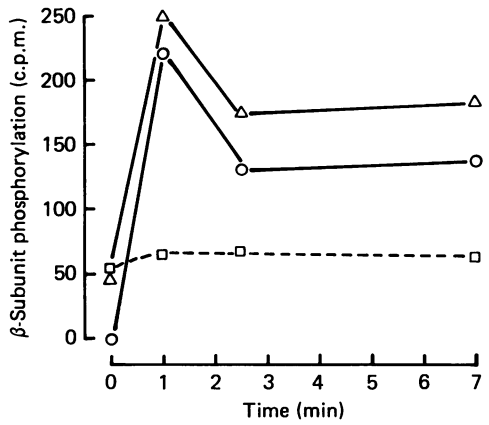


**Fig. 4. Quantitative analysis of the insulin-receptor β-subunit phosphorylation as a function of exposure time to insulin**

The radioactive bands observed in Fig. 3 and corresponding to the insulin-receptor β-subunit were removed from the gel; the radioactivity was eluted and measured by Čerenkov counting. As detailed in the legend to Fig. 3, the insulin receptors were subjected to (i) a single immunoprecipitation with either anti-receptor antibodies (△) or anti-phosphotyrosine antibodies (○), or (ii) a sequential immunoprecipitation with first anti-phosphotyrosine antibodies and thereafter anti-receptor antibodies (□). The amount of radioactivity (c.p.m.) found in the receptor β-subunit is shown as a function of the period of exposure to insulin.

ing in the supernatant after exposure of the cellular extract to anti-phosphotyrosine antibodies showed that these receptors were phosphorylated exclusively on serine residues (Fig. 5*b*). This indicates that all the insulin receptors phosphorylated on tyrosine residues were immunoprecipitated by the anti-phosphotyrosine antibodies.

## DISCUSSION

In intact freshly isolated rat hepatocytes, insulin stimulates its receptor β-subunit phosphorylation in a dose-dependent manner, with half-maximal effect being reached at 2 nM. This concentration is comparable with that causing half-maximal insulin-stimulated amino acid transport (1.3 nM), and is also consistent with the equilibrium constant for dissociation of insulin from its receptor on hepatocytes (0.6 nM) (Fehlmann *et al.*, 1981). This stimulatory action of insulin on its receptor phosphorylation appears extremely rapidly, within 1 min. A similar time course has been reported by Pang *et al.* (1985) and White *et al.* (1985). Taken together, the promptness of the insulin effect on its receptor phosphorylation and the physiological hormone concentration producing this effect are consistent with the notion that insulin-receptor phosphorylation is involved in mediating biological responses to insulin in hepatocytes.

A key finding of the present study is that in intact normal (non-transformed) rat hepatocytes insulin initially

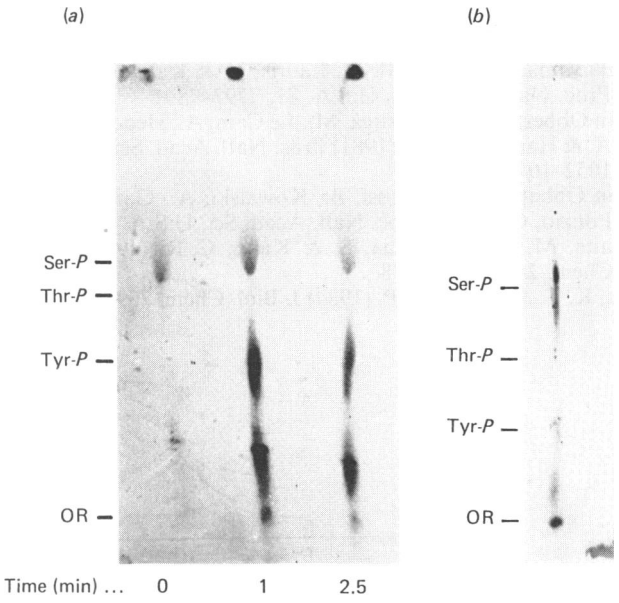


**Fig. 5. Phosphoamino acid analysis of insulin receptors precipitated by anti-receptor antibodies**

Hepatocytes labelled with [$^{32}$P]$P_i$ were exposed to insulin (100 nM) for 0, 1 and 2.5 min. Partially purified insulin receptors prepared from those cells were subjected to either a single immunoprecipitation with anti-receptor antibodies (*a*; serum from patient B7; 50 $\mu$g of IgG/ml), or to a sequential immunoprecipitation consisting of a first immunoprecipitation with anti-phosphotyrosine antibodies (20 $\mu$g of IgG/ml) followed by a second precipitation with anti-receptor antibodies (*b*). Finally, immunoprecipitated receptors were analysed by SDS/polyacrylamide-gel electrophoresis, and after elution from the gel the phosphoamino acid content in the receptor $\beta$-subunit was determined as described in the Materials and methods section. OR, origin.

stimulates phosphorylation of its receptor on tyrosine residues. This tyrosine phosphorylation which we report here contrasts with our earlier work, which led to the suggestion that in intact hepatocytes insulin stimulated predominantly serine phosphorylation of its receptor (Gazzano *et al.*, 1983). We ascribe this difference to the experimental procedure used here, which was intended to minimize dephosphorylation during the purification of the receptors, and to the 15 min insulin exposure which was used in our previous study.

Labelling of the insulin-receptor $\beta$-subunit on tyrosine residues reached a maximum within 1 min after the addition of insulin, and thereafter declined despite the continuous presence of the hormone. This swift dephosphorylation indicates the presence of phosphatases capable of reversing the insulin activation of the receptor kinase. Our observations could be explained by internalization of the insulin–receptor complex, resulting in exposure of the tyrosine-phosphorylated receptors to compartmentalized phosphatases. In any event, these brisk kinetics of insulin-receptor phosphorylation found in intact target cells suggest a closely regulated process with physiological relevance.

In the absence of insulin, receptors were phosphorylated exclusively on serine residues. Addition of insulin leads to the appearance of a significant amount of phosphotyrosine-containing receptors. However, not all receptors undergo this tyrosine phosphorylation, since a distinct receptor population, which cannot be precipitated by anti-phosphotyrosine antibodies, contains exclusively phosphoserine residues. At least two different classes of phosphorylated insulin receptors coexist in hepatocytes exposed to insulin. A first population bears only phosphotyrosine residues or concomitantly phosphoserine residues, whereas a second set of receptors is phosphorylated exclusively on serine residues. Those results could be explained by the following mechanisms. According to one hypothesis, insulin receptors that are incapable of undergoing phosphorylation on tyrosine residues would not be exposed at the cell surface and would be unable to bind insulin. This view is consistent with our findings that in freshly isolated rat hepatocytes only 60% of the cellular receptors reside at the cell surface (E. Van Obberghen & F. Fehlmann, unpublished work). Another possibility would be that receptor phosphorylation on serine residues inhibits the insulin-induced receptor tyrosine autophosphorylation. This hypothesis is supported by the observation that agents which stimulate phosphorylation of insulin receptors on serine residues concomitantly inhibit the ligand-induced receptor tyrosine kinase activity (Takayama *et al.*, 1984; Pessin *et al.*, 1985; Häring *et al.*, 1986).

In summary, our present findings demonstrate that, in normal hepatocytes, insulin initially stimulates insulin-receptor phosphorylation almost entirely on tyrosine residues. The demonstration of an operational insulin-receptor tyrosine kinase activity in normal hepatocytes provides strong support for the concept that the receptor kinase plays a vital role in intracellular transmission of the insulin signal.

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