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Tyrosine phosphorylation of the receptor for insulin-like growth factor II is inhibited in plasma membranes from insulin-treated rat adipocytes

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Insulin action in intact adipocytes leads to a rapid increase in the concentration of receptors for insulin-like growth factor (IGF) II on the adipocyte cell surface, and to a decrease in the [^{32}P]phosphate content of these receptors on the plasma membrane [Corvera & Czech (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7314–7318]. It has been previously shown that the receptor for IGF-II can be phosphorylated on tyrosine residues by a kinase activity which is expressed in isolated adipocyte plasma membranes. It is now shown that IGF-II-receptor phosphorylation in vitro, in plasma membranes derived from insulin-treated cells, is markedly decreased compared with the phosphorylation of the receptor in membranes from control cells. This effect of insulin cannot be attributed to an increase in the activity of phosphotyrosyl phosphatase in the membranes. The tyrosine kinase that catalyses the phosphorylation of IGF-II receptors is associated with a fraction of the plasma membrane which is insoluble in Triton X-100. Removal of the Triton X-100-soluble components of the membrane markedly enhances receptor phosphorylation. Moreover, the expression of the inhibitory effect of insulin requires the presence of one or several Triton X-100-soluble components of the plasma membrane.

INTRODUCTION

One of the major effects of insulin on its target cells is a rapid increase in the expression of several membrane proteins at the cell surface. This effect involves a redistribution of these proteins from an intracellular membrane compartment (low-density microsomal fraction) to the cell surface membrane. Three of the proteins that are known to be affected in this manner in response to insulin are the glucose transporter [1–3], the IGF-II receptor [4,5] and the transferrin receptor [6].

Isotopic labelling experiments using [^{32}P]phosphate have shown that IGF-II receptors in plasma membranes are phosphorylated in vivo on serine and threonine residues, to a higher stoichiometry than the receptors found in low-density microsomal membranes. The redistribution of IGF-II receptors that occurs on exposure of intact cells to insulin correlates with a rapid decrease in the phosphoserine and phosphothreonine contents of the receptors which reside on the plasma membrane [7,8]. The phosphorylation of the IGF-II receptor in isolated rat adipocyte plasma membranes has also been studied. It has been found that, on incubation of the membranes with [γ-^{32}P]ATP, the receptors become phosphorylated on tyrosine residues. This phosphorylation is catalysed by a potent tyrosine kinase activity endogenous to the adipocyte plasma membrane [9].

In the present paper we report that insulin treatment of intact adipocytes results in a pronounced and rapid inhibition of tyrosine phosphorylation of IGF-II receptors in subsequently isolated plasma membranes.

MATERIALS AND METHODS

Preparation of cells and plasma membranes

Plasma membranes were prepared from isolated adipocytes obtained from 75–100 g Sprague–Dawley rat (Taconic Farms) epididymal fat-pads as previously described [10]. Where indicated, cells were exposed to 0.1 μM-insulin for 10 min at 37 °C. The cell suspensions were then centrifuged (500 g for 2 min), and the buffer was aspirated. Cells were homogenized in 8 vol. of a buffer containing 0.25 M-sucrose, 10 mM-Tris/HCl, 5 mM-EDTA and 100 μM-phenylmethanesulphonyl fluoride, pH 7.4 at 5 °C. Plasma membranes were separated as described previously [4,11]. Protein concentrations were determined by the Bradford [12] procedure (Bio-Rad).

Phosphorylation of the IGF-II receptor

(a) Intact plasma membranes (final concn. 2 mg of protein/ml) were resuspended in a buffer (phosphorylation buffer) containing 25 mM-Hepes, 80 mM-β-glycerophosphate, 10 mM-MgCl$_2$, 2 mM-MnCl$_2$, 100 μM-phenylmethanesulphonyl fluoride and 10 mM-ammonium molybdate (Sigma), pH 7.4 at 5 °C. Portions (50 μl) of the membrane suspension were used for each sample. Phosphorylation reactions were started by the addition of 5 μl of a 1 mM stock of [γ-^{32}P]ATP (Amersham) to give a final concentration of 100 μM (10 μCi/nmol), and continued for 10 min at 5 °C. Reactions were terminated by addition of 500 μl of solubilizing buffer, containing 25 mM-Hepes, 1.5% Triton X-100, 1% sodium deoxy-

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cholate, 0.1% SDS, 1% bovine serum albumin, 0.5 M-NaCl, 50 mM-NaF, 100 μM-Na$_3$VO$_5$, 50 mM-Na$_4$P$_2$O$_7$, 5 mM-EDTA and 100 μM-phenylmethanesulphonyl fluoride, pH 7.4 at 5 °C.

(b) Triton X-100-solubilized plasma membranes were prepared by vigorously vortex-mixing intact membranes (2 mg/ml) in the phosphorylation buffer described above, containing 1% Triton X-100 (Bio-Rad), for 30 min at 55 °C. The phosphorylation reactions were started by the addition of [γ-^{32}P]ATP as described above. After incubation for the times indicated in each Figure legend, the reactions were stopped by the addition of 500 μl of solubilizing buffer.

(c) The experiments shown in Figs. 4 and 6 illustrate the phosphorylation of the IGF-II receptor present in the Triton X-100-insoluble membrane fractions. For these experiments, plasma membranes were resuspended (2 mg/ml) in phosphorylation buffer containing 1% Triton X-100. After incubation for 30 min at 5 °C, the suspensions were centrifuged at 5000 g for 10 min. Supernatants were removed, and the Triton X-100-insoluble pellets were gently resuspended in 50 μl of phosphorylation buffer at 5 °C, with a 27½-gauge needle and syringe. Phosphorylation reactions were performed as described above.

Immunoprecipitation and SDS/polyacrylamide-gel electrophoresis

After the addition of solubilizing buffer, approx. 10 μg of rabbit polyclonal anti-(IGF-II receptor) immunoglobulin coupled to 20 μl of Affi-Gel 10 (Bio-Rad) was added to each sample. Immunoadsorptions were performed overnight at 4 °C on an end-over-end mixer. The resin was then washed twice with solubilizing buffer and once with 25 mM-Hepes/0.15% Triton X-100/0.1% SDS, pH 7.4. The samples were then boiled for 2 min in 50 mM-Tris/HCl/3% SDS/0.005% Bromophenol Blue/20% (v/v) glycerol, pH 6.8, and separated on a 6%-polyacrylamide slab gel by the Laemmli [13] method. Gels were then stained with Coomassie Brilliant Blue, dried and exposed to Kodak X-OMAT film at −70 °C.

Phosphoamino acid analyis

Gel slices corresponding to autoradiography bands were excised and subjected to tryptic digestion in 0.1 M-N-ethylmorpholine acetate, pH 8.3, for 24 h at 37 °C. The tryptic peptides were freeze-dried to dryness, resuspended and hydrolysed in 6 M-HCl for 75 min at 110 °C. The hydrolysates were dried, resuspended in a solution containing phosphoserine, phosphothreonine and phosphotyrosine (1 mg/ml each), and spotted on thin-layer cellulose plates (Brinkman Instruments). Electrophoresis was then performed for 120 min at 1000 V with a running buffer of acetic acid/pyridine/water (10:1:189, by vol.). Plates were then dried, spots detected by ninhydrin staining, and the plates autoradiographed at −70 °C.

RESULTS

Fig. 1 shows the effect of brief insulin treatment of intact adipocytes on the subsequent phosphorylation of IGF-II receptors in plasma membranes prepared from these cells. Triton X-100-solubilized plasma membranes were employed because in previous experiments it was found that the detergent enhanced the amount of
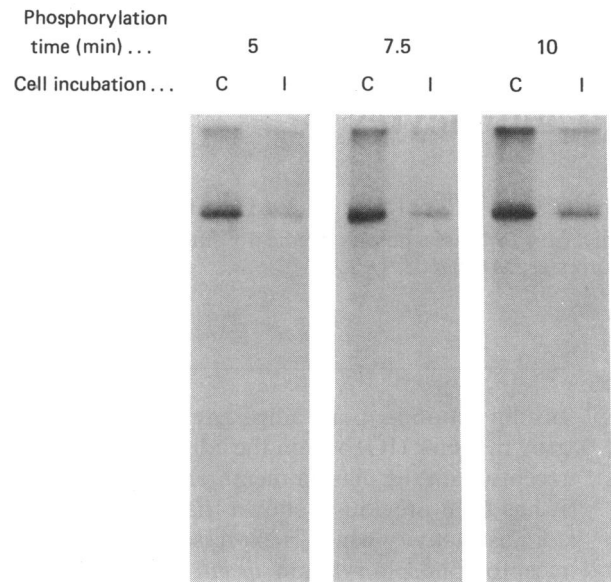

Fig. 1. **Time course of IGF-II-receptor phosphorylation in membranes from control or insulin-treated rat adipocytes**

Plasma membranes were prepared from control cells (C) or cells treated with 0.1 μM-insulin (I) as described in the Materials and methods section. Plasma membranes were resuspended in phosphorylation buffer containing 1% Triton X-100. After 30 min at 5 °C, [γ-^{32}P]ATP was added, to a final concentration of 100 μM. The reactions were terminated after incubation at 5 °C for the times indicated (5, 7.5 and 10 min), by the addition of a 10-fold excess of solubilizing buffer as described in the Materials and methods section. The receptor immunoprecipitates were applied on a 6%-polyacrylamide gel. Autoradiographs of such gels showed one main band at M_r 230000, which corresponds to the value for the IGF-II receptor. Some of the radioactivity remained at the top of the separating gel, possibly representing an aggregation product of the solubilized immunoprecipitated receptor.

[^{32}P]phosphate incorporated into the IGF-II receptor [9]. After addition of 100 μM-[γ-^{32}P]ATP to the membranes, IGF-II-receptor phosphorylation increased linearly over a 10 min period at 5 °C. The rate at which ^{32}P was incorporated into IGF-II receptors was greatly decreased in membranes derived from insulin-treated cells compared with those derived from control cells.

The identities of the phosphorylated residues on the IGF-II receptor were investigated by thin-layer electrophoresis of an acid hydrolysate of the protein. Fig. 2 shows that incubation of plasma membranes from control cells with [γ-^{32}P]ATP resulted in the incorporation of [^{32}P]phosphate into tyrosine residues. The phosphoamino acid analysis of the receptor in membranes derived from insulin-treated cells was complicated by the very low amount of [^{32}P]phosphate incorporated. However, prolonged exposure of the thin-layer plates revealed the presence of phosphotyrosine. No phosphoserine or phosphothreonine was detected. In a previous paper [9] and in some of the experiments described below, it was shown that the tyrosine kinase that phosphorylates the IGF-II receptor is tightly bound to the Triton X-100-insoluble fraction of the plasma membrane, and that it preferentially phosphorylates the IGF-II receptors contained within that fraction. Fig. 3
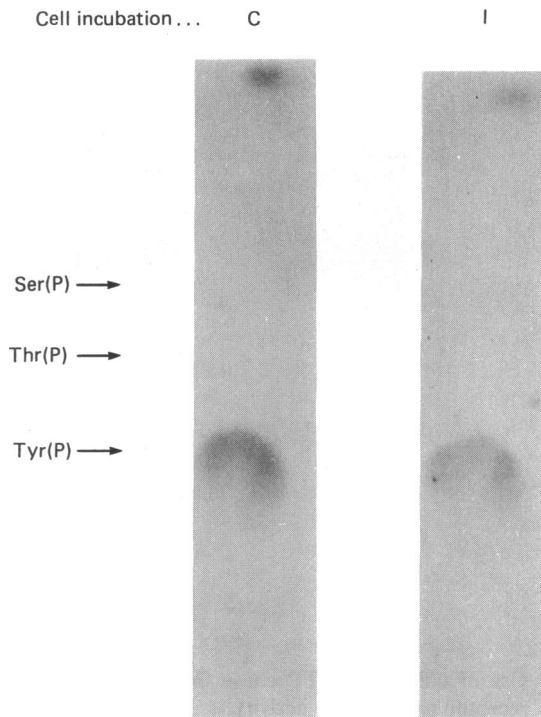
Cell incubation... C I

Ser(P) ⟶

Thr(P) ⟶

Tyr(P) ⟶

Fig. 2. Phosphoamino acid analysis of the IGF-II receptor

Intact plasma membranes from control cells (C) or cells treated with 0.1 μM-insulin (I) were incubated with [γ-³²P]ATP (25 μCi/nmol) as described in the Materials and methods section. The IGF-II receptor was immunoprecipitated from these membranes and phosphoamino acid analysis was performed as described in the Materials and methods section. The positions of the phosphoamino acid standards [phosphoserine, Ser(P), phosphothreonine, Thr(P) and phosphotyrosine Tyr(P)] are indicated. Exposure of the thin layers was 12 h (C) or 72 h (I).

Membranes... Intact Triton-soluble Triton-insoluble

$10^{-3} \times M_r$

200 →
110 →
90 →
68 →
45 →

Fig. 3. Protein composition of intact, Triton X-100-soluble and Triton X-100-insoluble fractions of adipocyte plasma membranes

Plasma membranes from control cells were isolated and electrophoresed on a 6–16%-polyacrylamide slab gel. The left lane contains 50 μg of membrane protein. The middle lane is the fraction of this preparation that is soluble in 1% Triton X-100, and the right lane is the Triton X-100-insoluble material obtained from the same preparation. Positions of M_r standards are indicated.

shows that a large majority of the protein found in this preparation of adipocyte plasma membranes is soluble in 1% Triton X-100, whereas only few major proteins compose the Triton X-100-insoluble structure. Thus the activity of the tyrosine kinase toward the IGF-II receptor can be studied in the presence or absence of detergent-soluble membrane components, which might potentially regulate the phosphorylation reaction.

The results shown in Fig. 4 compare the magnitude of ³²P incorporation into IGF-II receptors when the phosphorylation reaction was performed with (1) intact plasma membranes, (2) Triton X-100-solubilized plasma membranes, (3) the Triton X-100-insoluble fraction of the plasma membrane and (4) the Triton X-100-soluble fraction of the plasma membrane, all derived from control or insulin-treated cells. Several important findings were observed. First, as described previously [9], addition of Triton X-100 to the plasma membranes dramatically increased the incorporation of ³²P into the IGF-II receptor (exposure times are indicated in the legend to Fig. 4).

Second, the effect of insulin to inhibit ³²P incorporation into IGF-II receptors was most pronounced when the phosphorylation reaction was carried out with intact plasma membranes. On solubilization of the membranes in Triton X-100, the i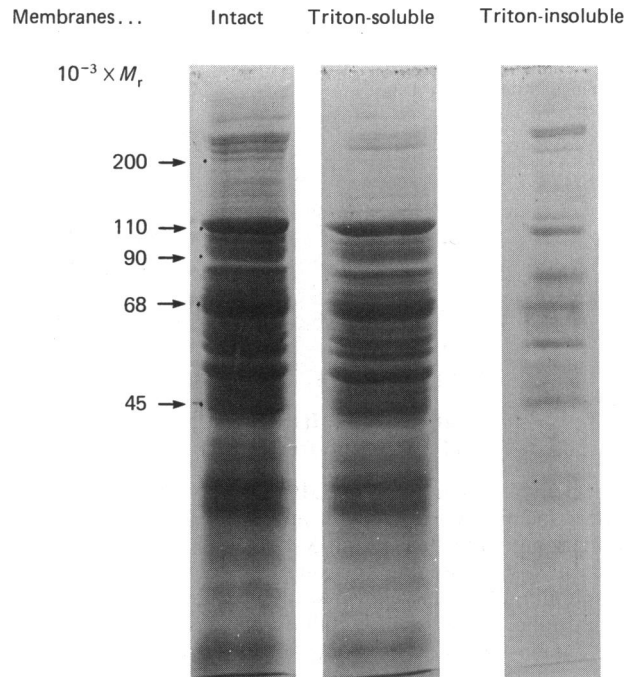nhibitory effect persisted, but was less pronounced than that observed in intact membranes. However, when the Triton X-100-soluble components were removed from the insoluble fraction before initiation of the phosphorylation reaction, the inhibitory effect of insulin was completely abolished. Under these conditions the amount of ³²P associated with the receptor band was actually increased in the receptors obtained from insulin-treated cells compared with controls. This effect could be accounted for by the increased number of receptors that exist in plasma membranes derived from insulin-treated cells [4,5]. Taken together, these results strongly suggest that the inhibition of ³²P incorporation into IGF-II receptors observed in membranes from insulin-treated cells is conferred by a component of the plasma membrane which is soluble in Triton X-100.

Another observation made in this experiment is that ³²P incorporation into the receptors present in the Triton X-100-soluble fraction of the plasma membrane could not be observed, neither when [³²P]ATP was added to the soluble portion after it was separated from the insoluble structure (Fig. 4) nor when the [γ-³²P]ATP was added before separation of the fractions (results not shown). These results confirm that only the receptors present in the Triton-insoluble structure can become tyrosine-phosphorylated under the conditions of these experiments, perhaps reflecting a strict steric requirement for this reaction to occur. Analysis of the binding of ¹²⁵I-IGF-II to these fractions revealed that approx. 20% of
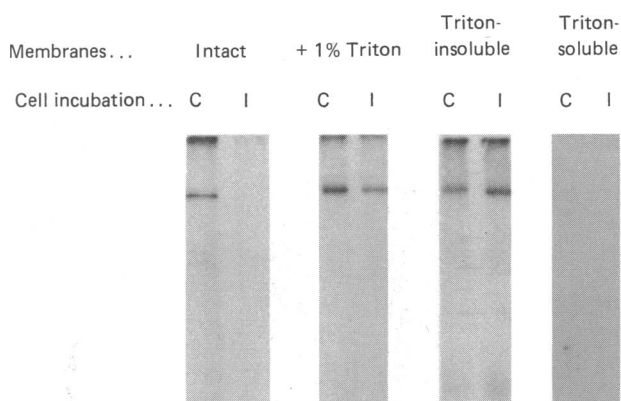
Fig. 4. Phosphorylation of the IGF-II receptor in membranes from control and insulin-treated cells

The phosphorylation of the IGF-II receptor was studied in intact membranes (intact), in membranes resuspended in buffer containing 1 % Triton X-100 (+1 % Triton), in the Triton X-100-insoluble fraction of the membrane (Triton-insoluble), or in the Triton X-100-soluble fraction of the membrane (Triton soluble), all obtained from control (C) or insulin-treated (I) adipocytes. The phosphorylation buffer and the methods employed to obtain the different membrane fractions are described in the Materials and methods section. Phosphorylation reactions were started with 100 μM-[γ-^{32}P]ATP, and continued for 10 min at 5 °C. The IGF-II receptor was then immunoprecipitated, separated on a 6%-polyacrylamide slab gel, and detected by autoradiography. The exposure time for the experiment carried out in intact plasma membranes was 72 h; for the rest of the experiments it was 20 h. Exposure of the gels for up to 5 days did not reveal ^{32}P incorporation into receptors obtained from the Triton X-100-soluble membrane fraction. Similar results were obtained in five successive experiments, with different cell preparations.

the total number of receptors in the plasma membrane were contained in the Triton-insoluble structure (results not shown).

The possibility that the Triton X-100-soluble component that mediates that inhibitory effect of insulin could be a phosphotyrosyl phosphatase activity was investigated. An experiment was performed in which a 10-fold excess of unlabelled ATP was added after incubation of the membranes with [^{32}P]ATP. The activity of a receptor phosphotyrosyl phosphatase would be reflected, under these conditions, by a decrease in the amount of ^{32}P in the receptor. The results of this experiment (Fig. 5) show that incubation of the membranes with unlabelled ATP for 10 min resulted in a small decrease in the amount of ^{32}P in the IGF-II receptor, demonstrating the presence of a phosphotyrosyl phosphatase activity. However, the decrease was small, and no difference could be observed between membranes from control or insulin-treated cells. These results do not support the hypothesis that activation of a phosphotyrosyl phosphatase is responsible for the inhibitory effect of insulin. However, the possibility remains that the addition of a high concentration of ATP could cause a competitive inhibition of the enzyme [14] or alter the activities of other kinases or phosphatases that could be involved in the modulation of IGF-II-receptor phosphorylation. For this reason, a different experiment
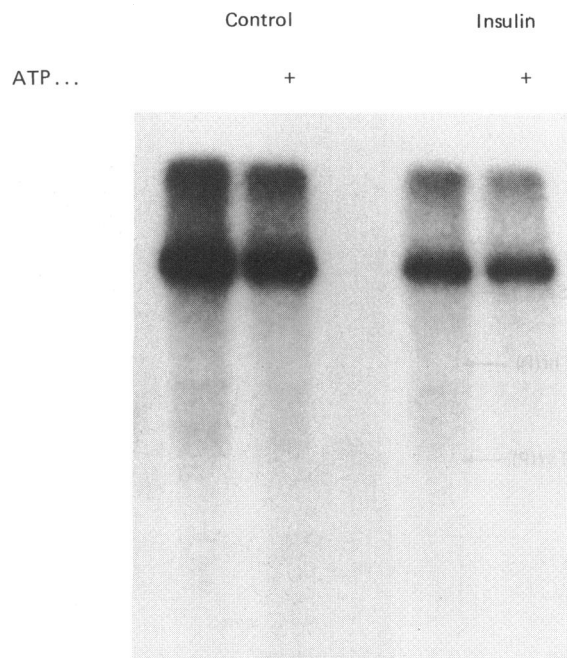

Fig. 5. Use of unlabelled ATP chase for detection of IGF-II-receptor phosphatase activity

Plasma membranes were obtained from control (Control) or insulin-treated cells (Insulin) and resuspended in phosphorylation buffer containing 1 % Triton X-100. Phosphorylation reactions were started with 100 μM-[γ-^{32}P]ATP and continued for 10 min. Where indicated (+), unlabelled ATP was then added (final concn. 2 mM). After 10 min of further incubation, the reactions were stopped and the IGF-II receptor was immunoprecipitated, electrophoresed and detected by autoradiography.

was performed in which Triton X-100-insoluble membrane fractions containing ^{32}P-labelled IGF-II receptor were used as substrates to measure the phosphotyrosyl phosphatase activity of the Triton X-100-soluble membrane extracts from control or insulin-treated cells. No phosphatase activity could be observed on incubation of the ^{32}P-labelled receptors with the Triton X-100-soluble extracts from either control or insulin-treated cell membranes (Fig. 6). These results are consistent with those obtained by using an excess of unlabelled ATP to detect the presence of phosphotyrosyl phosphatase activity (Fig. 5), and suggest that under these conditions the activity of receptor phosphotyrosyl phosphatases is negligible.

It is important to note that all the experiments described above were performed in the presence of 80 mM-β-glycerophosphate and 10 mM-molybdate. Among several inhibitors tested (fluoride, zinc, β-glycerophosphate, vanadate and molybdate), these two were the most effective in promoting ^{32}P incorporation into the IGF-II receptor.

It was decided to investigate the effect of these phosphatase-inhibitory agents on the effect of insulin to inhibit IGF-II-receptor phosphorylation in isolated membranes. The results shown in Fig. 7 compare the effects of molybdate, vanadate and fluoride on the phosphorylation of the IGF-II receptor in membranes from control and insulin-treated cells, and show that

Buffer...   +   +

Soluble fraction...   C   I
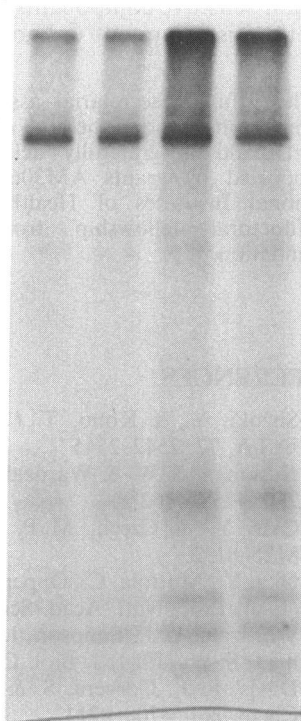
Incubation time (min)...   0   10   10   10

Fig. 6. Assay for the presence of IGF-II-receptor phosphatase activity in Triton X-100-soluble extracts of adipocyte plasma membranes

The Triton X-100-insoluble fraction of the plasma membrane was obtained from control cells as described in the Materials and methods section, and incubated in phosphorylation buffer with 100 μM-[γ-^{32}P]ATP for 10 min. The suspension was then centrifuged for 10 min and the supernatant was removed. The detergent-insoluble pellets were gently resuspended in 50 μl of phosphorylation buffer (+) containing 1% Triton X-100 or in 50 μl of Triton X-100-soluble extracts derived from plasma membranes obtained from control (C) or insulin-treated (I) cells. The suspensions were incubated for the time indicated above each lane, after which the IGF-II receptor was solubilized, immunoprecipitated, electrophoresed and detected by autoradiography.

Inhibitor...   MoO$_3$ Na$_3$VO$_5$ NaF
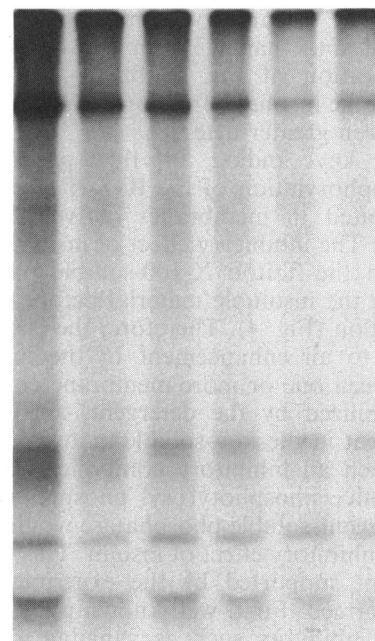
Cell incubation...   C   I   C   I   C   I

Fig. 7. Effect of different phosphatase inhibitors on IGF-II-receptor phosphorylation

Plasma membranes were obtained from control (C) or insulin-treated (I) cells and resuspended in phosphorylation buffer containing 1% Triton X-100 from which the ammonium molybdate was omitted. After 30 min of incubation, ammonium molybdate (MoO$_3$), sodium vanadate (Na$_3$VO$_5$) or sodium fluoride (NaF) was added (final concn. 10 mM, 100 μM and 50 mM respectively). Phosphorylation reactions were then started with [γ-^{32}P]ATP and continued for 10 min. The IGF-II receptor was then immunoprecipitated, electrophoresed and detected by autoradiography.

vanadate is almost as effective as molybdate in promoting the basal incorporation of [^{32}P]phosphate into the receptor. Fluoride, a selective phosphoseryl phosphatase inhibitor, is much less effective in promoting IGF-II-receptor phosphorylation. Interestingly, whereas the decrease in IGF-II-receptor phosphorylation in membranes from insulin-treated cells persisted in the presence of vanadate, it became undetectable when fluoride was employed. Fluoride is known to have multiple effects on membrane components, such as the stimulation of adenylate cyclase activity [15] and of photoreceptor phosphodiesterase [16], through its interaction with GTP-binding proteins [16]. However, an intriguing possibility is that fluoride could block the effect of insulin on tyrosine phosphorylation of the IGF-II receptor

by inhibiting an insulin-sensitive phosphoserine phosphatase, which might in turn modulate the activity of the tyrosine kinase.

DISCUSSION

It has previously been shown that the receptor for IGF-II is phosphorylated in intact cells on serine and threonine residues [7,8]. In contrast, when isolated fat-cell plasma membranes are incubated with [γ-^{32}P]ATP, the phosphorylation occurs principally on tyrosine residues on the receptor [9]. This phosphorylation reaction appears to be catalysed by a potent tyrosine kinase activity, which is tightly bound to the Triton X-100-insoluble portion of this membrane, and not from an autophosphorylation reaction [9]. Interestingly, the Triton X-100-insoluble fraction of several other cell types has been found to contain elevated tyrosine kinase activity [17–19], although phosphorylation of proteins on tyrosine residues occurs rarely in these cells. These findings suggest that the steady-state extent of tyrosine phosphorylation is greatly suppressed in intact cells, and is increased only under particular physiological circumstances or in response to specific stimuli.

Interestingly, removal of Triton X-100-soluble components of the plasma membrane greatly enhances the phosphorylation of the IGF-II receptor on tyrosine residues. Fig. 4 shows that phosphorylation of the receptor occurs to a much lesser extent in intact membranes than in Triton X-100-solubilized membranes. Separation of the Triton X-100-soluble components from the insoluble fraction enhances phosphorylation to an even greater extent.

A key finding in this paper is that tyrosine phosphorylation of the IGF-II receptor is dramatically inhibited in membranes derived from insulin-treated cells. The inhibitory effect of insulin cannot be observed when the Triton X-100-soluble proteins are removed from the insoluble material before the phosphorylation reaction (Fig. 4). Therefore, the effect of insulin can be due to an enhancement of the inhibitory interaction between one or more membrane components which are solubilized by the detergent, and the tyrosine kinase present in the non-soluble material.

Such an inhibitory component could be an insulin-sensitive phosphotyrosyl phosphatase. Removal of the detergent-soluble phosphatase would therefore eliminate the inhibitory effect of insulin. This possibility, however, is not supported by the experiments that have been performed. First, well-known phosphotyrosyl phosphatase inhibitors such as vanadate do not suppress the inhibitory effect of insulin (Fig. 7). Second, incubation of phosphorylated IGF-II receptors with unlabelled ATP (Fig. 5) or Triton X-100 extracts from control or insulin-treated membranes (Fig. 6) does not result in a marked dephosphorylation of the receptor, suggesting that IGF-II-receptor phosphotyrosine phosphatases are not active under the experimental conditions used.

An alternative possibility is that the effect of insulin may be indirectly mediated through the activation of a phosphoseryl/phosphothreonyl phosphatase. This possibility is raised by the finding that fluoride, which is a relatively selective inhibitor of such phosphatases, is the only compound which can completely suppress the inhibitory effect of insulin on IGF-II-receptor phosphorylation. Moreover, we have found that in intact cells, labelled with [^{32}P]phosphate, insulin decreases the phosphorylation of the receptor on threonine and serine residues [7,8], suggesting that insulin may stimulate the activity of a phosphoseryl/phosphothreonyl phosphatase.

The physiological relevance of tyrosine phosphorylation of the IGF-II receptor is not known. However, it is interesting that both the phosphorylation of the receptor in vivo on serine and threonine residues, and the phosphorylation in vitro on tyrosine residues, are decreased in response to insulin. These results raise the possibility that similar mechanisms might be involved in mediating these effects. The enzymes involved in the

phosphorylation of the receptor may be modulated in similar ways by insulin-sensitive kinases or phosphatases, or by the presence of stimulatory or inhibitory factors. The nature of the serine, threonine and tyrosine kinases that phosphorylate the IGF-II receptor, and of the insulin-sensitive components of the Triton X-100-soluble fraction, is at present unknown.

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