Insulin Activation of Insulin Receptor Tyrosine Kinase in Intact Rat Adipocytes

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We have studied the effect of incubation of intact cells with insulin on insulin receptor kinase activity. Following exposure of rat adipocytes to insulin, cells were solubilized and insulin receptors purified by specific immunoprecipitation or by insulin affinity chromatography. Kinase activity of the receptors, as measured by phosphorylation of histone 2B, was then determined. Insulin treatment of the cells resulted in a 10–20-fold increase in histone kinase activity of the subsequently isolated insulin receptors. The insulin effect was half-maximal at 3 s and maximal within 15 s of exposure, was dose-dependent (EC50 = 21 ng/ml), and was rapidly reversible following dissociation of insulin from the cells. The insulin effect in intact cells on insulin receptor kinase activity could be partially reversed in vitro by dephosphorylation of the isolated receptors by alkaline phosphatase. It is proposed that: 1) in intact cells, insulin causes alterations in insulin receptors, such that their kinase activity toward non-receptor substrates increases; 2) increased insulin receptor kinase activity following insulin stimulation in intact cells is, at least in part, the result of an increased phosphate content of the receptors; and 3) effects of insulin on insulin receptors in intact cells can be preserved during receptor isolation and thus can be measured in a cell-free system.

It is well accepted that the biological effects of insulin are initiated by interaction of the hormone with specific cell surface receptors such that one or more intracellular signaling mechanisms are activated (1–3). One possible signaling mechanism involves phosphorylation and dephosphorylation of cellular proteins (4–10). That phosphorylation reactions may play a role in mediating insulin action is supported by the finding that the highly purified insulin receptor expresses insulin-stimulable kinase activity directed toward tyrosine residues of its own β-subunit (autokinase activity) as well as toward tyrosine residues of exogenous (non-receptor) substrates (11–15). Recent evidence from cell-free systems suggests that insulin does not directly activate the receptor kinase toward non-receptor substrates. Instead, insulin appears first to activate the kinase toward tyrosine residues of the β-subunit. Phosphorylation of the β-subunit then results in the receptor becoming a more active kinase toward non-receptor substrates (16–18). These findings in cell-free systems suggest that in intact cells similar mechanisms might activate the insulin receptor kinase toward non-receptor substrates and thereby couple insulin binding to some, or all, of insulin's biologic effects.

Studies conducted with intact cells labeled with orthophosphate have revealed that insulin stimulates insulin receptor phosphorylation in vivo1 as well as in a cell-free system (19–24). Recently, Pang et al. (23) and White et al. (24) found that the addition of insulin to rat hepatoma cells initially stimulates insulin receptor phosphorylation almost entirely on tyrosine residues, indicating that receptor phosphorylation during the initial phase of exposure to insulin is catalyzed by the receptor-associated insulin-stimulable tyrosine kinase. At later time points, insulin may also have an effect on the phosphorylation of serine and threonine residues of its receptor (19, 20, 23, 24). Since the purified insulin receptor does not express kinase activity toward these residues, the phosphorylation of these sites is most likely caused by other kinases in the cellular environment of the receptor. Recent studies have shown that the phosphorylation content of the serine- and threonine-containing sites of the insulin receptor can be increased by incubation of cells with phorbol esters (21, 25) and that phosphorylation of these sites might inhibit the effect of insulin to promote autophosphorylation at tyrosine residues (23, 25). Upon dissociation of insulin from cells, the phosphorylation content of insulin receptors decreases rapidly, most likely due to phosphatase and protein kinase activity distinct from the insulin receptor (20).

The pattern of phosphorylation of sites on the insulin receptor observed following insulin stimulation of intact cells differs markedly from the phosphorylation pattern observed following insulin-stimulated autophosphorylation in a cell-free system (24). Thus, the regulation of receptor kinase activity toward non-receptor substrates as observed in vitro, resulting from autophosphorylation, may not reflect the regulation which occurs in intact cells. To assess the effect of insulin receptor phosphorylation in vivo on the receptor kinase activity toward non-receptor substrates, we have developed a technique to isolate insulin receptors from cells under conditions designed to preserve the cellular phosphorylation

1 In this report, the term "in vivo" refers to intact cells, whereas the term "in vitro" refers to a cell-free system.
state of the receptors and to subsequently measure the ability of these isolated receptors to phosphorylate non-receptor substrates. We demonstrate that incubation of intact adipocytes with insulin causes alteration in the receptor protein, that these changes can be preserved during receptor isolation, and that they result in an increase in the intrinsic tyrosine kinase activity of the receptor. Furthermore, we show that this "in vivo" activation of the insulin receptor kinase is due to changes in the phosphorylation state of the receptor.

EXPERIMENTAL PROCEDURES

Materials—Porcine monocomponent insulin and [3H-]Tyr A and [3H-]Thr A were generously supplied by Drs. Bruce Frank and Ronald Chance of Lilly. Serum from a patient with anti-receptor antibodies (26) was kindly supplied by Dr. Lawrence Mandarin (La Jolla, CA). [7-32P]-ATP (3000 Ci/mmol) was purchased from Amer sham Corp. Collagenase was purchased from Worthington, bovine serum albumin (fraction V) from Armour, Protein A (formalin-fixed Staphylococcus aureus cells) from Bethesda Research Laboratories, CanBr-activated Sepharose 4B from Pharmacia, and histone 2B from Sigma. Materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad Laboratories. Porcine monocomponent insulin and [3H-]Tyr A and [3H-]Thr A were generously supplied by Drs. Bruce Frank and Ronald Chance of Lilly.

Preparation and Insulin Treatment of Isolated Adipocytes—Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats (200-250 g each) as previously described (27). The cells were then incubated at 37 °C in the presence or absence of insulin in DMEM (5.2 mM KCl, 1.2 mM CaCl2, 1.3 mM MgSO4, 1.3 mM KH2PO4, 1 mM NaHPO4, 1 mM glucose, and 1% bovine serum albumin, pH 7.4).

Solubilization of Adipocytes and Partial Purification of Insulin Receptors—Following the incubation of adipocytes in Krebs-Ringer phosphate buffer, an ice-cold solubilization mixture was added to the cells to yield final concentrations of 1% Triton X-100, 2.5 mM phenylmethylsulfonyl fluoride, 8 mg/ml bacitracin, 1 mg/ml benzamidine, 800 trypsin inhibitor units/ml aprotinin, 8 mM EDTA, 160 mM NaF, 10 mM sodium pyrophosphate, 0.2 mM sodium vanadate, 2 mM dichloroacetic acid, and 20 mM HEPES, pH 7.4. The cells were immediately shaken vigorously, and cell solubilization was allowed to continue for 30 min at 4 °C. Under these conditions, about 90% of the insulin receptors are extracted (28). Following solubilization, the cellular extract was centrifuged at 11,000 X g for 10 min at 4 °C, the cellular lipids were removed, and nonsoluble material was separated by a second spin at 120,000 X g for 30 min at 4 °C. The supernatant was diluted 1:3 with buffer A (0.5% Triton X-100, 100 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 0.1 mM sodium vanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 4 mM EDTA, 10 mM glycerol, and 20 mM HEPES, pH 7.4) and applied to a wheat germ-agarose column at 4 °C (29). The column was washed with 30 volumes of buffer A, and the glycoprotein eluates were collected with buffer B containing 0.3 M N-acetyl-D-glucosamine. Protein content was determined by the Bradford (30) dye method using Bio-Rad reagent. The average protein concentration was about 250 μg/ml, and no difference in protein yield was observed between wheat germ-eluted insulin receptor preparations, adipocytes were isolated under conditions designed to preserve their phosphorylation state, i.e. in the presence of potent kinase and phosphatase inhibitors. The receptors were immobilized on insulin-agarose and washed to remove non-receptor proteins and inhibitors. The ability of the agarose-bound insulin receptors to stimulate phosphorylation of histone 2B was then assayed. The results shown in Fig. 1 demonstrate that the kinase activity of receptor-agarose preparations from insulin-treated cells (lane B) was higher than than that from control cells (lane A). In three experiments, the amount of 32P incorporated into histone by receptors from insulin-treated cells was 10-20-fold higher than by receptors from control cells. When duplicate samples were processed as in lanes A and B (Fig. 1), except that binding of the receptors to insulin-agarose was prevented by the addition of 100 μg/ml insulin, no effect of insulin incubation of the cells on histone phosphorylation was observed (Fig. 1, lanes A* and B*). This demonstrates that the increase in kinase activity in the receptor-agarose from insulin-treated cells compared to receptor agarose from control cells was the result of an increased insulin receptor kinase activity rather than an increased activity of other kinases present in the preparation. Phosphoamino acid analysis of the phosphorylated histone protein revealed exclusively phosphotyrosine (data not shown), consistent with the known tyrosine kinase activity of the insulin receptor.

As seen in Fig. 2, phosphorylation of histone by insulin-agarose-bound receptors from cells treated with and without insulin was linear for at least 60 min. The procedures used to prepare receptors remove essentially all free and bound insulin from the preincubation; nevertheless, even if some bound insulin were carried over, this could not affect the results since receptor kinase activity was measured while all receptors were bound to insulin-agarose.

To compare the kinase activity of receptors from insulin-
were incubated in the absence (lanes A, A*, C, and C*) or presence (lanes A*-D*) of 500 ng/ml insulin for 20 min. Incubations were terminated by addition of ice-cold solubilization mixture. The extracted insulin receptors, partially purified by wheat germ-agglutinin chromatography, were incubated with insulin-agarose in the absence (lanes A-D) or presence (lanes A*-D*) of 100 μg/ml added insulin. After 16 h at 4 °C, the agarose was washed and suspended in buffer containing 5 mM MnCl₂ and 12 mM MgCl₂ (lanes A, A*, B, and B*) or 5 mM MnCl₂, 12 mM MgCl₂, and 1 mM ATP (lanes C, C*, D, and D*). After 1 h at 4 °C, the agarose was extensively washed, and phosphorylation reactions were initiated by the addition of 4 μM [γ-32P]ATP, 1 mg/ml histone 2B, 5 mM MnCl₂, and 12 mM MgCl₂ (final concentrations). Reactions were terminated after 30 min as described under "Experimental Procedures," and phosphoproteins were identified by SDS-PAGE and autoradiography. Histone (H2B) counts/min detected in lanes A-D* were 176, 621, 1636, 1646, 155, 172, 163, and 182, respectively. To calculate histone phosphorylation activity of the receptors bound to insulin-agarose, duplicate samples were processed as in lanes A and B (Fig. 1), except that the receptor-agarose was incubated with 1 mM ATP, 5 mM MnCl₂, and 12 mM MgCl₂ at 4 °C for 60 min and washed before histone kinase activity was measured. As can be seen in lane C (Fig. 1), unactivated receptors from control-cells could be activated in vitro. Furthermore, activated receptors from insulin-treated cells could be further activated in vitro (Fig. 1, lane D). In three experiments, the kinase activity of insulin receptors from cells incubated with or without insulin was 24 ± 3 and 1.5 ± 0.8%, respectively, of that obtained by subsequent activation of the receptors in vitro.

The purpose of these studies was to assess the ability of insulin to stimulate the tyrosine kinase activity of the insulin receptor in intact cells. Thus, it was necessary to show that the in vitro assay conditions did not significantly alter the existing activity state of the receptors. The [γ-32P]ATP, which is used as a substrate to measure phosphorylation of histone, might also serve as a substrate for autophosphorylation of the receptor, thereby further activating the receptor kinase. We minimized this problem by using a low concentration of ATP (4 μM) so that the extent of autophosphorylation was not great enough to appreciably affect the ability of the receptor-agarose preparations to phosphorylate histone. In the experiments depicted in Fig. 3, receptors immobilized on insulin-agarose were preincubated with different concentrations of unlabeled ATP for 20 min at 4 °C. Histone kinase activity of the extensively washed receptor-agarose beads was then measured at an ATP concentration of 4 μM. Preincubation with unlabeled ATP increased the histone kinase activity of the immobilized insulin receptors with a maximal effect at 0.2 mM. At ATP concentrations lower than 4 μM, very little activation was observed during a 20-min period. Addition of histone 2B (1 mg/ml) to the preincubation inhibited the ATP-induced activation of the receptor kinase at low ATP concentrations, most likely due to competitive inhibition of receptor

| Lane | Insulin (cells) | Insulin (beads) | ATP |
|------|----------------|----------------|-----|
| A    | -              | -              | -   |
| B    | +              | -              | +   |
| C    | +              | -              | +   |
| D    | -              | +              | +   |
| A*   | -              | +              | +   |
| B*   | +              | +              | +   |
| C*   | +              | -              | +   |
| D*   | -              | -              | +   |

Fig. 1. Kinase activity of insulin receptors purified from cells incubated with or without insulin. Isolated rat adipocytes were incubated in the absence (lanes A, A*, C, and C*) or presence (lanes A*-D*) of 500 ng/ml insulin for 20 min. Incubations were terminated by addition of ice-cold solubilization mixture. The extracted insulin receptors, partially purified by wheat germ-agglutinin chromatography, were incubated with insulin-agarose in the absence (lanes A-D) or presence (lanes A*-D*) of 100 μg/ml added insulin. After 16 h at 4 °C, the agarose was washed and suspended in buffer containing 5 mM MnCl₂ and 12 mM MgCl₂ (lanes A, A*, B, and B*) or 5 mM MnCl₂, 12 mM MgCl₂, and 1 mM ATP (lanes C, C*, D, and D*). After 1 h at 4 °C, the agarose was extensively washed, and phosphorylation reactions were initiated by the addition of 4 μM [γ-32P]ATP, 1 mg/ml histone 2B, 5 mM MnCl₂, and 12 mM MgCl₂ (final concentrations). Reactions were terminated after 30 min as described under "Experimental Procedures," and phosphoproteins were identified by SDS-PAGE and autoradiography. Histone (H2B) counts/min detected in lanes A-D* were 176, 621, 1636, 1646, 155, 172, 163, and 182, respectively. To calculate histone phosphorylation activity of the receptors bound to insulin-agarose, duplicate samples were processed as in lanes A and B (Fig. 1), except that the receptor-agarose was incubated with 1 mM ATP, 5 mM MnCl₂, and 12 mM MgCl₂ at 4 °C for 60 min and washed before histone kinase activity was measured. As can be seen in lane C (Fig. 1), unactivated receptors from control-cells could be activated in vitro. Furthermore, activated receptors from insulin-treated cells could be further activated in vitro (Fig. 1, lane D). In three experiments, the kinase activity of insulin receptors from cells incubated with or without insulin was 24 ± 3 and 1.5 ± 0.8%, respectively, of that obtained by subsequent activation of the receptors in vitro.

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Fig. 2. Time course of histone phosphorylation by insulin receptors bound to insulin-agarose. Insulin receptors from isolated cells incubated with or without (C) 500 ng/ml insulin were bound to insulin-agarose. Phosphorylation reactions were initiated as described in the legend to Fig. 1 and terminated at the indicated times. Radioactivity in histone in samples in which receptor binding to insulin-agarose had been prevented by an excess of free insulin was subtracted as background.

Fig. 3. Effect of autophosphorylation "in vitro" on insulin receptor kinase activity. Aliquots of insulin receptors bound to insulin-agarose were exposed to the indicated concentrations of unlabeled ATP for 20 min in the absence (○) or presence (●) of 1 mg/ml histone 2B. The receptor-agarose was then extensively washed, and histone phosphorylation was measured in a phosphorylation reaction with 4 μM [γ-32P]ATP, 1 mg/ml histone 2B, 5 mM MnCl₂, and 12 mM MgCl₂. After 10 min at 4 °C, reactions were terminated by boiling in Laemmli sample buffer, and phosphoproteins were separated by SDS-PAGE. Gel pieces containing histone were excised, and radioactivity was determined by liquid scintillation. Background was subtracted as described in the legend to Fig. 1.
autophosphorylation. These results demonstrate that histone phosphorylation by receptors linked to agarose beads can be measured in the presence of 4 μM [γ-32P]ATP without appreciable changes during the time of the assay in the activity state of the insulin receptors as a consequence of added ATP.

Activation of the histone kinase activity of receptor-agarose beads by 1 mM ATP (in the presence of 5 mM MnCl₂ and 12 mM MgCl₂) was completely prevented by the inhibitors present in the buffer used to solubilize the adipocytes (data not shown). This shows that the inhibitors were able to prevent further activation of cellular insulin receptors after adipocyte solubilization.

**Dose Response of Insulin Activation of Insulin Receptor Kinase**—Control experiments indicated that only about 15–30% of the receptors present in the wheat germ lectin preparations were immobilized on insulin-agarose (data not shown). To circumvent this problem and to confirm the above results with an independent method, immunoprecipitation was used in most of the following experiments to purify and immobilize the insulin receptors. This method routinely achieved 85–95% recovery of receptors (31). To investigate the dose-response relationship of insulin activation of insulin receptors in intact cells, adipocytes were incubated with various insulin concentrations. Fig. 4, A and B, shows the amount of 32P incorporated into histone 2B by the immunoprecipitated receptors of these cells. The kinase assay was terminated after 10 min and during this period, 32P incorporation into histone was linear using receptors from both control and insulin-stimulated cells (data not shown). The results indicate that activation of the insulin receptor kinase is dose-dependent with half-maximal stimulation at 21 ± 6 ng/ml. 32P incorporation was also observed into the 95-kDa subunit of the insulin receptor (Fig. 4A and open circles in Fig. 4B); the dose-response curve for autophosphorylation was quite similar to that of histone phosphorylation.

The possibility can be raised that some of the insulin initially bound to the receptors of the intact cells was still present during and after the receptor purification steps. This is unlikely due to the extensive washing and dilution steps during these procedures. Nevertheless, to control for this possibility, 5 μg/ml insulin (final concentration) was added to all samples immediately after cell solubilization. Any effect of residual insulin on the measurement of kinase activity would be equal in all samples, but no effect of this insulin addition after cell solubilization was observed.

**Time Course of Insulin Activation of Insulin Receptor Kinase**—Cells were incubated with 500 ng/ml insulin at 37°C, and the results in Fig. 5, A and B, indicate that the activation of insulin receptor kinase toward histone and toward autophosphorylation sites on the receptor occurs extremely rapidly. Half-maximal effects are reached after about 3 s with complete activation by ~5 s. Although these times do not include the time period over which activation might continue after the addition of solubilizing buffer, this latter time period must be extremely short, because 5 μg/ml insulin added immediately after the solubilizing buffer failed to activate the receptor kinase (Fig. 5A, left lane). In additional experiments, insulin receptor kinase activity was measured following incubation with 500 ng/ml insulin for 5, 10, 20, and 40 min. No further changes in kinase activity were observed after the 5-min time point.

**Deactivation of Insulin Receptor Kinase**—The reversibility of insulin activation of the insulin receptor kinase was studied following dissociation of prebound insulin from intact cells. Adipocytes were incubated for 20 min with 50 ng/ml insulin. Subsequently, the insulin-containing buffer was removed, and the cells were resuspended in insulin-free buffer. Deactivation of insulin receptor kinase activity and dissociation of cell-associated 125I-insulin were measured under comparable conditions. The results shown in Fig. 6 indicate that deactivation of the insulin receptor kinase occurs rapidly following removal of insulin. The time course of deactivation (tₜₐ = 4.1 ± 0.8 min) appears to be slightly more rapid than the loss of insulin from the cells (tₜₐ = 5.6 ± 0.8 min). A possible explanation for this observation is that loss of cell-associated insulin represents dissociation of insulin from surface receptors as well as release of internalized radioactivity from the cells. Since the

![Fig. 4. Dose response of insulin activation of insulin receptor kinase. Isolated rat adipocytes were incubated at various insulin concentrations for 20 min. Incubations were terminated by the addition of ice-cold solubilization buffer and rapid disruption of the cells by vigorous shaking. Insulin (final concentration 5 μg/ml) was added to all samples shortly after initiation of solubilization. Aliquots of partially purified insulin receptors of the cells were incubated with serum containing anti-insulin receptor antibody (ARA) or control serum (NHS) for 16 h at 4°C, followed by the addition of 60 μl of Protein A (20%, w/v). Phosphorylation reactions were performed for 10 min using the immunoprecipitated receptors, and proteins were separated by SDS-PAGE as described under "Experimental Procedures." A, autoradiograph of the labeled phosphoproteins in a representative experiment; B, phosphorimaging of histone 2B (○) and the 95-kDa β-subunit of the insulin receptor (○) by insulin receptors from cells incubated with the indicated insulin concentrations. Radioactivity detected in corresponding protein bands of samples processed with control serum was subtracted as background. The results represent the mean ± S.E. of three separate experiments.](image)
latter process is slower than dissociation from receptors (36), it is possible that the time course of insulin dissociation from receptors and deactivation of kinase activity are comparable.

Effect of Alkaline Phosphatase on Kinase Activity—The previous experiments indicate that incubation of intact cells with insulin modifies the receptor protein such that its kinase activity increases. To further investigate the nature of this modification, insulin receptors from insulin-treated cells were immobilized on insulin-agarose and exposed to alkaline phosphatase. The results in Fig. 7 demonstrate that treatment with alkaline phosphatase led to a 65% decrease in the kinase activity of the receptors (lanes A and C). To show that this loss of activity was indeed due to dephosphorylation of sites on the receptor and not caused by some other effect of alkaline phosphatase, immobilized receptors treated with or without alkaline phosphatase were incubated with 1 mM ATP. After 1 h at 4 °C, the agarose beads were again extensively washed, and phosphorylation of histone by the receptor-agarose was measured. Histone counts/min detected in lanes A–D were 4,011, 14,251, 1,383, and 16,695, respectively.
intact cells is caused by \textit{in vivo} phosphorylation of sites on the receptor protein.

**DISCUSSION**

It has been proposed that phosphorylation of cellular substrates by the insulin receptor kinase might be involved in the transmission of the insulin signal (11, 16). Thus far, the effect of insulin on the kinase activity of the insulin receptor toward substrates other than its own \(\beta\)-subunit (non-receptor substrates) has only been studied in cell-free systems. These studies have indicated that insulin stimulates autophosphorylation, leading to an increase in the phosphorys content of tyrosine-containing sites on the \(\beta\)-subunit of the receptor, thereby activating the receptor kinase toward non-receptor substrates (16–18). For intact cells, the pattern of phosphorylation of sites on the insulin receptor following insulin stimulation markedly differs from the phosphorylation pattern following autophosphorylation of insulin receptors \textit{in vitro} (24). Furthermore, regulation of the phosphorylation state of the receptor is more complex in intact cells. Phosphatases are present which readily dephosphorylate the receptors (20), and changes in the phosphorus content of serine and threonine residues on the insulin receptors occur (19, 20, 23, 25) that might influence their functional properties. The goal of the present study was to investigate the \textit{in vivo} regulation of the insulin receptor kinase activity toward non-receptor substrates. To perform these studies, it was necessary to isolate insulin receptors from cells under conditions that preserve their kinase activity state. Under the assumption that the cellular activity state of the receptor kinase could be maintained by preserving the phosphorylation state of the receptor, insulin receptors were isolated by rapidly solubilizing cells in the presence of potent kinase and phosphatase inhibitors. Following receptor purification by immobilization on insulin-agarose or by immunoprecipitation, these inhibitors were removed, and the kinase activity of the receptors, measured using histone 2B as a model substrate, was determined. In the kinase assays, a low ATP concentration (4 \(\mu\)M) was used to prevent significant increases in the receptor kinase activity due to autophosphorylation during the assay period. Using these procedures, we detected a 10–20-fold increase in the kinase activity of insulin receptors from insulin-treated, compared to control cells. These results indicated that incubation of intact cells with insulin modifies the period. Using these procedures, we detected a 10–20-fold increase in the kinase activity of the receptors, thereby activating the receptor kinase toward non-receptor substrates (16–18). For intact cells, the pattern of phosphorylation of sites on the insulin receptor following insulin stimulation markedly differs from the phosphorylation pattern following autophosphorylation of insulin receptors \textit{in vitro} (24). Furthermore, regulation of the phosphorylation state of the receptor is more complex in intact cells. Phosphatases are present which readily dephosphorylate the receptors (20), and changes in the phosphorus content of serine and threonine residues on the insulin receptors occur (19, 20, 23, 25) that might influence their functional properties. The goal of the present study was to investigate the \textit{in vivo} regulation of the kinase activity toward non-receptor substrates. To perform these studies, it was necessary to isolate insulin receptors from cells under conditions that preserve their kinase activity state. 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These results indicated that incubation of intact cells with insulin modifies the insulin receptors such that their kinase activity toward histone is maximally active kinase activity state. In aggregate, these findings strongly suggest that the increase in kinase activity of insulin receptors from insulin-treated adipocytes is due to an \textit{in vivo} increase in receptor phosphorus content, most likely due to an insulin-induced stimulation of receptor autophosphorylation.

At present, we do not know why the kinase activity of receptors from cells treated with maximal insulin concentrations accounted only for 20–30% of the maximal kinase activity that could be reached by phosphorylating receptors \textit{in vitro} with high concentrations of unlabeled ATP (Fig. 1). It is possible that phosphatases are present \textit{in vivo} which prevent the maximal activation of the insulin receptor kinase or that additional regulatory phosphorylation sites exist which are only available when receptors are phosphorylated in a cell-free state. Furthermore, it cannot be excluded that some receptors were dephosphorylated during their isolation from intact cells despite the presence of phosphatase inhibitors. Another possibility relates to the subcellular distribution of insulin receptors. When adipocytes are incubated with high concentrations of insulin at physiological temperatures, a portion of the receptors are internalized (28); conceivably only occupied receptors on the cell surface retain their activated kinase properties.

The activation of the insulin receptor kinase by insulin in intact cells occurred extremely rapidly. The half-maximal effect was reached within 3 s with full activation within 15 s. This is more rapid than the time course observed for overall \(32^P\) incorporation into insulin receptors of \(32^P\)-labeled Fao hepatoma cells, as observed by Haring et al. (20), where nearly 30 min was required to reach maximum incorporation of \(32^P\) into the receptors. More recently, however, Pang et al. (23) and White et al. (24) reported that insulin-stimulated phosphorylation of tyrosine residues of insulin receptors in these cells reached a maximum level within 20–60 s and plateaued thereafter. Insulin-stimulated receptor phosphorylation after this time appeared to be predominantly on serine and/or threonine residues (23). The concordance between the time course of \textit{in vivo} tyrosine phosphorylation of insulin receptors (23, 24) and the time course of \textit{in vivo} activation of the receptor kinase presented here supports the view that phosphorylation of tyrosine residues of the insulin receptor serves to activate the receptor kinase toward non-receptor substrates. In any event, the \textit{in vivo} time course data presented in Fig. 5 indicate that, after 10–15 s of insulin exposure, autophosphorylation has proceeded to a degree which allows full activation of the receptor kinase; ongoing receptor phosphorylation (20, 23, 24) after that point might be due to continuing self-phosphorylation or the action of other cellular kinases. Although these subsequent phosphorylations may subserve functional properties, they do not seem to further stimulate the kinase activity (at least as far as substrates represented by histone 2B are concerned). Conceivably, the longer term changes in receptor phosphorylation which occur \textit{in vivo} after insulin treatment may represent phosphorylation events subsequent to receptor internalization. The role of insulin receptor phosphorylation (if any) in targeting receptors for internalization and recycling remains to be defined. The kinase activity of receptors from insulin-treated cells was increased toward sites on the \(\beta\)-subunit of the receptor as well as toward histone. This finding indicates that insulin-stimulated phosphorylation of tyrosine-containing sites on the insulin receptor activates the receptor kinase toward histone as well as toward other tyrosine-containing sites on the \(\beta\)-subunit. Most probably, these latter sites are only phosphorylated \textit{in vitro} and are not accessible to phosphorylation \textit{in vivo}.

Dissociation of prebound insulin from adipocytes led to rapid deactivation of the receptor kinase activity. This pro-
Kinase Activity of Insulin Receptors Activated in Vivo

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mechanisms, much of the cell-associated insulin is internalized (38, 37), and although intact insulin dissociates rapidly from cell surface receptors, insulin-derived radioactivity is more slowly released from the intracellular compartment (36). Thus, loss of cell-associated radioactivity consists of two components, and the more rapid rate of dissociation of insulin from cell surface receptors may closely approximate the rate of deactivation of receptor kinase activity. In any event, the rapid decrease in insulin-stimulated receptor kinase activity following removal of insulin indicates the presence of ample cellular phosphatases capable of reversing the activation. This suggests a closely regulated biologic process. Possibly the phosphatase activity is subject to regulation providing a means to modulate insulin’s biologic activity. The rapid decline in receptor kinase activity following removal of insulin raises the possibility that the internalized receptors are no longer active as kinases, since one might not expect their activity to decline so rapidly following removal of insulin from the extracellular and surface compartments.

The kinase activity of insulin receptors was dependent on the insulin concentration used during the cell incubations. Half-maximal effect on histone kinase activity was reached at 21 ± 6 ng/ml insulin, a concentration comparable to that causing half-maximal insulin receptor autophosphorylation in Fao hepatoma cells labeled with ortho-32P phosphate (20). Compared to most biologic effects of insulin (38, 39), the dose-response curve for stimulation of receptor kinase activity is somewhat shifted to the right, suggesting that the ability of insulin to activate the receptor kinase is closely linked to fractional receptor occupancy (40, 41), consistent with an intramolecular reaction in which each occupied receptor undergoes activation by a process involving self-phosphorylation of specific tyrosine residues.

Our data indicate that the insulin-induced functional changes in insulin receptor kinase activity that have been observed in cell-free systems also occur in the physiological cellular environment. Thus, these results are consistent with the concept that insulin binding to receptors in intact cells initially causes a rapid increase in the phosphorylation of tyrosine-containing sites on the receptor and that this covalent modification in turn enhances the activity of the receptor toward non-receptor substrates that might mediate some or all of insulin’s biologic effects. As these events appear to occur quite rapidly, they might be involved in triggering even the initial cellular responses to insulin such as stimulation of glucose transport.

This sequence of events might also provide a mechanism to modulate insulin action at a post-binding site. The efficiency of transduction of the insulin signal could be modulated by kinases and phosphatases in the cellular environment of the insulin receptor that, in addition to the insulin-stimulated self-phosphorylation, might also affect the phosphorylation state of the receptor. This view is supported by recent observations showing that phorbol esters, possibly by stimulating protein kinase C, increase the phosphoserine and phosphothreonine content of insulin receptors in intact cells and that phosphorylation of these sites might inhibit insulin-promoted tyrosine autophosphorylation (23, 25). The methods described in this report of assessing the kinase activity of receptors stimulated in vivo may provide a valuable tool to further investigate these ideas.

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