ATP sensitizes the insulin receptor to insulin

(tyrosine kinase/N-ethylmaleimide alkylation/human placental insulin receptor purification/avidin-biotin affinity chromatography/receptor signaling)

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ABSTRACT Insulin receptor with high insulin binding and tyrosine kinase activities has been prepared from human placenta. Based on a molecular mass of 306 kDa for the receptor (the value obtained from the sum of the amino acid residues), this preparation is capable of binding 1.48 mol of insulin per mol of receptor. The receptor is free from phosphatase and ATPase activity and is not stimulated by sodium vanadate. Autophosphorylation is linear with respect to receptor concentration, and the 32P incorporated is stable even in the presence of a 100-fold excess of unlabeled ATP. The Km for ATP is 208 μM. N-Ethylmaleimide inhibits autophosphorylation. Alkylation with 3H-labeled N-ethylmaleimide results in the incorporation of 1.13 ± 0.37 mol of N-ethylmaleimide per mol of insulin binding activity exclusively into the β subunit of the receptor. The nonhydrolyzable ATP analog adenosine 5'-[β,γ-imido]triphosphate stimulates autophosphorylation of the receptor, an effect that is evident at ATP concentrations below 1 mM. The stimulatory effect of adenosine 5'-[β,γ-imido]triphosphate is the result of increasing the binding of the receptor to the α subunit, and this reflects itself in a shift to the left of the insulin dose–response curve for autophosphorylation. The same is true for ATP. As a consequence, it is now possible to reconcile the concentration of insulin necessary for stimulating the autophosphorylation reaction with physiological levels and with the levels of insulin required for its classical biological effects.

The tyrosine kinase activity of the human placental insulin receptor has been the subject of numerous investigations. The majority of these studies were performed with wheat germ agglutinin-purified receptor (wheat germ receptor) that contains only ~0.3% of the insulin receptor (1). It appears that this preparation of the receptor was used rather than affinity-purified material because the latter, in spite of high insulin binding activity, exhibits very poor tyrosine kinase activity. However, the wheat germ receptor, which has a relatively high tyrosine kinase activity, can be expected to contain phosphate-metabolizing enzymes such as ATPases and phosphatases, which are likely to interfere with accurate kinetic measurements. The disadvantages inherent in both of these preparations prompted the present investigation, which had as its primary goal the development of methodology for the preparation of highly purified receptor exhibiting tyrosine kinase activity comparable to that of the wheat germ receptor. This goal was achieved by the use of avidin-biotin technology and the development of an improved method for eluting the receptor from the affinity resin. The ensuing preparations, which exhibited a high level of insulin binding activity and a high degree of tyrosine kinase activity, appeared to be well suited for accurate kinetic measurements. Such studies are the subject of this paper.

MATERIALS AND METHODS

Nα-[β,γ-imido]triphosphosphate (AMP-PNP), ATP, GTP, ADP, guanosine 5'-[β,γ-imido]triphosphate (GMP-PNP), and PhosPHate F were purchased from Sigma; bacitracin was from Upjohn; Heps was from United States Biochemical; Triton X-100 was from Aldrich; and octyl β-D-glucoside was from Pierce. N,N'-Dihydroxyethylenbisacrylamide and protein standards for PAGE were purchased from Bio-Rad; N-[ethyl-2-3H]-ethylmaleimide and [14C]acetic acid were purchased from New England Nuclear; Na[125I] was from Amersham. Crystalline bovine insulin was a gift from Eli Lilly. All other chemicals were reagent grade. Sodium orthovanadate was purified as described (3). Ovalbumin (10 mg), from Sigma, was acetylated with [14C]acetic anhydride (Amersham) in 0.2 M borate buffer (pH 9.0) for 90 min at 4°C. Unreacted [14C]acetic anhydride was removed by chromatography on Sephadex G-25 in 50 mM NH4HCO3. The specific activity of the product was 7.74 μCi/mg (1 Ci = 37 GBq). An equal amount of NaDODSO4 (4%) in sodium phosphate buffer (50 mM) at pH 7.0 containing diothiothreitol (0.2 M) was added, and the solution was heated at 100°C for 2 min. NEM was added to 250 mM, the solution was incubated for 15 min at room temperature, and unreacted NEM was removed by chromatography on Sephadex G-100 in 50 mM NH4HCO3.

Preparation of the Receptor with Octyl β-Glucoside at pH 7.6 (pH 7.6 Receptor). A wheat germ agglutinin eluate was prepared in the usual manner (2). The ligand Nα-[β,γ-imido]triphosphosphate (AMP-PNP), adenosine 5'-[β,γ-imido]triphosphate (AMP-PNP), and guanosine 5'-[β,γ-imido]triphosphate (GMP-PNP), and PhosPHate F were purchased from Sigma; bacitracin was from Upjohn; Heps was from United States Biochemical; Triton X-100 was exchanged for octyl β-glucoside by washing with one column volume of 50 mM Heps, pH 7.6/1 M NaCl/0.1% Triton X-100/0.1 mM Phosphate F. The wheat germ agglutinin eluate (800 pmol of insulin binding activity) was adjusted to 1 M with NaCl and was cyclized over the column for 1 h at a flow rate of 0.2 ml/min. The wash was washed with 50 column volumes of Heps/salt/Triton buffer containing 0.1 mM Phosphate F at a flow rate of 50 ml/hr. Triton X-100 was exchanged for octyl β-glucoside by washing with one column volume of 50 mM Heps, pH 7.6/1 M NaCl/0.6% octyl β-glucoside buffer containing bacitracin (2 mg/ml), antipain (1.5 μg/ml), and Phosphate F (0.1 mM). The column was equilibrated to room temperature and was eluted with the same buffer. Fractions (20, 2 ml each) were collected into 1 ml of 50 mM Heps, pH 7.6 (chilled to 4°C), pooled, and concentrated in an Amicon stirred cell (PM-10 filter) to a receptor concentration of ~0.1 mg of protein per ml.

Detergent Exchange. The Amicon concentrate was diluted with 50 ml of 50 mM Heps, pH 7.6/0.1% Triton X-100 and

Abbreviations: AMP-PNP, adenosine 5'-[β,γ-imido]triphosphate; GMP-PNP, guanosine 5'-[β,γ-imido]triphosphate; NEM, N-ethylmaleimide.

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concentrated as before. The dilution and concentration process were repeated once more. Evidence that the receptor was in a Triton X-100 rather than an octyl β-glucoside environment was obtained by measuring insulin-stimulated autophosphorylation as a function of insulin concentration.

**Insulin Binding Measurements.** The binding of 125I-labeled insulin was determined by the polyethylene glycol assay (4) as described (5), and the results were subjected to Scatchard (6) analysis. The charcoal assay of Williams and Turtle (7) was used to detect binding activity in column eluates.

**Autophosphorylation Assays.** Receptor (2–4 pmol of insulin binding activity) was incubated in the presence or absence of insulin (at the concentrations indicated) with MnCl2 (5 mM) for 45 min at room temperature (preincubation). Autophosphorylation was initiated by addition of [γ-32P]ATP (0.36 µCi/nmol) at the concentrations indicated. Total reaction volume was 50 µl. The reaction proceeded for 10 min or as otherwise indicated and was terminated by addition of stopping buffer as described (2). Samples were analyzed by NaDodSO4/PAGE as described by Laemmli (8) with a 5% stacking and a 7.5% resolving gel. The electrophoresis, the gels were incubated for 10 min in 10% (wt/vol) trichloroacetic acid, washed with water, and incubated with 1 M KOH for 1 hr at 56°C to determine incorporation into phosphotyrosine (9). Autoradiograms were obtained with Kodak XAR-5 film at room temperature. Areas corresponding to exposed film were excised and quantitated by Cerenkov (10) radiation. Background was determined on a similar gel area.

**NEM Alkylation Procedure.** Insulin receptor (10 pmol of insulin binding activity) was incubated with [3H]NEM (specific activity of 2.5 Ci/mmol, 250 µM) for 1 hr at room temperature. The reaction was terminated by adding a 100-fold excess of unlabeled NEM, and [14C]ovalbumin (14 nCi) was added as an internal standard. The volume was adjusted to 100 µl with 50 mM Hepes (pH 7.6) and the solution was extracted with chloroform/methanol according to the method of Wessling and Flügge (11). The precipitate was dissolved in NaDodSO4/PAGE reducing sample buffer and heated at 100°C for 2 min. Electrophoretic separation was performed on N,N'-dihydroxyethylenebisacrylamide cross-linked gels by using a 4% stacking and a 10% resolving gel. The gels were stained with 0.5% (wt/vol) Serva blue dye in isopropanol/acetic acid/water, 3:1:6 (vol/vol) and electrophoretically destained with acetic acid/methanol/water, 0.5:1.7:7.8 (vol/vol). The gels were washed three times with water, cut into 2-mm slices, and incubated for 16 hr at 30°C in 25 mM periodic acid (12). [14C]Ovalbumin was added as an internal standard to estimate recovery.

**ATP Assay.** Autophosphorylation assays were performed in the usual manner except that, before addition of stopping buffer, aliquots (10 µl) were withdrawn, chilled to 4°C, and mixed with 200 µl of bovine serum albumin (6.25 mg/ml in 50 mM Hepes at pH 7.6) and 1 ml of 10% trichloroacetic acid, both at 4°C. After 10 min at 4°C, the suspensions were centrifuged (2000 × g for 15 min), an aliquot of the supernatant (100 µl) was withdrawn, and [γ-32P]ATP and [32P] were quantitated according to the procedure of Avron (13).

**Other Assays and Procedures.** Protein was determined with fluorescamine (14). Receptor labeling was performed as described (5).

**RESULTS AND DISCUSSION**

**Properties of the pH 7.6 Receptor.** Fig. 1 depicts the yield of 125I-labeled insulin binding activity of human placental receptor eluted from the affinity column under various conditions. The yields of receptor retrieved at 4°C with Triton X-100 at pH 5.0 ([pH 5.0 receptor] (1) or at room temperature with octyl β-glucoside at pH 7.6 (pH 7.6 receptor) are comparable. Generally 50–60% of the bound receptor is eluted with an average specific insulin binding capacity of 1.48 mol of 125I-labeled insulin bound per mol of receptor. However, as can be seen in Fig. 2, the pH 5.0 receptor exhibits low autophosphorylating activity. Elution of the affinity resin with octyl β-glucoside at pH 7.6 and 25°C provides receptor preparations exhibiting autophosphorylation activity comparable to those obtained with wheat germ eluates (Fig. 2). The level of 32P incorporation into the pH 5.0 and pH 7.6 receptors was unchanged when the gels were incubated with alkali to destroy phosphoserine and phosphothreonine. This was not true for the wheat germ receptor. The autoradiogram of NaDodSO4/PAGE of the pH 7.6 receptor under reducing conditions (Fig. 1 Inset) indicates the purity of this preparation and documents its tyrosine kinase activity.

The nature of the detergent employed exerts a marked influence on the strength of the binding of insulin to its receptor. This is illustrated in Fig. 3, which compares the dose–response curves for autophosphorylation of receptor in octyl β-glucoside and Triton X-100. Autophosphorylating

![Fig. 1. Elution of human placental insulin receptor from insulin affinity columns by various methods.](image1)

![Fig. 2. Autophosphorylating activity of insulin receptor purified by different methods.](image2)
activity of the pH 5.0 receptor in Triton X-100 reaches a maximum at an insulin concentration of 100 nM (15), whereas the pH 7.6 receptor in octyl β-glucoside reaches maximum activity only at insulin concentrations of 250 nM (Fig. 3A); however, if the octyl β-glucoside is exchanged for Triton X-100 (detergent-exchanged receptor), the dose-response curve returns to the position characteristic for Triton X-100-solubilized material (Fig. 3B).

In addition to the excellent tyrosine kinase activity of the pH 7.6 receptor, the autophosphorylation activity was linear as a function of a wide range of receptor concentrations (Fig. 4). In previous reports (15, 16), autophosphorylation activity was found to be linear for both the wheat germ and pH 5.0 receptors at receptor concentrations considerably below 1 pmol. In this study, where a broader range of receptor concentrations was examined, linearity was obtained only with the pH 7.6 receptor.

A study of the time course for receptor autophosphorylation revealed that 32P incorporation was complete by 10 min and no phosphotyrosine phosphatase activity could be demonstrated. This was true both at ATP concentrations at or below the K_m. Furthermore, addition of a 100-fold excess of unlabeled ATP did not reduce 32P incorporation when incubation was continued for as long as 35 min (data not shown).

**NEM Alkylation of the pH 7.6 Receptor.** NEM alkylation of the pH 7.6 receptor inhibited autophosphorylation as shown in Fig. 5. The linearity of the 1/v (where v = velocity) versus inhibitor plot (Fig. 5 Inset) suggests that the decrease in tyrosine kinase activity results from modification of a single sulfhydryl group although modification of a class of sites with similar inhibition constants could provide the same result (17).

When the [3H]NEM-alkylated pH 7.6 receptor was subjected to NaDodSO4/PAGE under reducing conditions, radioactivity was found exclusively at the position corresponding to the β subunit. Quantitative analysis of the radioactivity in the β subunit indicated that 1.13 ± 0.37 mol (mean ± SD, n = 13) of NEM were incorporated per mol of insulin binding activity.

Shia et al. (18) observed that dithiothreitol stimulated and NEM inhibited autophosphorylation. They suggested that a reduced sulfhydryl group(s), at or near the active site, was necessary for maximal activity. Inhibition of autophosphorylation by sulfhydryl alkylation has subsequently been confirmed by others (16, 19).

Wilden et al. (20) have incorporated [3H]NEM into the receptor and have concluded that only the β subunit of the native receptor is alkylated. The same laboratory (21) has shown that autophosphorylation is not inhibited when iodoacetamide is used to alkylate the receptor, suggesting that inhibition by NEM is due to steric interference.

**The Effect of AMP-PNP on Autophosphorylation.** We attempted to examine autophosphorylation in the presence of a nonhydrolyzable substrate analog, AMP-PNP, with the expectation that occupation of the site by the ATP analog might prevent alkylation. At an AMP-PNP concentration of 1 mM, inhibition of alkylation (63%) was observed. To determine the concentration of AMP-PNP necessary to fill the ATP binding site, we measured autophosphorylation as a function of AMP-PNP concentration (Table 1) and found, surprisingly, that concentrations of AMP-PNP up to 500 µM not only did not inhibit 32P incorporation but, on the contrary, stimulated autophosphorylation. This role for AMP-PNP could not be subserved by GMP-PNP, GTP, or ADP (data not shown).

Two possible reasons for the effect of AMP-PNP were considered: (i) that AMP-PNP stimulated autophosphorylation in an "indirect" manner (e.g., by inhibiting ATPase activity present in the pH 7.6 receptor preparation thereby increasing the concentration of ATP available for the autophosphorylation reaction) or (ii) that AMP-PNP had a "direct" stimulatory effect on the receptor.

The possibility that ATPase activity copurified with the receptor could not be overlooked especially in light of the hypothesis that insulin activation of the membrane bound
Na⁺, K⁺, and Mg²⁺-ATPases are responsible for some of its biological effects (22). This consideration prompted an investigation of the effects of known ATPase inhibitors on insulin-stimulated autophosphorylation. Ouabain at 1 mM, a concentration capable of inhibiting 90% of Na⁺, K⁺-ATPase activity (23), had no effect on the level of ³²P incorporation into tyrosine residues of the β subunit (data not shown).

An alternative inhibitory effect of AMP-PNP activity, and one that has been observed to have an insulin-like effect, is vanadate. The insulin-mimetic action of vanadate has variously been ascribed to its ability to inhibit phosphatases (24) and ATPases (25–27) or to its ability to stimulate autophosphorylation of the receptor itself (28).

We have examined the effect of vanadate on the pH 7.6 receptor by measuring basal and insulin-stimulated autophosphorylation in the presence of vanadate at concentrations ranging from 1 μM to 1 mM. Neither activation nor inhibition of autophosphorylation was observed (data not shown). We also measured basal and insulin-stimulated autophosphorylation in the presence of vanadate at several concentrations of ATP and compared the effect of vanadate with that of AMP-PNP (Fig. 6). Vanadate alone had neither a stimulatory nor inhibitory effect, regardless of the substrate concentration. In contrast AMP-PNP, at concentrations of ATP below and at the Km, stimulated ³²P incorporation into tyrosine residues of the β subunit. The lower the substrate concentration, the more pronounced was the AMP-PNP stimulation. At 1 mM ATP, where substrate is in excess, the effect was no longer evident. The AMP-PNP effect was less pronounced in the absence of insulin. Adding vanadate did not enhance or diminish the AMP-PNP effect.

The disappearance of ATP was also measured directly (Fig. 7). The production of ³²P₁ from radiolabeled ATP was compared for the wheat germ and pH 7.6 receptors in the presence of vanadate or AMP-PNP. A profound decrease in ATP was observed when wheat germ receptor was incubated with ATP for periods up to 30 min. Vanadate diminished but did not abolish the formation of ³²P₁ from ATP. In contrast, hydrolysis of ATP was barely detectable with the pH 7.6 receptor. Furthermore, AMP-PNP had no effect on this reaction. Thus, AMP-PNP stimulation of autophosphorylation of the pH 7.6 receptor is not due to inhibition of ATPase activity.

The alternative explanation, namely that AMP-PNP stimulates autophosphorylation by acting directly on the receptor, was explored by measuring its influence on the velocity of the reaction as a function of substrate concentration (Fig. 8). Addition of AMP-PNP increases maximum reaction velocity and lowers the Km for ATP. In the absence of AMP-PNP, a Vmax of 0.13 pmol of ³²P₁ incorporated per pmol of insulin binding activity per min and a Km of 208 μM were obtained; with AMP-PNP added, the Vmax was 0.25 and the Km was 129 μM.

Since binding of insulin to the receptor is reported to increase the Vmax for autophosphorylation (30), we hypothesized that the effect of AMP-PNP on these kinetic parameters might actually be due to a change in insulin binding. For this reason, we examined the effect of AMP-PNP on the binding of ¹²⁵I-labeled insulin to the receptor (Fig. 9). Binding studies were performed in the same manner as the preincubation reaction of the autophosphorylation assay. Scatch-
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