A Pertussis Toxin-sensitive G-protein Mediates Some Aspects of Insulin Action in BC3H-1 Murine Myocytes*

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The involvement of G-proteins in the insulin signal transduction system has been studied in detail using the murine BC3H-1 myocyte system. Pertussis toxin (PT) treatment, previously shown to attenuate some of the metabolic effects of insulin in this cell line (Luttrell, L. M., Hewlett, E. L., Romero, G., and Rogol, A. D. (1988) J. Biol. Chem. 263, 6134–6141), abolished insulin-induced generation of diacylglycerol and inositolglycan mediators with no effects on either the autophosphorylation of the insulin receptor or the phosphorylation of the major endogenous substrates for insulin-stimulated tyrosine kinase activity (pp185 and pp42–45). In vitro ADP-ribosylation and immunoblotting studies suggest that the major PT substrate is a 40-kDa protein of the G family. This protein band did not exhibit detectable tyrosine phosphorylation upon stimulation of either intact cells or cell membranes with insulin. In the presence of low concentrations of GTP, insulin treatment of isolated myocyte plasma membranes resulted in a small (30–40%) but significant stimulation of GTP hydrolysis. This effect was best observed in the presence of small concentrations of sodium dodecyl sulfate. The rate of guanosine 5'-O-(3-thiotriphosphate) (GTPγS) binding to BC3H-1 membranes was also significantly increased in the presence of insulin. The effects of insulin on GTP hydrolysis and GTPγS binding were found to be dependent on the concentration of insulin. These effects were not detected in plasma membranes prepared from PT-pre-treated BC3H-1 myocytes. In contrast, pretreatment with the B (inactive) subunit of PT did not alter the response of myocyte membranes to insulin. High affinity binding of [125I]iodoinsulin to myocyte plasma membranes was reduced by 60–70% in the presence of guanine nucleotides. Similar effects on insulin binding were produced by PT pretreatment of the cells. In contrast, adenine nucleotides had no effect on insulin binding. Scatchard analysis of the binding data showed that the observed effects of guanine nucleotides and PT on insulin binding resulted either from a reduction in the number of high affinity insulin binding sites or from a significant reduction of the affinity of insulin for its receptor. Low affinity binding sites did not appear to be affected by either guanine nucleotides nor PT pretreatment. These results provide substantial evidence suggestive of a noncovalent interaction between the insulin receptor and a regulatory G-protein system during the process of insulin signaling.

During the past few years a substantial amount of evidence suggestive of a certain role of regulatory G-proteins in the mechanisms of insulin signaling has been generated (1–7). Most of the available evidence, however, is limited to observations on the effects of bacterial toxins that catalyze the ADP-ribosylation of specific G-proteins, such as the Bordetella pertussis and Vibrio cholera toxins. The metabolic effects of insulin are attenuated by pretreatment of intact adipocytes (1), BC3H-1 myocytes (2), or hepatocytes (3) with pertussis toxin (PT). These effects are apparently correlated with the ADP-ribosylation of a 40-kDa protein, presumably the α-subunit of a G-protein heterotrimer (1–3). The involvement of a smaller (25 kDa), cholera toxin-sensitive GTP binding protein has also been suggested (4).

The insulin receptor is a heterotetramer composed of two classes of subunits (α and β). Insulin binding to the receptor induces the activation of the β-subunit tyrosine kinase which then autophosphorylates and catalyzes the phosphorylation of other cellular substrates (for a review see Ref. 8). In contrast, most of the receptors associated with G-protein systems are monomeric and consist of seven transmembrane domains (see, for example, Ref. 9). The latter interact with G-protein systems in a complex equilibrium involving noncovalent complexes consisting of the receptor, the G-protein heterotrimer, guanine nucleotides, and specific effectors which are responsible for the generation of the second messenger of the hormone. Since the activated insulin receptor is a tyrosine kinase, the association of the receptor with a G-protein system might be expected to occur by two different mechanisms, namely: (i) insulin may modulate the function of G-proteins by altering their phosphorylation state; and/or (ii) insulin may affect noncovalent interactions between the insulin receptor and a G-protein system in a manner analogous to that observed with other G-protein-associated receptors.

We report here the results of our observations on the mechanisms of interaction of insulin and its receptor with G-protein systems using as a model plasma membranes isolated...
from BC3H-1 murine myocytes. According to our observations, insulin does not alter the state of tyrosine phosphorylation of G-protein α-subunits. However, the interactions of GTP binding proteins with guanine nucleotides are significantly modulated by insulin and, vice versa, guanine nucleotides have significant effects on the interactions of insulin with the receptor. This suggests that the interactions of the insulin receptor and G-protein regulatory system are remarkably similar to those observed with the β-adrenergic receptor and other related proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—** Pertussis toxin was a generous gift from Dr. E. L. Hewlett (University of Virginia). An enriched preparation of the inactive B protomer of pertussis toxin was also supplied by E. L. Hewlett. This preparation was slightly contaminated with about 1% active PT holotoxin. Affinity purified rabbit anti-phosphotyrosine immunoglobulin was provided by Dr. J. T. Parsons (University of Virginia) (10). Polyclonal rabbit antibodies raised against synthetic peptides common to several G-protein α-subunits (antiserum J-881) and α (antiserum A-584) and α (antiserum U-46), respectively, were a gift from Dr. S. Mumby and Dr. A. G. Gilman (University of Texas Southwestern Medical Center) (11). Porcine insulin, 1,2-dimyristoyl- phosphatidylcholine-coated enzymes purchased from Sigma (product no. 3099), and other reagents were obtained as described (12). Nitrocellulose filters were blocked overnight at room temperature in blocking solution (25 mM Tris, 150 mM NaCl, 0.1% bovine serum albumin, and 0.05% Tween 20, and twice again with rinsing buffer. Filters were then incubated with 1 μCi/ml [35S]protein A for 1 h and the washing sequence repeated.

The α-subunit of G-proteins was detected by the immunoblotting procedure described by Munday et al. (11). Briefly, following transfer to nitrocellulose, filters were blocked for 1 h in low-detergent blotting buffer (50 mM Tris, pH 8, 2 mM CaCl2, 80 mM NaCl, 5% nonfat dry milk, 0.2% Nonidet P-40, and 0.02% azide) at room temperature, incubated for 1 h with the appropriate antiserum (1:200, diluted in the low-detergent blotting solution) and washed three times (10 min each) with the low detergent solution. Filters were next incubated for 1 h with 1 μCi/ml [35S]-goat anti-rabbit Fab' in a high detergent blotting solution containing 2% Nonidet P-40 and 0.2% SDS, washed three times with the low detergent solution and twice with 50 mM Tris (pH 8), 2 mM CaCl2, and 80 mM NaCl.

**Cell Stimulation and Insulin Receptor Immunoprecipitation—** Intact myocytes were cultured as described (2). Prior to stimulation or plasma membrane preparation, cultures of differentiated myocytes were serum-starved for 24 h in Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, 0.1% bovine serum albumin, and 2 mM glutamine. PT-treated myocytes were serum-starved in the presence of 100 ng/ml PT, conditions that result in a complete blockade of in vitro PT-catalyzed ADP-ribosylation of the predominant 40-kDa PT substrate in the myocyte (2). In most experiments, cells were pretreated with PT for 20 h. Shorter treatments (6 h) were used occasionally as reported under "Results." Plasma membranes were prepared from 20-100 mm dishes of differentiated cells. The cells were scraped, pelleted by centrifugation, and resuspended in plasma membrane isolation buffer (50 mM Hepes, pH 7.6) containing 8% sucrose, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. The cell extracts were layered on 35% sucrose in Hepes buffer (pH 8), 2 mM MgCl2 and either 200 μM GTPγS or 1 mM MgCl2, 0.5 mM MnCl2, 1 mM dithiothreitol, 0.2 mM isobutylmethylxanthine, 0.1% bovine serum albumin, 5 mM phosphocreatine, 50 units/ml creatine kinase, and 0-30 mM 4'-P]GTP. The final volume of the incubation mixture was 100 μl. Small amounts of SDS were included in the assays as shown in the corresponding figures, as suggested by Willard et al. (13). The reaction was stopped by addition of 1 ml of phosphate (20 mM, pH 7.4) containing 5% activated charcoal, 0.1% dextran, and 0.5% bovine serum albumin. The samples were centrifuged at 20,000 g for 10 min and 0.5-ml aliquots of the supernatant were removed and counted. The rate of GTP hydrolysis was calculated from the slope of the time course of the reaction.

**Assay of Insulin-stimulated GTP Hydrolysis—** GTP hydrolysis was determined on sucrose gradient purified plasma membranes by the method of Okajima et al. (14). Briefly, 15-20 μg of membrane protein were incubated for 2-10 min at 37 °C in 25 mM Tris (pH 7.5), 1 mM EGTA, 1 mM ATP, 0.5 mM MgCl2, 0.5 mM MnCl2, 1 mM dithiothreitol, 0.2 mM isobutylmethylxanthine, 0.1% bovine serum albumin, 5 mM phosphocreatine, 50 units/ml creatine kinase, and 0-30 μM [γ-32P]GTP. The final volume of the incubation mixture was 100 μl. Small amounts of SDS were included in the assays as shown in the corresponding figures, as suggested by Willard et al. (13). The reaction was stopped by addition of 1 ml of phosphate (20 mM, pH 7.4) containing 5% activated charcoal, 0.1% dextran, and 0.5% bovine serum albumin. The samples were centrifuged at 20,000 g for 10 min and 0.5-ml aliquots of the supernatant were removed and counted. The rate of GTP hydrolysis was calculated from the slope of the time course of the reaction.

**Assay of Insulin-stimulated Binding of GTPγS—** The binding of GTPγS was measured as described by Asano and Ross (16) with some modifications. All incubations and assays were performed on ice. Membranes (150-300 μg of protein/ml) were preincubated in the presence of Hepes buffer (pH 8), 2 mM MgCl2, and either 200 μM GTPγS or 1 mM MgCl2, 0.5 mM MnCl2, 1 mM dithiothreitol for no further additions as indicated. After 30 min, 5 μM GDP, and 200 nM insulin were added and the incubation continued for a further 5 min. Assays were then initiated by addition of 200 nM GTPγS (34 μCi/μmol). The final volume of the incubation mixture was maintained at 1 ml. Samples (100 μl) were removed at appropriate times and quenched in 10 volumes of ice-cold 20 mM Tris-HCl (pH 8), containing 100 mM NaCl, 10 mM MgCl2, 1 mM β-mercaptoethanol, and 0.1 mM GTPγS. Membrane-bound nucleotide was separated by filtration on cellulose acetate filters. The filters were rapidly washed with 16 ml of ice-cold buffer (20 mM Tris, pH 8, 100 mM NaCl, 10 mM MgCl2) and dissolved in 2-mercaptoethanol prior to the addition of scintillation fluid. Nonspecific binding, determined in the presence of 100 μM GTPγS, was less than 10% of total binding. We report specific binding defined as the difference between total and nonspecific binding. The binding of GTPγS was found to be linear during the first 3 min of the reaction. The rate of GTPγS binding was estimated from linear regression analysis of the time course of the reaction during the initial 90-120 s. Four to six data points were used for each analysis; the rates for each individual experiment were calculated with less than 10% error.

**Other Procedures—** The insulin-stimulated generation of [H]methyl-DAG by Rh C-1 cells was determined as described (2, 12). The release of pyruvate dehydrogenase stimulatory mediators to the extracellular medium was determined as described earlier (12). Insulin-stimulated activation of endogenous pyruvate dehydrogenase activity was determined by the method of Taylor et al. (17).

Insulin binding was determined using radiolabeled insulin as described elsewhere (18). Bicistronic (1 μM) was included in all binding assays to inhibit the proteolytic degradation of insulin by endogenous membrane proteases (18). Nonspecific binding was de
fined as the amount of iodoinsulin bound in the presence of 10 μM unlabeled insulin. Binding data were analyzed by nonlinear regression analysis of Scatchard plots assuming a two-component model.

In vitro ADP-ribosylation of purified plasma membranes was performed as described (2) using the procedure of Bokoch et al. (19).

RESULTS

The incubation of BC3H-1 myocyte plasma membranes with insulin stimulates the tyrosine phosphorylation of several protein bands. The most obvious are a 95-kDa protein (the β-subunit of the insulin receptor), a 185-kDa protein and several bands of approximately 42–45 kDa (Fig. 1, A and B). The 185-kDa band is most likely related to p185, the 185-kDa endogenous substrate for the receptor tyrosine kinase first reported by White et al. (20), whereas the 42–45-kDa protein may be related to p42–45, a protein recently identified in 3T3-L1 cells as microtubule-associated protein kinase by Rossmann et al. (21). The time course of tyrosine phosphorylation of these peptides in the presence of 100 nM insulin is shown in Fig. 2. As shown, PT pretreatment of the myocytes had no significant effect on the time course of tyrosine phosphorylation of the receptor β-subunit (Fig. 2, A and B), p185 or p45 (Fig. 2, C and D). In contrast, as shown previously, PT pretreatment of the cells reduced insulin-stimulated generation of [3H]myristate DAG and release of pyruvate dehydrogenase-stimulating mediator to the medium (2, 11) (Fig. 2, E and F). Thus, under conditions of maximal stimulation of cellular responses, PT pretreatment blocks insulin-stimulated mediator and DAG generation without affecting the tyrosine kinase activity of the receptor. These observations clearly show that receptor tyrosine kinase activity is not sufficient per se to regulate all metabolic functions modulated by insulin and that the signaling pathway involves additional biochemical components.

We have previously shown that the blockade of insulin-induced generation of DAG and mediator activity by PT occurs under conditions that parallel the inhibition of several biochemical actions of insulin and that these effects appear to be correlated to the ADP-ribosylation of a protein of approximately 40 kDa (2). On the basis of these and other observations we proposed that at least some of the actions of insulin were mediated by a G-protein system. Similar suggestions have been made by others (1, 3–7) although, to date, a conclusive demonstration of the linkage between insulin signaling and G-proteins has not been reported. We decided first to investigate some of the classical criteria used to establish the relationships between hormone receptors and G-protein systems. One of these has been the hormone-stimulated hydrolysis of GTP. In preliminary experiments, the GTPase activity of purified BC3H-1 plasma membranes was found to respond very weakly to insulin. However, as shown by Williamson et al. (15) for the fMet-Leu-Phe receptor system, the presence of low concentrations of SDS may enhance significantly the response of the receptor-linked GTPase activity. We thus decided to use a similar approach in our study of insulin-stimulated GTP hydrolysis.

As shown (Fig. 3A), in the presence of very low concentrations of SDS, insulin stimulated the rate of GTP hydrolysis by as much as 40% when compared to control. This effect was most apparent at micromolar SDS concentrations. The insulin-dose response of the effect measured in the presence of 2 μM SDS is shown in Fig. 3B. As shown, the half-maximal effect is observed in the presence of 0.5 nM insulin, well within

![Fig. 1. Detection of tyrosine-phosphorylated proteins, Gα, and pertussis toxin substrates in BC3H-1 cells. A, differentiated BC3H-1 myocytes were serum-starved and stimulated with insulin for 0–30 min with 200 nM insulin as indicated. Cells were lysed in situ by addition of boiling Laemmli sample buffer. Proteins were resolved by SDS-PAGE on 9% gels as described. Tyrosine-phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antibodies followed by development with radiolabeled protein A and autoradiography. B, plasma membranes purified from BC3H-1 myocytes were solubilized in Laemmli buffer and resolved by SDS-PAGE in 9% gels as described. Gα proteins were detected by immunoblotting with rabbit polyclonal antisera specific against a common peptide sequence found in most G-protein α subunits (J-881) or with polyclonal antisera specific for α4 (A-584) and α5 (U-46). C, purified BC3H-1 membranes were ADP-ribosylated in vitro with radiolabeled NAD in the presence of activated pertussis toxin as described prior to resolution by SDS-PAGE in 9% gels. ADP-ribosylated proteins were detected by autoradiography as described.](http://www.jbc.org/)

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G-proteins and Insulin Signaling

Fig. 2. Comparison of the time courses of insulin-stimulated tyrosine phosphorylation and second messenger generation in pertussis toxin-treated and untreated BCaH-1 myocytes. Data obtained from PT-treated myocytes are shown on the right. Controls are shown on the left. Cells were treated with 200 nM insulin. Tyrosine phosphorylation levels were determined by immunoblotting with anti-phosphotyrosine IgG followed by densitometric analysis of the respective autoradiograms. Data shown represent the average of three separate experiments. A and B, tyrosine phosphorylation of the β-subunit of the insulin receptor (p95). Insulin receptors were immunoprecipitated as described under “Experimental Procedures.” C and D, tyrosine phosphorylation of p185 (Ο, ●) and p45 (△, ▲). E and F, insulin-stimulated generation of [3H]myristate-labeled DAG (Ο, ●) and release of pyruvate dehydrogenase (PDH) stimulatory mediator to the culture medium (≯, ▲). These results are normalized with respect to time 0.

The normal range of response to the hormone. These results are consistent with previous reports demonstrating the activation of GTP hydrolysis by insulin in a platelet membrane system (7).

The kinetics of the insulin-stimulated hydrolysis of GTP by BCaH-1 plasma membranes was further characterized (Fig. 4). As shown (Fig. 4A), the time course of hydrolysis of GTP (0.3 μM) in the presence of 2 μM SDS was essentially linear over 10 min of incubation. The rate of GTP hydrolysis as a function of the concentration of the substrate is shown in Fig. 4B. In consistence with other reports, the basal hydrolysis of GTP did not follow saturation kinetics, probably due to the contributions of low affinity and nonspecific nucleotide triphosphatases in the plasma membrane (14, 22). In contrast, insulin-stimulated GTPase activity reached a plateau at about 1 μM GTP, a result similar to those reported for the Met-Leu-Phe receptor of rabbit neutrophil membranes (14). Table I compares control, insulin-, and isoproterenol-stimulated GTP hydrolysis in BCaH-1 plasma membrane preparations obtained from control and PT-treated cells. As shown, insulin and isoproterenol stimulated the hydrolysis of GTP by 33 and 28%, respectively, when untreated plasma membranes were used as enzyme sources. PT treatment did not affect the basal or isoproterenol-stimulated rates of GTP hydrolysis. However, the effect of insulin was completely blocked by pretreatment of the cells with PT. This provides substantial supportive evidence to our hypothesis that PT attenuates the action of insulin by altering the interactions between the receptor
Effects of pertussis toxin treatment on hormone-stimulated GTPase activity

Membraones were incubated with GTP (1 μM) in the presence of 2 μM SDS as described above. The rate of GTP hydroxysis was calculated as described under "Experimental Procedures." 

Table I

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Pretreatment</th>
<th>GTPase activity</th>
<th>Increase over basal</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/mg/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>78.5 ± 6.8</td>
<td>(n = 5)</td>
<td>100</td>
</tr>
<tr>
<td>Insulin, 100 nM</td>
<td></td>
<td>104.5 ± 4.7</td>
<td>(n = 5)</td>
<td>133.1</td>
</tr>
<tr>
<td>Isoproterenol, 10 μM</td>
<td></td>
<td>100.8 ± 5.6</td>
<td>(n = 5)</td>
<td>128.4</td>
</tr>
<tr>
<td>None</td>
<td>PT, 100 ng/μl</td>
<td>79.4 ± 10.7</td>
<td>(n = 5)</td>
<td>101.2</td>
</tr>
<tr>
<td>Insulin, 100 nM</td>
<td>PT, 100 ng/ml</td>
<td>79.8 ± 9.9</td>
<td>(n = 5)</td>
<td>101.7</td>
</tr>
<tr>
<td>Isoproterenol, 10 μM</td>
<td>PT, 100 ng/ml</td>
<td>92.5 ± 10.9</td>
<td>(n = 4)</td>
<td>117.8</td>
</tr>
</tbody>
</table>

* Greater than basal (p < 0.05).
* Not significant (p < 0.5).

Effects of insulin on the rate of binding of GTPyS

Plasma membranes were incubated with or without insulin in the presence of ATP (0.2 mM) and MnCl₂ (2 mM), AMP-PCP (1 mM) or in the absence of nucleotide and the rate of GTPγS binding was determined as described under "Experimental Procedures." PT-pretreated cell membranes were prepared from cells incubated in the presence of 100 ng/ml pertussis toxin for 6 h. Where indicated, cells were treated with the B protomer of pertussis toxin (100 ng/ml) for 6 h. The B protomer preparation used contained a small but measurable (1%) contamination with the active holotoxin.

Table II

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Additions</th>
<th>Rate of GTPγS binding pmol/min/mg</th>
<th>% control</th>
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<tr>
<td>None</td>
<td>None</td>
<td>1.4 ± 0.32 (n = 5)</td>
<td>100</td>
</tr>
<tr>
<td>Insulin, 200 nM</td>
<td>None</td>
<td>2.6 ± 0.26 (n = 5)*</td>
<td>185.7</td>
</tr>
<tr>
<td>None</td>
<td>ATP/MnCl₂</td>
<td>0.66 ± 0.15 (n = 5)*</td>
<td>100</td>
</tr>
<tr>
<td>Insulin, 200 nM</td>
<td>ATP/MnCl₂</td>
<td>1.1 ± 0.26 (n = 5)*</td>
<td>166.7</td>
</tr>
<tr>
<td>None</td>
<td>AMP-PCP, 1 mM</td>
<td>0.35 ± 0.11 (n = 4)</td>
<td>100</td>
</tr>
<tr>
<td>Insulin, 200 nM</td>
<td>AMP-PCP, 1 mM</td>
<td>0.7 ± 0.11 (n = 6)*</td>
<td>166.7</td>
</tr>
<tr>
<td>None</td>
<td>PT-pretreated</td>
<td>1.47 ± 0.26 (n = 2)</td>
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<tr>
<td>Insulin, 200 nM</td>
<td>PT-pretreated</td>
<td>1.68 ± 0.38 (n = 2)</td>
<td>114.3</td>
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<tr>
<td>None</td>
<td>PT B protomer</td>
<td>1.37 ± 0.2 (n = 5)</td>
<td>100</td>
</tr>
<tr>
<td>Insulin, 200 nM</td>
<td>PT-pretreated</td>
<td>2.06 ± 0.2 (n = 5)*</td>
<td>150.0</td>
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</table>

* Greater than control (p < 0.01).

The mechanism by which most hormone receptors stimulate GTP hydrolysis appears to be related to the effects of the hormone-receptor complex on the dissociation of GDP from the α-subunit of the G-protein (23). Thus, agonist-receptor interactions in most of these systems are generally associated to enhanced rates of exchange of guanine nucleotides. To confirm that the interactions of the insulin receptor with G-proteins were of a similar nature, we also studied the effects of insulin on the rate of binding of [35S]GTPγS to BC₃H₁ plasma membranes. The basal rate of binding of GTPγS to membranes was found to be very fast at room temperature, the binding reaction being over in less than 1 min (not shown). To increase the resolution of the analysis, the effects of insulin were studied at much lower temperatures (4 °C). As shown (Fig. 5A), the rate of association of GTPγS to BC₃H₁ myocytes at 4 °C is greatly enhanced by insulin. The initial rate of GTPγS was determined from the initial slope of the time course of the binding process. Initial slopes were determined by linear least squares analysis of the early (<120 s) time course of the reaction.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** The effect of insulin on the time course of GTPγS binding to purified BC₃H₁ membranes. GTPγS binding was assayed as described under "Experimental Procedures" in the presence of 200 μM ATP and 2 mM MnCl₂. A, the time course of GTPγS binding in the presence (●) or absence (○) of 200 nM insulin. Data shown are representative of experiments reproduced by triplicate. B, insulin dose dependence of the rate of GTPγS binding. The rate of GTPγS binding was determined from the initial slope of the time course of the binding process. Initial slopes were determined by linear least squares analysis of the early (<120 s) time course of the reaction.

...and a specific G-protein system.

To examine the role of the tyrosine kinase activity of the receptor on its interactions with the G-protein system we also tested the influence of ATP/Mn²⁺ and nonhydrolyzable ATP analogues on the effects of insulin on the initial rate of association of GTPγS to BC₃H₁ plasma membranes. As shown (Table II), the addition of adenine nucleotides reduced the rate of GTPγS binding, probably as a result of nonspecific binding of the unlabeled nucleotide. However, the stimulatory effects of insulin on the rate of binding of GTPγS were insensitive to the presence of either ATP/Mn²⁺ or AMP-PCP. The ATP and Mn²⁺ used in these experiments were sufficient to induce the tyrosine kinase activity of the β-subunit of the receptor to maximal levels and, conversely, AMP-PCP was used at concentrations sufficient to block autophosphorylation of the receptor (27). Therefore we suggest that the tyrosine kinase activity of the insulin receptor is not required...
for the interactions of the receptor with G proteins.

The effects of nucleotides and PT pretreatment on the binding of insulin to purified plasma membranes were also studied. Table III compares the effects of PT pretreatment and purine nucleotide addition on the binding of iodinated insulin to purified B&H-1 plasma membranes. These experiments were performed in the presence of 0.1 nM [125I] insulin. As shown, GTP, GTPγS, and GDPβS reduced insulin binding by about 60-70% under the conditions of the experiment. Low concentrations of GDP (0.1 mM) had no effect on insulin binding although higher concentrations (1 mM) significantly reduced insulin binding. This suggests that the affinity of GDP for the receptor-G-protein complex is significantly lower that of GTP. The presence of ATP and ADP had no apparent effect on insulin binding. Scatchard analysis of competition binding data (Fig. 6, Table III) indicated that the observed inhibition was the result of an apparent reduction of the number of high affinity insulin binding sites, with little effect on the affinity and number of the low affinity insulin binding sites in the membrane. These results are consistent with negative cooperative interactions among the receptor, a G-protein system and insulin binding. PT pretreatment of the cells also reduced significantly insulin binding to purified plasma membranes (Table III) by apparently a similar mechanism, as shown in the Scatchard plot of Fig. 6D. Some effects of PT in certain cells have been associated to an increase in the basal contents of cAMP. However, the cAMP contents of B&H-1 myocytes is not affected by PT pretreatment (data not shown). Thus, the observed effects of PT appear to be correlated to the ADP-ribosylation of specific G-proteins. Furthermore, since the effects of guanine nucleotides and PT pretreatment appear to be limited to the high affinity insulin binding sites, our observations suggest that the linkage among guanine nucleotides, PT action, and insulin binding are mediated by the direct coupling of the insulin receptor to a G-protein system.

**DISCUSSION**

We have demonstrated here a direct, most likely functional, correlation between the interaction of insulin with its receptor and a G-protein system in B&H-1 myocyte plasma membranes. This report strongly supports previous hypothesis set forth by us (2) and others (1, 3–7) on the involvement of G-proteins in the signaling mechanisms of insulin.

The G-protein system involved in the insulin signaling

**TABLE III**

*Effect of purine nucleotides and pertussis toxin treatment on insulin binding*

Plasma membranes obtained from naive or PT-pretreated cells were purified as described and incubated with 0.1 nM [125I] labelled insulin in the presence of the indicated purine nucleotides. Insulin binding was determined as described under "Experimental Procedures." B, as described under "Experimental Procedures." B, except that the binding assay was performed in the presence of 100 nM GTPγS. C, as A, except that the binding was performed in the presence of 1 mM GDP. D, as A, except that the membranes were prepared from myocytes pretreated for 20 h with pertussis toxin (100 ng/ml). The solid lines represent the best fit of the data to a two-site binding model.

![Figure 6](http://www.jbc.org/)

**FIG. 6.** Scatchard analysis of the binding of insulin to purified B&H-1 membranes. A, aliquots of B&H-1 membranes were incubated with 0.1 nM [125I] insulin in the presence of 0.1-1000 nM total insulin. Specific insulin binding was determined as described under "Experimental Procedures." B, as A, except that the binding assay was performed in the presence of 100 nM GTPγS. C, as A, except that the binding was performed in the presence of 1 mM GDP. D, as A, except that the membranes were prepared from myocytes pretreated for 20 h with pertussis toxin (100 ng/ml). The solid lines represent the best fit of the data to a two-site binding model. The binding constants for the high and low affinity sites were calculated as 1.8 (±0.0) and 36 (±22) nM. The number of high affinity binding sites was estimated at 2.7 (±0.6) × 10^11 mol of sites/mg protein for the control experiment. This number was reduced by 82 and 84% in the presence of 0.1 mM GTPγS and 1 mM GDP, respectively (B and C) and by 61% in the case of the PT-treated membranes (D). The number of low affinity binding sites calculated from these experiments was 4.5 (±4.3) × 10^11 mol of sites/mg membrane protein. This number was not significantly affected by any of the treatments shown. Data shown represent two to six independently performed experiments.

**Mechanism of insulin action.**

The mechanism of insulin action is complex and involves multiple signaling pathways. Insulin binding to its receptor on the cell surface initiates a series of events that lead to the activation of intracellular signaling molecules and ultimately affect cellular metabolism. The G-protein system plays a crucial role in this process, acting as a transducer between the extracellular signal (insulin binding) and the intracellular response.

Insulin binding to its receptor leads to the activation of the insulin receptor tyrosine kinase, which can phosphorylate various substrates on tyrosine residues. These include the insulin receptor itself, other tyrosine kinases, and non-receptor tyrosine kinases. The activated insulin receptor can then recruit and activate signaling molecules such as the G-protein coupled receptors, which are involved in various cellular functions including metabolism, proliferation, and survival.

The G-proteins are heterotrimeric proteins composed of α, β, and γ subunits. In the absence of ligand, G-proteins are bound to GDP. Ligand binding causes the dissociation of GDP, allowing for the exchange of GDP for GTP, resulting in the activation of the G-protein. This activated G-protein can then dissociate into α and βγ subunits and interact with various target proteins, leading to downstream signaling events.

In the context of insulin signaling, the G-protein system, including the Gαi, Gαs, and Gα12/13 subunits, plays a significant role in mediating the effects of insulin. Gαi subunits are involved in the inhibition of adenyl cyclase, leading to decreased cAMP levels, while Gαs subunits activate adenyl cyclase, increasing cAMP levels. Gα12/13 subunits activate small GTPases such as RhoA and Rac, which can modulate cell motility and growth.

The experiments described in the paper support the idea of a direct coupling between the insulin receptor and a G-protein system. The inhibition of insulin binding by nucleotides and PT pretreatment suggests that the interaction between insulin and its receptor is mediated by the G-protein system. The reduction in insulin binding in the presence of GDP and the increase in binding with GTPγS support the involvement of a G-protein, likely a Gαi subunit.

Overall, the findings provide strong evidence for a direct coupling between the insulin receptor and a G-protein system, which is critical for the proper insulin signaling pathway. This understanding is crucial for the development of therapeutic strategies to target insulin resistance in diseases such as diabetes.
Scatchard analysis of the data shows that this effect is consistent with the disappearance of high affinity insulin binding sites from the membrane. Since insulin binds to insulin-like growth factor-I receptors with lower affinity, our results suggest that the observed effects of guanine nucleotides and PT are mediated by the insulin receptor. These effects are typical of the interactions of G-proteins with their associated receptors.

There are several hypothetical mechanisms by which the insulin receptor may communicate with a G-protein system. The simplest is a mechanism similar to that described for receptors of the rhodopsin class. In these systems, the receptor-ligand complex interacts with the G-protein holotrimer, catalyzes the exchange of GDP bound to the a-subunit of the G-protein complex with GTP, and thus promotes the activation of effector enzyme(s) which then generate(s) a secondary message (for a review, see Gilman (26)). These systems do not require covalent modifications of any of the proteins involved in the signal transduction pathway, although the potential role of protein phosphorylation/dephosphorylation in the fine tuning of the system has been acknowledged (26). Alternatively, since the insulin-insulin receptor complex behaves as a tyrosine kinase, it is conceivable that tyrosine phosphorylation of a certain set of substrates may generate a signal that results in the activation of a G-protein system.

Our data suggest that the insulin-insulin receptor complex interacts directly with G-proteins and that the tyrosine kinase activity of the receptor may not play a significant role in the communications established between the receptor and the G-protein system. This conclusion is based primarily on the lack of effects of ATP, Mn²⁺ and nonhydrolyzable ATP analogs on the insulin-induced acceleration of the binding of GTPγS to BChI-I membranes. Additional support to this hypothesis comes from our failure to detect insulin-induced tyrosine phosphorylation of the PT-sensitive 40-kDa G, apparently involved in insulin action and from the lack of effect of PT pretreatment on the pattern of phosphorylation of endogenous substrates (including the β-subunit of the receptor) in the presence of maximal concentrations of insulin. Observations made in other laboratories support this view as well. For instance, insulin stimulates GTP hydrolysis by platelet plasma membranes in the presence of 1 mM AMP-PNP (7). These results are in marked contrast with the hypothesis that receptor kinase activity is an absolute requirement for the biochemical actions of insulin (8), including insulin mediator generation (27). These observations thus suggest the existence of two parallel pathways by which insulin communicates with the target cell: on one hand, the tyrosine kinase activity of the receptor initiates a cascade of events in which peptide phosphorylation plays a central role; on the other, direct interactions of the receptor with G-proteins lead to activation of specific effectors one of which is most likely a specific phospholipase C involved in the generation of insulin mediators (2, 12). Apparently, both pathways must be fully activated for the generation of insulin mediators as well as for many of the biochemical functions that characterize the full effects of insulin in intact cells and tissues. Whether any of the metabolic effects of insulin may be triggered independently by one of this pathways is not known.

Acknowledgments—We are grateful to Dr. A. G. Gilman and Dr. S. Mumby who supplied us with specific anti-G, antibodies, to Dr. J. T. Parsons for his gift of anti-phosphotyrosine antibodies, and to Dr. E. L. Hewlett for his gift of B. pertussis toxin.

REFERENCES
A pertussis toxin-sensitive G-protein mediates some aspects of insulin action in BC3H-1 murine myocytes.
L Luttrell, E Kilgour, J Larner and G Romero


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