Journal of Cellular Biochemistry 53:181–189 (1993)

ARTICLES

Insulin Stimulates GDP Release From G Proteins in the Rat and Human Liver Plasma Membranes

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Abstract Plasma membranes (1–2 mg protein) prepared from the livers of adult male rats and human organ donors were incubated with 0.6 μM [α-^{32}P] guanosine triphosphate (GTP) in an adenosine triphosphate (ATP)-regenerating buffer at 37°C for 1 h; during this incubation, the [^{32}P]GTP is hydrolyzed and the nucleotide that is predominantly bound to the membranes is [^{32}P] guanosine diphosphate (GDP). [^{32}P]GDP release from the liver membranes was proportional to the protein concentration and increased as a function of time. At 5 mM, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} maximally inhibited GDP release by 80–90%, whereas, 5 mM Cu^{2+} maximally stimulated the reaction by 100%. Therefore, cations were not included in the buffer used in the GDP release step. One μM Gpp(NH)p (5'-guanylylimidodiphosphate), a nonhydrolyzable analog of GTP, maximally stimulated [^{32}P]GDP release in the liver membranes by up to 30%. Although 10 nM Gpp(NH)p had no effect on GDP release, it appeared to stabilize the hormonal effect by blocking further GDP/GTP exchange.

In the rat membranes, 1–100 nM glucagon (used as a positive control) stimulated [^{32}P]GDP release by about 17% ($P < .05$); similarly, 0.1–100 nM insulin stimulated [^{32}P]GDP release by 10–13% ($P < .05$). In the human membranes, 10 pM to 100 nM insulin stimulated [^{32}P]GDP release by 7–10%. In the rat membranes, 10 nM insulin stimulated [^{32}P]GDP release by 17 and 24% at 2 and 4 min, respectively ($P < .05$); in the human membranes, 10 nM insulin stimulated [^{32}P]GDP release by about 9% at 2 and 4 min. Normal rabbit IgG (used as a control for insulin receptor antibody) by itself stimulated the GDP release by rat and human membranes. However, the stimulation of the GDP release by insulin receptor antibody was consistently higher than that observed with normal rabbit IgG. Four to 15 μg of insulin receptor antibody stimulated [^{32}P]GDP release by 12–22% ($P < .05$) and 7–14% in rat and human membranes, respectively. These results indicate that ligand binding to the insulin receptor results in a functional interaction of the receptor with a guanine nucleotide-binding transducer protein (G protein) and activation of GTP/GDP exchange.

Key words: G proteins, liver, insulin, rat, human

Guanine nucleotide-binding transducer proteins (G proteins) are a family of closely related proteins involved in the transfer of information from surface receptors to biochemical effector mechanisms in a variety of systems. G proteins are heterotrimers, consisting of a variable α subunit (39–46 kDa) and β and γ subunits (35–36 and 8 kDa, respectively). Until recently it was thought that the α subunit interacts with effector molecules such as adenylyl cyclase and phospholipase C and β and γ subunits merely regulate the levels of the free α subunits. However, recent evidence emerging from various laboratories suggest that βγ subunits directly interact with receptor as well as effector molecules. For example, purified βγ subunits associated with β_1-adrenoceptor purified from turkey erythrocyte membranes, and somatostatin receptors from rat brain and AtT-20 pituitary cells. βγ subunits enhanced the agonist-stimulated receptor phosphorylation and desensitization in the β-adrenergic and rhodopsin systems. βγ subunits stimulated the type II adenylyl cyclase; specific β subunits couple muscarinic m4 and somatostatin receptors to voltage-dependent Ca^{2+} channels in rat pituitary GH_3 cells. Moreover, βγ subunits stimulated different isoforms of phospholipase C. These results suggest that an agonist binding to its receptor can stimulate

Received February 8, 1993; accepted July 13, 1993.

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one effector pathway through the α subunit and an entirely different pathway through the βγ subunits [reviewed in Spiegel et al., 1992].

Several species of G proteins have been identified: G_s mediates stimulation of adenylyl cyclase by β-adrenergic (and other) receptors, G_i mediates inhibition of adenylyl cyclase by muscarinic (and other) receptors, and G_q regulates phospholipase C in liver and brain. Gustducin is implicated in the transduction of taste in the rat taste buds [Spiegel et al., 1992].

Several lines of evidence suggest that insulin receptor is coupled to G protein(s). Pertussis toxin, which influences the function of G_i has been used to investigate insulin receptor coupling to G proteins. Insulin inhibited pertussis toxin-induced ADP-ribosylation of G proteins in plasma membranes prepared from rat adipocytes and liver [Rothenberg and Kahn, 1988] and pertussis toxin-catalyzed ADP ribosylation of G_i in rat hepatocytes [Pyne et al., 1989]. Pretreatment of BC_3H-1 murine myocytes with pertussis toxin abolished insulin-stimulated binding of GTPγS binding to these cell membranes [Luttrell et al., 1990]. On the other hand, pertussis toxin inhibited insulin-stimulated glucose transport and oxidation in BC_3H-1 murine myocytes [Moises and Heidenreich, 1990] and glucose oxidation in rat adipocytes [Goren et al., 1985]. Pertussis toxin inhibited [^{125}I]insulin binding to rat liver membranes [Burdett et al., 1990] and adipocyte membranes [Ciaraldi and Maisel, 1989]. Glucose transport by rat adipocytes became less sensitive to insulin after pertussis toxin treatment [Ciaraldi and Maisel, 1989]. $G_{i\alpha\text{-}2}$ from hepatocytes of diabetic rats was phosphorylated to a greater extent than $G_{i\alpha\text{-}2}$ from hepatocytes of normal animals [Bushfield et al., 1990]. G_i levels decreased dramatically in hepatocyte membranes from streptozotocin-induced diabetic rats [Gawler et al., 1987], whereas $G_{s\alpha}$ levels increased in the livers of streptozotocin-diabetic or BB/Wor spontaneously diabetic rats [Lynch et al., 1989]. There was no change in $G_{i\alpha}$ levels in liver [Allard et al., 1991] and adipocyte [Green and Johnson, 1991] plasma membranes obtained from streptozotocin-induced diabetic rats. Purified α-subunits of G_i, G_o and transducin were phosphorylated by insulin receptor kinase and this process was inhibited by GTPγS, suggesting that only the inactive GDP-bound α subunits can be phosphorylated by the insulin receptor kinase [Zick et al., 1986; O'Brien et al., 1987]. GTPγS inhibited [^{125}I]insulin binding to rat adipocyte plasma membranes [Davis and McDonald, 1990], but had no effect on the hormone binding to rat liver membranes [Burdett et al., 1990].

According to the generally accepted model of G protein function, the binding of an agonist to its receptor facilitates an exchange of GTP for GDP on the α subunit. The activated αGTP subunit dissociates from the βγ subunits, and interacts with effector molecules such as adenylate cyclase or phospholipase C. An intrinsic GTPase activity of the α subunit hydrolyzes GTP to GDP, releasing inorganic phosphate (Pi); αGDP then recombines with βγ, ending the activation cycle. Therefore, if insulin receptor is coupled to a G protein, insulin binding to its receptors should be followed by a rapid increase in GDP release, GTP binding, and GTP hydrolysis. In fact, insulin stimulated G protein GTPase activity in membranes from human platelets [Gawler and Houslay, 1987] and BC_3H-1 murine myocytes [Luttrell et al., 1990]. Insulin stimulated [^{35}S]GTPγS binding to membranes from fat cell and skeletal muscle [Kellerer et al., 1991] and BC_3H-1 murine myocytes [Luttrell et al., 1990], but did not stimulate the binding to rat liver membranes [Burdett et al., 1990]. This may reflect species specificity or differences in experimental conditions.

The stimulation of GDP release by insulin has not been reported. To assess insulin receptor-G protein coupling further, we examined the effect of insulin on [^{32}P]GDP release in rat and human liver plasma membranes and report here that insulin stimulated this first step in the G protein-mediated signal transduction pathway.

MATERIALS AND METHODS

Materials

Human recombinant insulin was a gift from Eli Lilly Co. (Indianapolis, IN). Polyethyleneimine-cellulose, thin layer chromatography (TLC) plates (catalog #T-6765) were purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled guanosine-5′-triphosphate ([α-^{32}P]; 1500 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). All other chemicals were purchased from Sigma Chemical Co. Polyclonal insulin receptor antibodies (B16) raised in rabbits against rat liver insulin receptor were used [Caro et al., 1987].

Plasma Membrane Preparation From Rat and Human Livers

Liver was obtained from two human organ donors; both were males aged 20 and 24 years weighing 70 and 72 kg, respectively. Adult male Sprague-Dawley rats (125–150 g; Charles River, Wilmington, MA) were housed in pairs in an environmentally controlled room with free access to food and water. Rats were killed by decapitation and their livers removed. All the subsequent procedures were conducted at 4°C. Routinely, liver was homogenized in buffer A [5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 2 μM leupeptin, 2 μM pepstatin, 1 mM PMSF, bacitracin (100 mg/L), and aprotinin (10 units/L) at pH 7.4] using a motor-driven Teflon-glass tissue grinder. The homogenate was centrifuged at 800 × *g* for 20 min. The supernatant was maintained on ice while the pellet was resuspended in buffer A and centrifuged at 800 × *g* for 20 min. The combined supernatants were then centrifuged at 100,000 × *g* for 1 h. The pellet was resuspended in buffer A containing 0.7 M sucrose and overlaid on to a discontinuous sucrose gradient and spun at 60,000 × *g* for 1 h; the gradient consisted of 4.5 mL of 1.3 M sucrose-buffer A, 2.5 mL of 1.0 M sucrose-buffer A, and 2.5 mL of sample. After centrifugation (60,000 × *g* for 1 h), 2.5 mL from the top of each tube was carefully collected, pooled, and washed with buffer A [Hubbard et al., 1983]. Protein content was estimated using the Bradford method [1976].

Labeling of Plasma Membranes With [^{32}P]GTP

Plasma membranes (1–2 mg protein) were incubated in an ATP-regenerating buffer (pH 7.4) containing 75 mM Tris-HCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM App(NH)p, 5 mM phosphocreatine, creatine phosphokinase (50 units/mL), 50 μg bovine serum albumin, 0.1 mM EDTA, 0.2 mM EGTA, 1 mM cAMP, 100 mM NaCl, and 0.6 μM [α-^{32}P]GTP at 37°C for 1 h. The labeling step is conducted in an ATP-regenerating system and manipulation of assay conditions such that only 10–20% of added GTP is hydrolyzed. The ATP-regenerating system inhibits GTP hydrolysis by nonspecific nucleosidases and the transfer of phosphate from GTP to ATP. The membranes were diluted with 10 mL ice-cold TED buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, and 1 mM DTT at pH 7.4) and washed four times with the same buffer by centrifugation (100,000 × *g* for 30 min at 4°C). The pellet was resuspended in TED buffer and immediately used in the GDP release assay [Cassel and Selinger, 1978].

[^{32}P]GDP Release Assay

[^{32}P] labeled plasma membranes were incubated in triplicate in TED buffer in a volume of 100 μL at 37°C for 4 min. The reaction was terminated by transferring the tubes to an ice bath, followed by centrifugation at 100,000 × *g* for 30 min. An aliquot (10 μL) from the sample was spotted on TLC plates and developed with 1.5 M LiCl. Spots corresponding to GTP, GDP, and GMP (identified using standards) were cut, placed in a scintillation vial containing 6 mL Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and the radioactivity content determined using a Beckman (Palo Alto, CA) liquid scintillation counter. In some cases the TLC plates were exposed to Kodak X-ray (X-Omat AR) films for 1 h and the autoradiographs developed [Cassel and Selinger, 1978].

Replication of Results and Statistics

[^{32}P]guanosine diphosphate release from a given membrane preparation is dependent upon the bound radioactivity and agreed within a batch of membranes. Since the amount of membrane-bound radioactivity varied among different batches of membrane preparation, results are presented as the percentage of control. All experiments with rat membranes were repeated at least three times with different batches of membrane preparation and mean ± SEM of three experiments are shown. Statistical differences among various groups were determined by analysis of variance and Fisher tests using the Statview program on a Macintosh IIci computer.

Tissue from the two human organ donors was processed separately, and the results obtained with these two batches of membranes are presented as the mean of duplicate experiments.

RESULTS

Preliminary Studies

Since the conditions for the GDP release assay in the liver have not been reported in the literature, we conducted preliminary studies to standardize and validate this assay for use with rat and human liver. The incorporation of radioactivity by the liver membranes increased with

increasing concentrations of [α-^{32}P]GTP present in the reaction mixture (Fig. 1). Since maximum incorporation was observed between 50–100 μCi [α-^{32}P]GTP, we used 100 μCi [α-^{32}P]GTP to label liver membranes (rat and human) in all the subsequent experiments. Incubation of liver membranes with [α-^{32}P]GTP in an ATP-regenerating buffer results in the hydrolysis of GTP by G proteins, and, therefore, the nucleotide that is predominantly bound to the membranes after the labeling step is GDP. Various amounts of [^{32}P] labeled rat liver membranes (10–80 μg) were incubated with 1 μM Gpp(NH)p, a nonhydrolyzable analog of GTP, and the release of GMP, GDP, and GTP monitored by TLC and autoradiography. A good separation of the nucleotides was observed (as revealed by TLC) up to 40 μg; beyond this protein concentration the system appears to be overwhelmed and the separation is not as good. The results obtained using autoradiography clearly demonstrated that the nucleotide that is predominantly bound to the membranes after the labeling step is GDP (Fig. 2). In another experiment, [^{32}P] labeled membranes were incubated with 1 μM Gpp(NH)p and it was observed that GMP, GDP, and GTP were 10, 80, and 10%, respectively, of the total radioactivity released by the rat membranes; similarly, with the human membranes GMP, GDP, and GTP were 3, 71, and 26%, respectively (data not shown). The amount of [^{32}P]GDP released was linear up to 40 μg of rat liver membrane protein; routinely, 10–30 μg protein per tube were used in the GDP release assay (Fig. 3). The effect of divalent cations on the GDP release process was next examined and it was observed that at 5 mM, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} maximally inhibited GDP release by 80–90%, whereas 5 mM Cu^{2+} maximally stimulated the reaction by 100% (data not shown). Therefore, cations were not included in the buffer used in the GDP release step.

Insulin Stimulation of [^{32}P]GDP Release

Hormone binding to its receptor results in receptor-G protein interaction and the subsequent release of GDP from the G protein. Since the affinity of Gpp(NH)p for G proteins is greater than that of GDP, the vacant nucleotide site (as a result of hormone-induced GDP release) is rapidly occupied by Gpp(NH)p and further GDP/GTP exchange is blocked. Therefore, GDP release was monitored in the presence of different concentrations of Gpp(NH)p. Gpp(NH)p (1 μM)

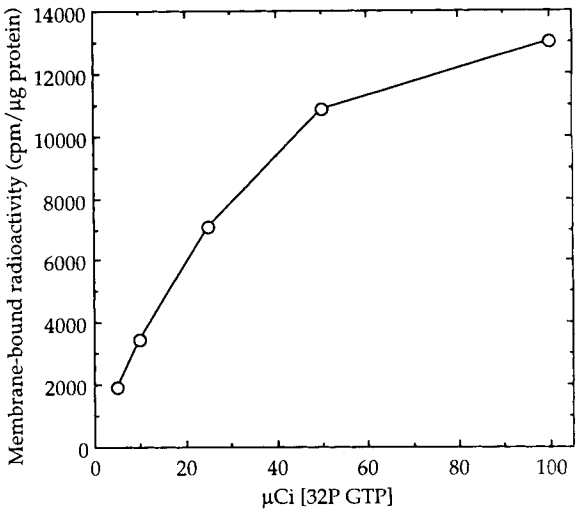

Fig. 1. Incorporation of radioactivity by rat liver membranes. One milligram of membrane protein was incubated with various amounts of [^{32}P]GTP for 1 h at 37°C. Then the membranes were washed four times and membrane-bound radioactivity was determined. Each point represents the mean of two determinations.

stimulated [^{32}P]GDP release by 30% in rat liver membranes (Fig. 4) ($P < .05$). When the effect of insulin or glucagon (used as a positive control) on [^{32}P]GDP release was examined in the presence of 1 μM Gpp(NH)p, a potentiation in the presence of both insulin (or glucagon) and Gpp(NH)p did not occur (data not shown). As these results did not allow us to evaluate the extent of the hormone-stimulated GDP release, experiments were conducted with insulin or glucagon in the absence of Gpp(NH)p. In the absence of Gpp(NH)p insulin or glucagon stimulation of [^{32}P]GDP release was not observed in rat membranes (Fig. 5). When 10 nM Gpp(NH)p, which by itself did not stimulate [^{32}P]GDP release, was included in the reaction mixture, consistent stimulation with glucagon or insulin was observed (Fig. 5) ($P < .05$); similar results were obtained with human liver membranes (data not shown). Figures 6 and 7 show the glucagon and insulin dose-response curves in the presence of 10 nM Gpp(NH)p. In the rat membranes, 1–100 nM glucagon stimulated [^{32}P]GDP release by about 17% (Fig. 6) ($P < .05$); 0.1–100 nM insulin stimulated [^{32}P]GDP release by 10–13% (Fig. 7) ($P < .05$). In the human membranes, 1–100 nM insulin stimulated [^{32}P]GDP release by about 10% (Fig. 7). Therefore, although at 10 nM Gpp(NH)p had no effect on GDP release (Figs. 4 and 5), it appeared to stabilize the hormonal effect. A time course study

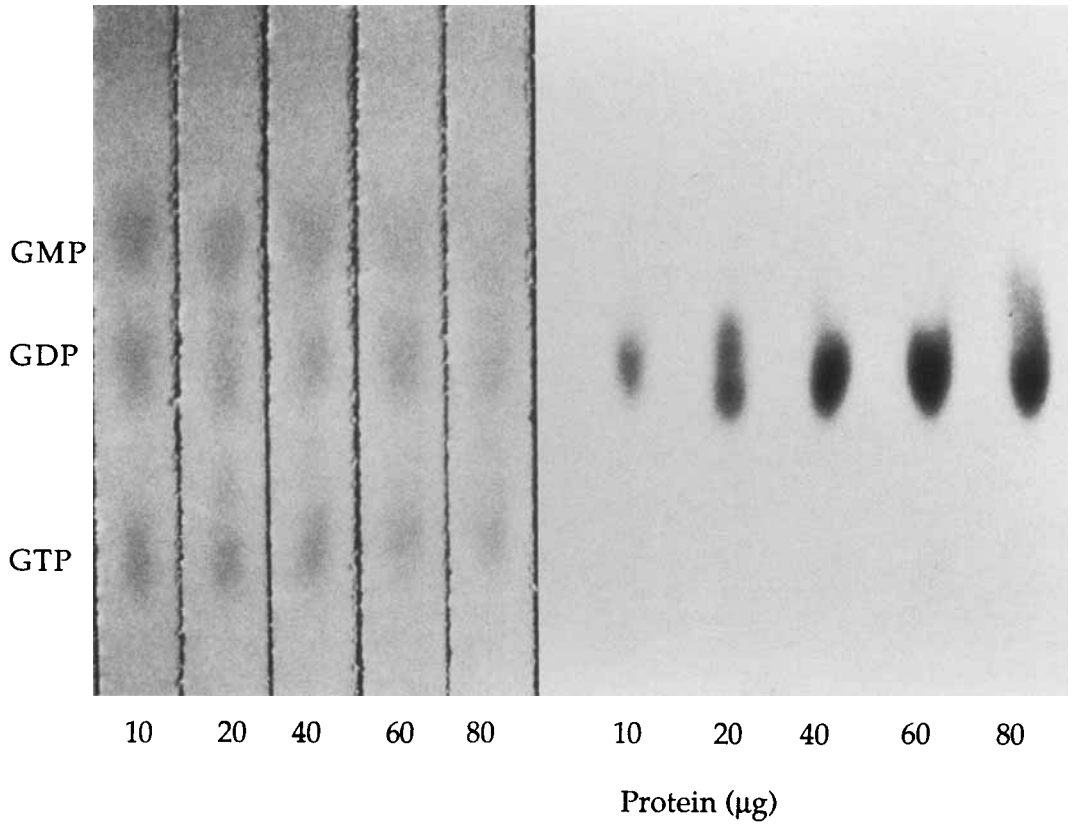

Fig. 2. Identification of nucleotides by TLC (left panel) and determination of labeling pattern by autoradiography (right panel). Various amounts of [^{32}P] labeled rat liver membranes were incubated with 1 μM Gpp(NH)p in Tris-HCl buffer (pH 7.4) for 4 min at 37°C and release of GMP, GDP, and GTP was monitored using TLC. After visualizing the nucleotides, the chromatogram was processed for autoradiography.

indicated that in the rat membranes, 10 nM insulin stimulated [^{32}P]GDP release by 17 and 24% at 2 and 4 min, respectively (Fig. 8) ($P < .05$); in the human membranes, 10 nM insulin stimulated [^{32}P]GDP release by about 9% at 2 and 4 min (Fig. 8). Routinely, GDP release experiments were conducted at 4 min. Finally, in order to demonstrate that the insulin effect was due to interaction with insulin receptor, we evaluated the effect of an insulin receptor antibody that mimics insulin action in rat and human liver. Normal rabbit IgG (used as a control for insulin receptor antibody) by itself stimulated the GDP release by about 10–20% in rat and human membranes (data not shown). However, the stimulation of the GDP release by insulin receptor antibody was consistently higher than that obtained with normal rabbit IgG. Four to 15 μg of anti-insulin receptor antibody stimulated [^{32}P]GDP release by 12–22% and 7–14% in rat and human membranes, respectively (Fig. 9).

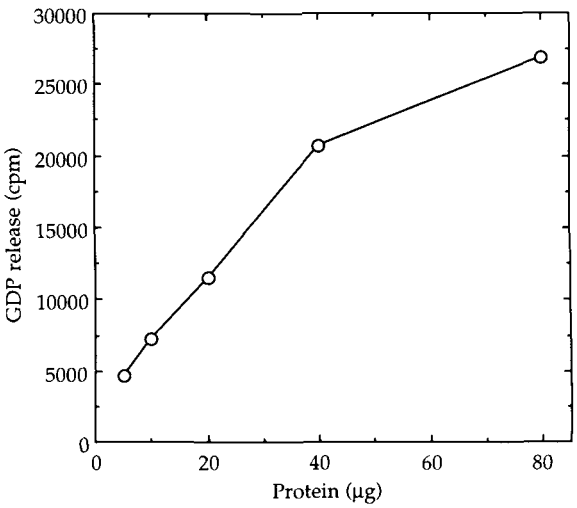

Fig. 3. Effect of protein concentration on [^{32}P]GDP release. Various amounts of labeled rat liver membranes were incubated with 1 μM Gpp(NH)p in Tris-HCl buffer (pH 7.4) for 4 min at 37°C and [^{32}P]GDP release was determined by TLC. Each point represents the mean of three determinations from a single batch of membrane preparation.

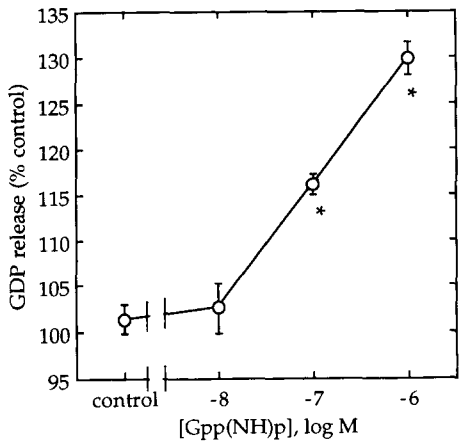

Fig. 4. Effect of Gpp(NH)p on [^{32}P]GDP release by rat liver membranes. Labeled membranes (10 μg protein) were incubated with various concentrations of Gpp(NH)p in Tris-HCl buffer (pH 7.4) for 4 min at 37°C and [^{32}P]GDP release was determined by TLC. Each point represents the mean ± SEM of three experiments using three different batches of membrane preparations; each experiment was conducted in triplicate. *Values significantly different from control level ($P < .05$).

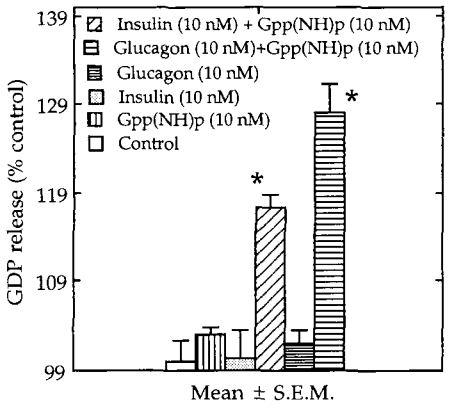

Fig. 5. Effect of Gpp(NH)p and hormones on [^{32}P]GDP release by rat liver membranes. Ten microgram labeled membranes were incubated, as indicated, in Tris-HCl buffer (pH 7.4) for 4 min at 37°C and [^{32}P]GDP release was determined by TLC. Each point represents the mean ± SEM of three experiments using three different batches of membrane preparations; each experiment was conducted in triplicate. *Values significantly different from control level ($P < .05$).

DISCUSSION

In the present study, the observation that binding of insulin to rat and human liver plasma membranes stimulates [^{32}P]GDP release indicates that a functional interaction exists between the insulin receptor and G protein(s). The stimulation of the GDP release ranged between 13–24% in the rat and 9–10% in the human organ donors. The modest amount of stimulation of [^{32}P]GDP release by physiological concentrations of insulin is consistent with our knowledge of other G protein-coupled receptor systems. For example, gonadotropin-releasing hormone (GnRH) and thyrotropin-releasing hormone

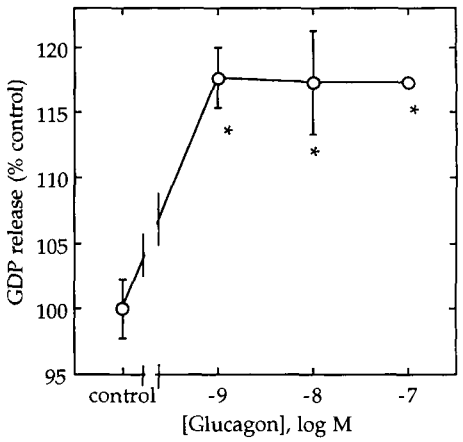

Fig. 6. Effect of glucagon on [^{32}P]GDP release by rat liver membranes. Ten microgram labeled membranes were incubated with various concentrations of glucagon in the presence of 10 nM Gpp(NH)p in Tris-HCl buffer (pH 7.4) for 4 min at 37°C and [^{32}P]GDP release was determined by TLC. Each point represents the mean ± SEM of three experiments using three different batches of membrane preparations; each experiment was conducted in triplicate. *Values significantly different from control level ($P < .05$). Where not shown, the error bar is smaller than the symbol representing the mean.

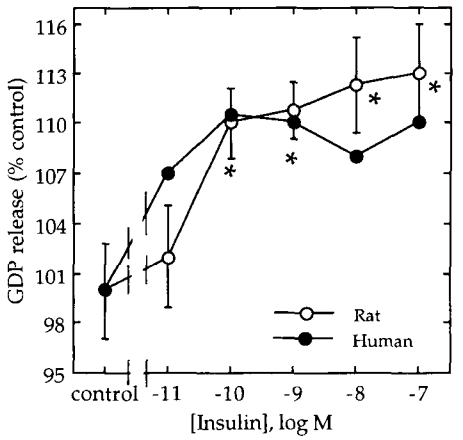

Fig. 7. Effect of insulin on [^{32}P]GDP release by rat and human liver membranes. Labeled membranes (rat—10 μg protein; human—13 μg protein) were incubated with various concentrations of insulin in the presence of 10 nM Gpp(NH)p in Tris-HCl buffer (pH 7.4) for 4 min at 37°C and [^{32}P]GDP release was determined by TLC. For rat membranes, each point represents the mean ± SEM of three experiments using three different batches of membrane preparations; each experiment was conducted in triplicate. *Values significantly different from control level ($P < .05$). For human membranes, each point represents the mean of two experiments using two different membrane preparations; each experiment was conducted in triplicate.

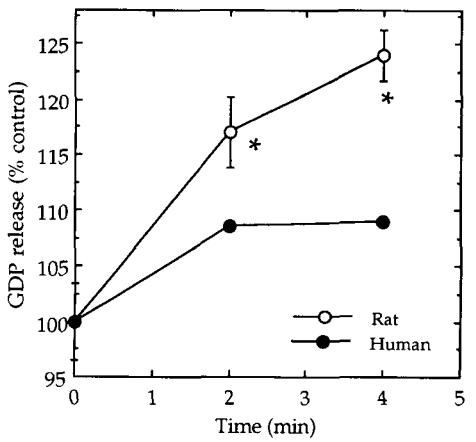

Fig. 8. Time course of insulin effect on [^{32}P]GDP release by rat liver membranes. Labeled membranes (rat—10 μg protein; human—13 μg protein) were incubated with 10 nM insulin in the presence of 10 nM Gpp(NH)p in Tris-HCl buffer (pH 7.4) at 37°C for the indicated periods of time and [^{32}P]GDP release was determined by TLC. For rat membranes, each point represents the mean ± SEM of three experiments using three different batches of membrane preparations; each experiment was conducted in triplicate. *Values significantly different from control level ($P < .05$). For human membranes, each point represents the mean of two experiments using two different membrane preparations; each experiment was conducted in triplicate.

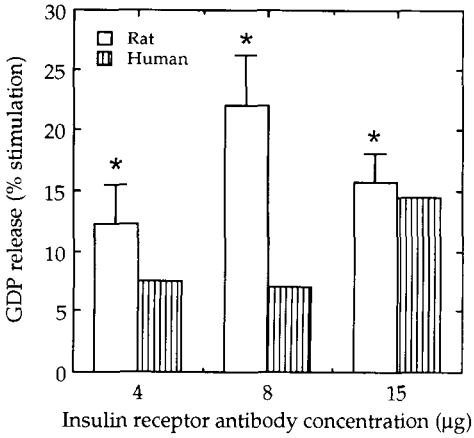

Fig. 9. Effect of insulin receptor antibody on [^{32}P]GDP release by rat and human liver membranes. Labeled membranes (10 and 13 μg protein of rat and human, respectively) were incubated with various concentrations of either normal rabbit IgG (a control for insulin receptor antibody) or insulin receptor antibody in Tris-HCl buffer (pH 7.4) at 37°C for 4 min and [^{32}P]GDP release was determined by TLC. Data obtained with rat and human membranes were calculated as the percentage of control values and expressed as the percentage of stimulation. For rat membranes, each point represents the mean ± SEM of three experiments using three different batches of membrane preparations; each experiment was conducted in triplicate. *Values significantly different from control level ($P < .05$). For human membranes, each point represents the mean of two experiments using two different membrane preparations; each experiment was conducted in triplicate.

(TRH) stimulated [^{32}P]GDP release from G protein in rat pituitary membranes by 15–40% [Ravindra and Aronstam, 1992]. GnRH and TRH stimulated [^{35}S]GTPγS binding to rat pituitary membranes by 25–35% and G protein GTPase activity by 40–50% [Ravindra and Aronstam, 1990a]. TRH stimulated G protein GTPase activity by 27% in GH_3 cells [Wojcikiewicz et al., 1986], and 10–50% in GH_4C_1 cells [Hinkle and Phillips, 1984]. In rat striatal membranes, acetylcholine stimulated [^{35}S]GTPγS binding by up to 45% [Ravindra and Aronstam, 1991a] and GTPase activity by about 50% [Ravindra and Aronstam, 1990b]. Insulin stimulated G protein GTPase activity by 30–40% in BC_3H-1 myocytes [Lutrell et al., 1990] and 60% in human platelets [Gawler and Houslay, 1987]. Insulin stimulated [^{35}S]GTPγS binding in fat cell and skeletal muscles by 50% [Kellerer et al., 1991] and 20% BC_3H-1 myocytes [Lutrell et al., 1990].

The conclusion that insulin receptor is functionally coupled to a G protein is further supported by the observation that insulin receptor antibody mimicked the effect of insulin on [^{32}P]GDP release in rat and human liver membranes. This antibody (B16) was observed to mimic insulin action in the rat and human liver (Caro, unpublished observations). The magnitude of the antibody effect was small and similar to that observed with insulin. Our observation that normal rabbit IgG stimulated the GDP release is similar to the effect of normal rabbit serum on low K_m GTPase activity in rat striatal membranes [Ravindra and Aronstam, 1990b]; it is not clear why normal rabbit IgG influences the GDP release process.

The time course of insulin effect on GDP release in rat and human liver membranes appears to compare well with other systems. For example, isoproterenol stimulation of GDP release in rat adipocytes was seen by 1 min, although maximal effects of the agonist were observed at 7 min [Murayama and Ui, 1984]. Isoproterenol stimulation of GDP release in turkey erythrocytes was also demonstrated between 1 and 2 min [Cassel and Selinger, 1978; Pike and Lefkowitz, 1981]. GnRH and TRH stimulation of GDP release in rat pituitary was evident by 1 min [Ravindra and Aronstam, 1992].

Since glucagon receptors are coupled to a G protein, we used glucagon as a positive control in the present study. Glucagon stimulated GDP release by rat liver membranes in a dose-depen-

dent manner, confirming previous observations in rat adipocyte membranes [Murayama and Ui, 1984].

Generally, the receptors known to couple to G proteins are made up of a single polypeptide chain that spans the plasma membrane seven times with the cytoplasmic loop between transmembrane regions 6 and 7 representing the G protein binding domain [Dohlman et al., 1991]. However, it has recently been demonstrated that IGF receptor is an exception to this in that, although this receptor does not span the plasma membrane seven times, it can still couple to G proteins [Okamoto and Nishimoto, 1991; Nishimoto et al., 1991]. Similarly, a possibility exists that insulin receptor is coupled to a G protein.

In turkey erythrocytes [Cassel and Selinger, 1978], frog erythrocytes [Pike and Lefkowitz, 1981], and rat adipocytes [Murayama and Ui, 1984], isoproterenol-stimulated [^{3}H]GDP release was potentiated by Gpp(NH)p. Similarly, in rod outer segment membranes, light-induced [^{3}H]GDP release was potentiated by GTP [Fung and Stryer, 1980]. In contrast, potentiation by Gpp(NH)p of hormone-stimulated [^{32}P]GDP release was not observed in liver membranes. The reason for this difference is not clear.

In previous studies, micromolar concentrations of agonist were included during the labeling of membranes with [^{3}H]GTP, resulting in an *increased amount* of [^{3}H]GDP that could be released in the second step of the assay [Cassel and Selinger, 1978; Pike and Lefkowitz, 1981; Murayama and Ui, 1984]. When insulin or glucagon was included in the labeling step, the amount of [^{32}P]GDP released was about 15% less compared to the membranes not exposed to either of the hormones. Moreover, the membranes were desensitized to subsequent exposure of hormones during the GDP release step (data not shown). Therefore, liver membranes were routinely labeled with [α-^{32}P]GTP in the absence of insulin. As a result, membranes were not refractory to hormones during GDP release step and a statistically significant stimulation was observed at 1 nM insulin or glucagon.

G_{ir} (66 kDa) (a G protein) coupled to the insulin receptor has recently been isolated from human placenta. GTPγS binding to G_{ir} and its phosphorylation was stimulated by insulin; GTPγS inhibited insulin binding to insulin receptor-G_{ir} complex. Anti-G_{ir} antibodies cross-reacted with $G_{s\alpha}$ and $G_{i\alpha}$ [Srivastava and Singh, 1990; Varma et al., 1992]. In rat adipocytes and human placenta two G proteins (41 and 67 kDa) that interact with insulin receptor have been identified and partially purified [Jo et al., 1992]. Future work should reveal if these novel G proteins can be demonstrated in other insulin-sensitive tissues.

Although it is known that GTP-GDP exchange by G proteins is important in signal transduction, the regulation of these initial biochemical steps is poorly understood. Recent work suggests that other GTP binding proteins, especially tubulin, might modulate the GTP-GDP cycle of G proteins in the muscarinic receptor system in the rat striatum [Ravindra and Aronstam, 1990b, 1991b] and GnRH/TRH receptor system in the rat anterior pituitary lobe [Ravindra and Aronstam, 1993]. Given the ubiquitous and highly conserved nature of both G proteins and tubulin, similar regulatory mechanisms may be operating in other systems, including the insulin receptor.

In summary, we demonstrated that insulin stimulates [^{32}P]GDP release in plasma membrane fraction from the rat and human livers. These results indicate an interaction between G protein(s) and insulin receptors. Future work should reveal if this interaction is direct or if other steps (such as influencing GTP hydrolysis via membrane-associated nucleotide phosphatase or a GTP-utilizing protein kinase) are involved in this process.

ACKNOWLEDGMENTS

This work was supported by USPHS grant RO1-DK-345592.

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