Insulin receptor function is inhibited by guanosine 5'-[γ -thio]triphosphate (GTP[S])

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The regulatory role of GTP-binding proteins (G-proteins) in insulin receptor function was investigated using isolated insulin receptors and plasma membranes from rat adipocytes. Treatment of isolated insulin receptors with 1 mM-guanosine 5'-[γ -thio]triphosphate (GTP[S]) inhibited insulin-stimulated phosphorylation of the β -subunit, histone Hf2b and poly(GluNa₄,Tyr.) by 22 %, 65 % and 65 % respectively. Phosphorylation of calmodulin by the insulin receptor kinase was also inhibited by 1 mM-GTP[S] both in the absence (by 88 %) and in the presence (by 81 %) of insulin. In the absence of insulin, 1 mM-GTP had the same effect on calmodulin phosphorylation as 1 mM-GTP[S]. However, when insulin was present, GTP was less effective than GTP[S] (41 % versus 81 % inhibition). Concentrations of GTP[S] greater than 250 μ M are necessary to inhibit phosphorylation. Although these concentrations are relatively high, the effect of GTP[S] is not due to competition with [³²P]ATP for the insulin receptor kinase since (1) other nucleotide triphosphates did not inhibit phosphorylation as much as did GTP[S] (or GTP) and (2) the V_{max} of the ATP-dependent kinase reaction was decreased in the presence of GTP[S]. GTP[S] (1 mM) also inhibited insulin binding to isolated receptors and plasma membranes, by 80 % and 50 % respectively. Finally, an antibody raised to a peptide sequence common to the α -subunits of G-proteins G_s, G_i, G_o and transducin detected G-proteins in plasma membranes but failed to detect them in the insulin receptor preparation. These results indicate that GTP inhibits insulin receptor function, but does so through a mechanism that does not require a conventional GTP-binding protein.

INTRODUCTION

Although the precise mechanism of the transduction of the insulin signal is not known, it is now well established that the insulin receptor contains a tyrosine kinase [1]. Binding of insulin to its receptor results in autophosphorylation of the β -subunit of the receptor and subsequent phosphorylation of numerous endogenous proteins [2,3]. Phosphorylation of target proteins is likely to play an important role in the mechanism by which insulin regulates their activity and function.

Compelling evidence has accumulated over the past 5 years suggesting that at least some of the actions of insulin are mediated through GTP-binding regulatory proteins (G-proteins). Physiological concentrations of insulin inhibit adenylate cyclase, but only in the presence GTP and Mg²⁺ [4]. In addition, Heyworth & Houslay [4] have suggested that insulin inhibits adenylate cyclase by stimulating an inhibitory G-protein which is different than G_i. These researchers have also demonstrated that G_i is decreased in insulin-resistant diabetes [5].

Pertussis and cholera toxins have been widely used as indirect probes for investigating the roles of G-proteins in signal transduction pathways. Before 1984, three groups of researchers [6–8] demonstrated that cholera toxin, which ADP-ribosylates primarily the stimulatory G-protein G_s, catalyses the ribosylation of putative G-proteins in isolated adipocyte plasma membranes. More recently, cholera toxin has been shown to enhance insulin-stimulated protein synthesis [9]. On the other hand, insulin has been shown to attenuate cholera toxin stimulation of adenylate cyclase [10]. There is also evidence that the insulin receptor interacts with a cholera toxin-sensitive G-protein which has tentatively been identified as having a 25 kDa α -subunit [11].

Pertussis toxin, which ADP-ribosylates G_i and G_o, has been used more extensively to study the involvement of G-proteins in insulin action. This toxin has been shown to inhibit many insulin-stimulated events such as activation of cyclic AMP phosphodiesterase [12], glucose oxidation [13], anti-lipolytic activity [13], protein synthesis [9], hexose transport [14], thymidine uptake [14] and hydrolysis of a recently discovered putative insulin second messenger precursor, phosphatidylinositol glycan [14]. Pertussis toxin also blocks the ability of insulin to inhibit adenylate cyclase [12]. On the other hand, insulin inhibits pertussis toxin-catalysed ADP-ribosylation of G_i [15].

The theory that G-proteins are involved in insulin action is further supported by the observation that the insulin receptor kinase can phosphorylate purified α -subunits of G_i, G_o and transducin [16–18]. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) inhibits the phosphorylation of the G-proteins, possibly by displacing GDP, which suggests that only the inactive GDP-bound α -subunits can be phosphorylated by the insulin receptor kinase. Recently, O'Brien *et al.* [19] have shown that insulin increases the phosphorylation, on tyrosine residues, of a 60 kDa protein which binds to GDP-agarose and which can be eluted by GDP. Schürmann *et al.* [20] have shown that GTP and GTP[S] can inhibit the reconstitution of glucose transport activity in membranes from insulin-treated adipocytes, and Burdett *et al.* [21] have shown that GTP[S], when added to electroporabilized cells, can inhibit the insulin-stimulated phosphorylation of a 160 kDa protein. Those authors found no effect of GTP[S] on insulin binding to hepatic plasma membranes. In the present report, we provide evidence that GTP[S] can interact directly with the insulin receptor to inhibit insulin-stimulated phosphorylation.

Abbreviations used: G-protein, guanine-nucleotide-binding regulatory protein; G_s, a G-protein that activates adenylate cyclase; G_i, a G-protein that inhibits adenylate cyclase; G_o, a G-protein of unknown function; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; IC₅₀, concn. causing 50 % inhibition.

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MATERIALS AND METHODS

Materials

Poly-L-lysine (30–70 kDa), wheat-germ lectin–Sephacrose 6MB, insulin–agarose, poly(GluNa₄Tyr₁), GTP[S] and all other nucleotides were obtained from Sigma (St. Louis, MO, U.S.A.). Histone Hf2b was purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.). Phosphocellulose (P81) filter paper was from Whatman. Unlabelled insulin was obtained from Eli Lilly (Indianapolis, IN, U.S.A.) and ¹²⁵I-labelled insulin was obtained from the Washington University Diabetes Research and Training Center (St. Louis, MO, U.S.A.). Ca²⁺-free porcine brain calmodulin was purchased from Ocean Biologics, Inc. (Edmonds, WA, U.S.A.). Protosol, Aquasol II, [γ -³²P]ATP, [α -³²P]GTP and [³⁵S]GTP[S] were obtained from DuPont–New England Nuclear (Boston, MA, U.S.A.).

Preparation of adipocyte plasma membranes and insulin receptors

The epididymal fat pads of male Sprague–Dawley rats (120–150 g; Sasco Inc., O'Fallon, MO, U.S.A.) were removed and adipocytes were isolated by the method of Rodbell [22]. Adipocyte plasma membranes were prepared by the method of Jarett [23], and insulin receptors were partially purified by wheat-germ lectin chromatography [24]. Insulin receptors prepared from rat livers, as described by Sacks & McDonald [25], were used in some experiments.

Protein phosphorylation assays

Unless otherwise stated, adipocyte plasma membranes (25 μ l, 25–30 μ g) or partially purified insulin receptors (25 μ l, 5–6 μ g) were preincubated in a final volume of 250 μ l in 50 mM-Tris/HCl (pH 7.5), 0.1 % (v/v) Triton X-100, 1 mM-EGTA, 0.8 mM-CaCl₂, 0.001 % (w/v) BSA, 1.2 μ M-calmodulin and 0.24 μ M-poly-L-lysine for 30 min at 22 °C. In some experiments, 67 nM-insulin was added. Other additions are indicated in the Figure legends. The reaction was then initiated by the addition of 250 μ M-ATP in 2 mM-MgCl₂ containing 10–30 μ Ci of [γ -³²P]ATP as a tracer. Histone phosphorylations were conducted under the same conditions except that calmodulin and poly-L-lysine were omitted and 1.8 μ M-histone Hf2b was added. Following incubation for 60 min (unless otherwise stated) at 30 °C, the reactions were stopped by adding 250 μ l of 10 % (w/v) trichloroacetic acid at 4 °C. The samples were centrifuged at 37000 *g* for 10 min at 4 °C, the supernatant was aspirated and solubilization buffer, containing 20 mM-Tris/HCl (pH 7.5), 2 % (w/v) SDS, 2 % (v/v) β -mercaptoethanol, 0.01 % (w/v) Bromophenol Blue, 0.25 M-sucrose and 2 mM-EDTA, was added to the pellets. The pH was then neutralized with NaOH. The samples were mixed, heated at 100 °C for 5 min and applied to the gels. Poly(GluNa₄Tyr₁) was phosphorylated in a system similar to that for calmodulin but in the absence of polybasic proteins. We used the method described by Sahal & Fujita-Yamaguchi [26] using P81 paper.

Gel electrophoresis and autoradiography

Proteins were separated by polyacrylamide-gel electrophoresis as described by Laemmli [27] on 10 % separating gels with 6 % stacking gels. Following staining with Coomassie Brilliant Blue solution and destaining, the gels were dried on filter paper and exposed at –70 °C to Kodak X-Omat AR film with DuPont Cronex Lightening Plus intensifying screens for autoradiography. Incorporation of ³²P into proteins was determined by cutting the appropriate protein band from the gel, solubilizing it in water/Protosol/Aquasol II (1:2:10, by vol.) and quantifying the radioactivity by liquid scintillation spectrometry. Background

radioactivity was determined by cutting out a similar-sized portion of the gel which did not contain enhanced radioactivity.

Insulin binding assays

Insulin binding to adipocyte plasma membranes (25 μ l) and partially purified insulin receptors (50 μ l) was quantified by using a modification of the method of Cuatrecasas [28] as described by Wong *et al.* [29]. Briefly, the assay conditions were the same as for the calmodulin phosphorylation experiments, except that 10 μ M unlabelled ATP and various concentrations of insulin with ¹²⁵I-insulin as a tracer were used. The final concentration of BSA was also increased to 0.1 % (w/v). The samples were preincubated for 3 min at 30 °C, then the plasma membranes or insulin receptors were added to initiate the reaction. After 60 min at 30 °C the reactions were terminated and the proteins were precipitated with poly(ethylene glycol). Following centrifugation, the supernatants were aspirated and the pellets were washed with poly(ethylene glycol). The samples were again centrifuged and the supernatants were aspirated. The pellets were then counted for radioactivity in a γ -radiation counter. Excess unlabelled insulin (1.34 μ M) was added to corresponding tubes to determine non-specific binding. The difference between the ¹²⁵I-insulin bound in the absence and presence of excess unlabelled insulin was defined as specific insulin binding to receptors. All assays were performed in triplicate.

Other methods

Protein concentration was determined by the method of Lowry *et al.* [30]. Immunoblotting was conducted as described by Mumby *et al.* [31] with a primary antibody which recognizes an epitope common to G_s, G_i, G_o and transducin (antibody J881). This antibody was kindly provided by Dr. Alfred Gilman and Dr. Suzanne Mumby of the University of Texas Southwestern Medical School, Dallas, TX, U.S.A. Detection of low-molecular-mass GTP-binding proteins with [α -³²P]GTP was conducted as described by Bhullar & Haslam [32].

RESULTS

Effects of GTP[S] on insulin receptor-dependent phosphorylation of the β -subunit and of histone

Since transduction of the insulin signal appears to proceed via autophosphorylation of the β -subunit of the insulin receptor and subsequent phosphorylation of other proteins, we have examined the effect of the slowly hydrolysable GTP analogue GTP[S] on insulin receptor-dependent protein phosphorylations. Initially we examined autophosphorylation of the β -subunit and phosphorylation of the commonly used exogenous substrate histone Hf2b. As shown in Fig. 1 (lanes 1 and 2), GTP[S] inhibited both β -subunit autophosphorylation and subsequent histone phosphorylation in the absence of insulin. When insulin was present (lanes 3 and 4), GTP[S] markedly inhibited histone phosphorylation (64.9 ± 5.1 %; $n = 3$) but inhibited autophosphorylation of the β -subunit by considerably less (by 22.3 ± 10.7 %; $n = 3$). We have seen comparable results with β -subunit autophosphorylation in the absence of any exogenous substrates (results not shown). Occasionally, an unidentified protein with a molecular mass of approx. 26 kDa was observed (arrow on right side of Figure). The phosphorylation of this protein appeared to be enhanced by insulin and GTP[S] (Fig. 1, lanes 3 and 4); however, this was not a consistent observation.

Presumably histone was moderately phosphorylated in the absence of insulin (lanes 1 and 2) due to partial insulin-independent preactivation of the insulin receptor kinase during preparation of receptors. This is supported by the small amount of β -subunit phosphorylation observed in lane 1. The apparent

Fig. 1. Inhibition of insulin receptor-dependent β -subunit autophosphorylation and histone Hf2b phosphorylation by GTP[S]

Partially purified rat adipocyte insulin receptors were incubated with histone Hf2b (1.8 μ M) for 60 min as described in the Materials and methods section. In the lanes indicated, 1 mM-GTP[S] and/or 67 nM-insulin was added. Following SDS/PAGE, the gels were dried and exposed to Kodak X-Omat film for approx. 22 h. The arrows on the left side of the Figure indicate the histone (His) and the β -subunit (β) bands. The arrow on the right side of the Figure indicates an unidentified protein (discussed in the text). Shown is a representative autoradiogram from one of three similar experiments. The bands corresponding to the β -subunit and histone were excised from the gel and the radioactivity was quantified by liquid scintillation counting.

Fig. 2. Inhibition of insulin receptor-dependent calmodulin (CaM) phosphorylation by GTP and GTP[S]

Partially purified rat adipocyte insulin receptors were incubated with calmodulin for 60 min as described in the Materials and methods section. Where indicated, 1 mM-GTP or -GTP[S] and/or 67 nM-insulin was added. Following SDS/PAGE and autoradiography the calmodulin band was excised from the gel and counted for radioactivity. A representative autoradiogram is shown.

discrepancy between the inhibitory effects that GTP[S] has on insulin-stimulated histone phosphorylation (65%, compare lanes 3 and 4) and on β -subunit phosphorylation (22%) suggests that GTP[S] may be acting at two levels. The first level is at the insulin-receptor interaction (leading to a partial decrease in insulin-stimulated receptor autophosphorylation) and the second is at the substrate or the substrate-receptor coupling step (resulting in further inhibition of substrate phosphorylation). We have further explored these possibilities by characterizing the effects of GTP[S] on both basal and insulin-stimulated phosphorylation of other receptor substrates, calmodulin and poly(GluNa₄,Tyr₁), and on insulin binding.

Effects of GTP[S] and GTP on calmodulin phosphorylation by the insulin receptor

We are interested in the insulin receptor-catalysed phosphorylation of calmodulin, a Ca²⁺-binding protein, so we examined the effect of GTP[S] on calmodulin phosphorylation. Calmodulin has been reported to be phosphorylated by the insulin receptor in the intact rat adipocyte [2], although this is controversial [33]. Calmodulin is phosphorylated *in vitro* using isolated receptors obtained from rat adipocytes [24,34], skeletal muscle [35], liver [25] and human placenta [36,37]. When using these receptor preparations, a basic protein (i.e. poly-L-lysine) is required for the phosphorylation of calmodulin by the insulin receptor kinase [25]. Histone can be phosphorylated without a cofactor and may also be used as a basic protein cofactor for calmodulin phosphorylation. The mechanism by which these

basic proteins function to couple the receptor to calmodulin phosphorylation is unknown. However, the effect of poly-L-lysine (and presumably other basic proteins) appears to be mediated, at least partially, by its ability to bind both the insulin receptor [37,38] and calmodulin [36]. The results shown in Fig. 2 demonstrate that 1 mM-GTP[S] markedly inhibited calmodulin phosphorylation catalysed by adipocyte insulin receptors, both in the absence (by $88.3 \pm 2.3\%$; compare lanes 1 and 3) and in the presence (by $80.6 \pm 1.8\%$; compare lanes 4 and 6) of insulin ($n = 8$). Insulin stimulated calmodulin phosphorylation by $191.0 \pm 17.5\%$.

In the absence of insulin, GTP had nearly the same effect as GTP[S] on insulin receptor-induced phosphorylation ($79.7 \pm 8.2\%$ inhibition; compare lanes 1 and 2). However, when insulin was present, the effect of GTP was diminished ($41.0 \pm 27.8\%$ inhibition; compare lanes 4 and 5; $n = 4$) compared with that of GTP[S] ($80.6 \pm 1.8\%$ inhibition, lane 6). This may be due to the presence of an insulin-sensitive GTPase in the receptor preparation which decreases the concentration of GTP in the incubations. Indeed, we have preliminary evidence that a high- K_m ($> 100 \mu$ M-GTP) insulin-sensitive GTPase is present in the adipocyte insulin receptor preparation (H. Jo, H. Davis & J. McDonald, unpublished work). In the absence of insulin, calmodulin is phosphorylated primarily on tyrosine residues by poly-L-lysine activation of the insulin receptor kinase [25]. In contrast, in the presence of insulin, calmodulin is phosphorylated by insulin receptors which are activated by both insulin and poly-L-lysine. Nevertheless, we have shown that either in the presence or absence of insulin the inhibition of calmodulin phosphorylation by GTP[S] is 80% or greater.

Calmodulin was also phosphorylated by enriched insulin receptors prepared from rat livers by wheat-germ lectin chromatography. The effect of GTP[S] on calmodulin phosphorylation using these receptors was similar to that obtained with the adipocyte receptors. The inhibition of liver receptor-catalysed calmodulin phosphorylation by GTP[S] was 90% in the absence of insulin and 94% in the presence of insulin (results not shown).

Fig. 3. Effect of GTP[S] concentration on insulin receptor-dependent phosphorylation of calmodulin

Insulin receptors were incubated as described in Fig. 2 in the presence of 67 nM-insulin and various concentrations of GTP[S].

The concentration of GTP or GTP[S] (1 mM) used in these studies was 1–2 orders of magnitude higher than the concentrations generally used for G-protein assays by other investigators. The requirement of these relatively high concentrations of GTP[S] was confirmed by examining the effect of increasing concentrations of GTP[S] on insulin receptor kinase-mediated phosphorylation of calmodulin (Fig. 3). There was very little effect of GTP[S] at concentrations of 100 μ M or lower, but there was substantial (approx. 50 %) inhibition of calmodulin phosphorylation at 500 μ M-GTP[S] (Fig. 3). This is well within the physiological range of GTP for most cells [39–45].

Inhibition of phosphorylation of poly(GluNa₄,Tyr₁) by GTP[S]

Since phosphorylations of histone Hf2b and calmodulin involve polybasic proteins, we examined the effects of GTP[S] on the phosphorylation of poly(GluNa₄,Tyr₁), a commonly used substrate for tyrosine kinases. Phosphorylation of this substrate by partially purified adipocyte insulin receptors was inhibited $65.4 \pm 1\%$ by 1 mM-GTP[S]. These results suggest that GTP[S] inhibits substrate phosphorylation at the receptor level and not by altering the substrate.

Inhibition of calmodulin phosphorylation by the insulin receptor by GTP[S] is not competitive

Since inhibition of receptor kinase-mediated calmodulin (or other protein) phosphorylation by GTP[S] could simply be due to competition with labelled ATP for the insulin receptor kinase, we examined the effects of various other nucleotide triphosphates. As shown in Fig. 4, unlabelled ATP and GTP were the most potent inhibitors of calmodulin phosphorylation. The inhibition by unlabelled ATP is expected, since it reduces the specific activity of the labelled ATP. Both ATP and GTP are purine triphosphates, so GTP may be expected to compete with ATP more readily than do pyrimidine nucleotides. However, ITP is also a purine triphosphate and it did not inhibit phosphorylation as much as did GTP (66 % versus 88 %; results not shown). Cassel & Selinger [46] have shown that both ATP and ITP inhibit GTPases whereas CTP and UTP (pyrimidines) have no effect. Therefore the purines may all interact similarly with the insulin receptor. It is possible that contaminating adenylate cyclase was present in the insulin receptor preparation and was activated by GTP[S]; the resulting cyclic AMP could then inhibit some of the insulin's actions. However, 100 μ M-cyclic AMP had no effect on calmodulin phosphorylation. In addition, we have demonstrated that, even when present at a high concentration (500 μ M),

Fig. 4. Effects of various nucleotides on insulin receptor-dependent calmodulin (CaM) phosphorylation

Insulin receptors were incubated as described in Fig. 2 in the presence of 67 nM-insulin. Various nucleotides: ATP (lane 2), CTP (lane 3), TTP (lane 4), UTP (lane 5), GTP (lane 6) and GDP[S] (lane 7) were added to a final concentration of 1 mM. No nucleotides were added to the sample run in lane 1. Shown is a representative autoradiogram from one of four experiments.

[³²P]GTP is not used as a phosphate donor by the insulin receptor kinase (results not shown).

We have also varied the ATP concentration to ascertain whether GTP[S] is competitively inhibiting ³²P incorporation into calmodulin. The results shown in Fig. 5 demonstrate that GTP[S] primarily reduces the $V_{\max}$ of the kinase reaction, suggesting that the effect of this nucleotide is not simple competition with ATP for the reaction site. We used the iterative method of Atkins [47] based on the Wilkinson analysis [48] of the Hill equation [49] to determine the effect of GTP[S] on the $V_{\max}$ and the $K_{0.5}$ of ATP-dependent insulin-stimulated calmodulin phosphorylation. The results of four experiments were as follows. The $V_{\max}$ in the absence of GTP[S] was $(5.1 \pm 2.9) \times 10^{-13}$ mol of phosphate/min per mol of calmodulin, versus $(1.3 \pm 0.9) \times 10^{-13}$ mol/mol in the presence of 1 mM-GTP[S]. The $K_{0.5}$ values were $(5.3 \pm 0.9) \times 10^{-5}$ M-ATP and $(7.5 \pm 1.7) \times 10^{-5}$ M-ATP in the absence and presence respectively of 1 mM-GTP[S].

Fig. 5. Effect of GTP[S] on the ATP dose dependence of calmodulin phosphorylation by the insulin receptor

Insulin receptors were incubated under the conditions described in the legend to Fig. 2 in the absence (●, ▲) or presence (○, △) of 67 nM-insulin and/or the absence (○, ●) or presence (△, ▲) of 1 mM-GTP[S]. Various concentrations of ATP were used. Following SDS/PAGE and autoradiography the sections of the gels corresponding to calmodulin were excised and counted for radioactivity.

Fig. 6. Effect of GTP[S] on insulin binding to partially purified insulin receptors and isolated adipocyte plasma membranes

(a) Insulin receptors (○, ●) or plasma membranes (△, ▲) were incubated as described in the Materials and methods section in the presence of various concentrations of ^{125}I -insulin and in the absence (○, △) or presence (●, ▲) of 1 mM-GTP[S] (50 μl of partially purified insulin receptors and 25 μl of plasma membranes were used). (b) Insulin binding to partially purified insulin receptors in the absence (○) or presence (●) of 400 μM -GTP[S] was determined. A 25 μl portion of insulin receptors was used in (b), which accounts for the lower amount of insulin bound than observed in (a). Each point is the mean of triplicate determinations.

Interestingly, the Hill coefficient suggests the presence of multiple sites or positive co-operativity when GTP[S] is added [$h = 1.28 \pm 0.16$ (absence of GTP[S]) versus 1.65 ± 0.13 (presence of GTP[S])]. Similar data were obtained when 500 μM -GTP[S] (approximately the IC_{50} , i.e. the concn. causing 50% inhibition) was used to inhibit calmodulin phosphorylation. The stoichiometry of calmodulin phosphorylation in this experiment was relatively low (0.015 mol of phosphate/mol of calmodulin or, if it is assumed that one tyrosine per calmodulin is phosphorylated [34], 1.5% of the calmodulin molecules are phosphorylated). Since this low percentage of phosphorylation may not be physiologically relevant, we altered the phosphorylation conditions [34] so that the stoichiometry of calmodulin phosphorylation was greater than 20% (or 0.2 mol of phosphate/mol of calmodulin). The results of these experiments indicate that the effect of GTP[S] on the phosphorylation of calmodulin is similar regardless of the stoichiometry (results not shown).

We have attempted to use aluminium fluoride (AlF_4^-) to further investigate the inhibition of insulin receptor function which is mediated by the putative GTP-binding protein. AlF_4^- , which is formed by the combination of AlCl_3 and NaF, binds GDP to produce a compound with a three-dimensional structure similar to that of GTP [50]. When the concentrations of AlCl_3 (30 μM) and NaF (10 mM) that are normally used by researchers [50–52] were employed, we saw no inhibition of calmodulin phosphorylation. Since high levels of GTP[S] are needed for the inhibition of calmodulin phosphorylation, we examined the effects of AlF_4^- formed with 300 μM - AlCl_3 and 100 mM-NaF. Under these conditions calmodulin phosphorylation was inhibited. However, at this concentration NaF alone inhibited the phosphorylation of calmodulin, so the effect was probably due to a non-specific inhibition of the kinase.

Effect of GTP[S] on insulin binding to partially purified insulin receptors and adipocyte plasma membranes

Since GTP[S] inhibits the insulin receptor-dependent phosphorylation of a variety of proteins, we assumed that the effect was mediated through the receptor and was not due to

modifications of the protein substrates. To examine this we evaluated the effect of GTP[S] on insulin binding to partially purified insulin receptors and adipocyte plasma membranes (Fig. 6a). As demonstrated, GTP[S] inhibited insulin binding at both submaximal and maximal insulin concentrations (i.e. 0.9 nM-insulin has been determined to be a maximal binding concentration in previous studies [29]). The inhibition by 1 mM-GTP[S] was evident both in the receptor preparation and in plasma membranes, although the effect was not as great in the latter preparation (>80% versus $\approx 50\%$ respectively). The reason for this is not known. A more complete insulin dose curve using a lower concentration (400 μM) of GTP[S] was performed to further characterize the GTP[S] effect on insulin binding (Fig. 6b). At both GTP[S] concentrations there was a clear inhibition of maximal binding (B_{max}). Scatchard analysis confirmed the effect on B_{max} , but the data do not adequately resolve the curvilinear nature of the Scatchard plot or the effect of GTP[S] on the affinity of insulin for its receptor. The IC_{50} of GTP[S] for insulin binding is approx. 500 μM (results not shown), which is in agreement with the IC_{50} of GTP[S] for calmodulin phosphorylation.

GTP[S] appears to be acting on the intracellular portion of the insulin receptor, since addition of 1 mM-GTP[S] to the outside of intact cells had no effect on insulin binding (results not shown). The inhibition of insulin binding by GTP[S] is not dependent on kinase activity, since GTP[S] inhibited insulin binding under conditions in which phosphorylation would not occur (e.g. in the absence of ATP; results not shown). Insulin binding in the absence of ATP was equivalent to that in the presence of 10 μM -ATP (normal condition), and 1 mM-GTP[S] inhibited binding by 79% and 75% in the presence and absence respectively of ATP.

As is the case with calmodulin phosphorylation, the effect of GTP on insulin binding appears to be rather specific. Although several of the nucleotides that we tested inhibited insulin binding by various degrees, none of them was nearly as potent as GTP. As such, insulin binding in the presence of 1 mM-ATP was 6 times greater than that in the presence of 1 mM-GTP. The values for other nucleotides were: GDP, 13.0 times; guanosine 5'-[β -thio]diphosphate (GDP[S]), 13.0 times; CTP, 7.1 times; cyclic

Fig. 7. Immunoblots of adipocyte plasma membranes and isolated insulin receptor preparations using an antibody directed towards a common α -subunit epitope of G-proteins

Plasma membranes or insulin receptor proteins were separated by SDS/PAGE and the proteins were transferred to nitrocellulose paper as described by Mumby *et al.* [31]. The G-proteins were probed with a common α -subunit antibody (J881), 1:500 dilution, and were detected with a second antibody containing ^{125}I -labelled goat anti-(rabbit IgG) followed by autoradiography for 18 h. The volume of plasma membranes (PM) or receptors used (μl) is denoted below the autoradiogram.

AMP, 7.6 times greater than the binding in the presence of GTP.

We have also shown that GTP[S] causes the dissociation of insulin from its receptors. The addition of 1 mM-GTP[S] after 45 min of insulin binding to receptors in the absence of GTP[S] resulted in the loss of 70 % of the bound insulin over 15 min, as compared with control receptors to which water was added (results not shown). These results indicate that at least part of the inhibition of insulin receptor-dependent phosphorylation by GTP[S] is due to inhibition of insulin binding to its receptor.

Immunoblots to detect G-proteins

In order to determine whether the effects of GTP[S] could be regulated through any of the well-characterized G-proteins (G_s , G_i or G_o), we used an antibody raised to an epitope that is common to these G-proteins. As shown in Fig. 7, the antibody recognizes two proteins (≈ 35 –40 kDa) in adipocyte plasma membrane preparations (lanes 4–6). However, the antibody fails to recognize anything in the isolated adipocyte insulin receptor preparation (lanes 1–3), even though there are at least half as many insulin receptors per volume as in the plasma membranes [as determined by insulin binding (see also Fig. 6a)]. Therefore comparable numbers of receptors would be in lanes 1 and 4, 2 and 5, and 3 and 6. We have also attempted to detect low-molecular-mass G-proteins by treating blots with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$. We could detect GTP-binding proteins of 20–24 kDa in 250 μl of the adipocyte plasma membranes and in platelet cytosol (kindly provided by Dr. J. Baldassare) but were unable to detect anything in 500 μl of the insulin receptor preparation (results not shown). These results suggest that the effects of GTP[S] are not mediated through a classical G-protein.

DISCUSSION

The classical model of the G-protein cycle indicates that GTP-binding proteins are activated by the replacement of GDP with GTP. GTP is then hydrolysed by an intrinsic GTPase to inactivate the G-protein [53,54]. Therefore, to obtain amplification of the

G-protein signal, non-hydrolysable (or slowly hydrolysable) GTP analogues such as GTP[S] are used. In the studies presented here, relatively high concentrations of GTP[S] were required to inhibit insulin receptor function. These concentrations of GTP[S] ($> 250 \mu\text{M}$) are considerably higher than those generally used to investigate G-protein action (10–100 μM), suggesting that a classical G-protein is not mediating the effects observed in these studies. Even though high concentrations of GTP[S] were used, they were not necessarily beyond the physiological range of GTP [41–45]. In most cells the basal concentration of GTP is in the high micromolar range (200–600 μM). Although adipocytes have been reported to have considerably lower basal concentrations of GTP [40], there may be localized compartments with markedly higher concentrations. We have shown that 1 mM-GTP[S] inhibits calmodulin phosphorylation by insulin receptors prepared from rat livers, a tissue in which the basal GTP concentration may be high.

Lambert *et al.* [40] have shown that the GTP concentration is increased in adipocytes that have been stimulated with β -agonists. Such an increase in GTP may be one mechanism responsible for β -agonist-induced inhibition of insulin action. This group has also shown that pertussis toxin increases GTP levels in adipocytes, presumably by inhibiting the GTPase activity associated with G_i [55]. It is therefore possible that β -agonists, which inhibit many of the actions of insulin, and bacterial toxins increase GTP in adipocytes to concentrations that could inhibit insulin receptor-dependent phosphorylation. Alternatively, insulin antagonists could cause a modification of the cellular milieu that would make the insulin receptor more sensitive to GTP. Such a change could also be partially responsible for the insulin resistance observed in non-insulin-dependent diabetes mellitus.

Burdett *et al.* [21] have shown that 1 mM-GTP[S] had no effect on insulin binding to crude membranes or plasma membranes isolated from rat livers. The difference between their results and ours may be due to the tissue used, although we have seen a greater than 80 % decrease in insulin binding to partially purified hepatic insulin receptors when 1 mM-GTP[S] was included in the assay (results not shown). We therefore feel that under our experimental conditions the GTP[S] effect on insulin binding and substrate phosphorylation is a general phenomenon for insulin receptors of all tissues.

Normally, when GTP[S] binds to G-proteins that are coupled to a receptor, it causes an effect that mimics that of agonist binding to the receptor [53,54]. However, GTP[S] added to the insulin receptor preparation results in antagonism of insulin action. Furthermore, GTP[S] causes a net reduction in the binding of insulin to its receptor. This appears to be due, at least in part, to increased dissociation of insulin from the receptor, since the addition of GTP[S] following insulin binding reduces the amount of insulin bound to receptors. It is well known that GTP, and its analogues, causes the dissociation of agonists from receptors [53,54,56,57]. In most cases GTP[S] causes the α -subunit of the G-protein to dissociate from the receptor and bind to the effector protein, resulting in signal transduction. In the present situation, however, GTP[S] appears to cause insulin dissociation from the receptor, which results in attenuation of the signal. We have also demonstrated that, in the presence of insulin, GTP[S] inhibits histone phosphorylation significantly more than it inhibits β -subunit autophosphorylation (65 % and 22 % inhibition respectively; Fig. 1). Other researchers [16–18] have obtained similar results when examining the phosphorylation of G-proteins (G_i , G_o and transducin) by the insulin receptor kinase, i.e. GTP[S] inhibited G-protein phosphorylation without a concomitant inhibition of β -subunit autophosphorylation. These similar observations in four different assay systems support the concept that GTP[S] is operating in all systems through

common mechanisms. GTP[S] could be operating at one or more of three distinct levels: binding of insulin to its receptor, autophosphorylation and/or receptor-substrate coupling.

A scenario to explain our observations may be that when insulin binds to its receptor it initiates autophosphorylation, but because GTP[S] induces dissociation from the receptor, the autophosphorylation cascade is not completed in the majority of the receptors. GTP[S] may also inhibit a conformational change in the receptor, thus interfering with an interaction between the two β -subunits in the holoreceptor; such an interaction has been proposed to be required for autophosphorylation [58]. Since it is known that certain tyrosines on the receptor must be phosphorylated for the transduction of a phosphorylation signal to occur [59,60], the incompletely autophosphorylated receptors may fail to phosphorylate substrates. Alternatively, GTP[S] may cause a compensatory increase in the number of serine and threonine residues that are phosphorylated in the insulin receptor. These serine and threonine phosphorylations are not sufficient for the phosphotransferase reaction to occur, but they would mask a decrease in autophosphorylation of tyrosine residues.

The data presented here demonstrate that GTP[S] (and GTP) inhibit insulin receptor function. These nucleotides do not appear to act through a conventional G-protein, but may react directly with the insulin receptor or operate through a unique GTP-binding protein that is closely coupled to the insulin receptor.

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