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MODULATION OF GUANINE NUCLEOTIDE EFFECTS ON THE INSULIN RECEPTOR BY $MgCl_2$

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Insulin binding to partially purified rat adipocyte insulin receptors is inhibited approximately 40-60 percent by 1 mM GTP-γ-S in the presence of 2 mM $MgCl_2$. However, in the presence of 10 mM $MgCl_2$, GTP-γ-S does not inhibit binding. Increasing $MgCl_2$ from 0.5 to 10 mM enhances the phosphorylation of calmodulin catalyzed by the insulin receptor but also reduces the inhibition seen with 500 μM GTP-γ-S. The reversal of the GTP-γ-S-induced inhibition of calmodulin phosphorylation by high concentrations of $MgCl_2$ appears to be due to an effect on the calmodulin molecule since $MgCl_2$ has little effect on the inhibition of phosphorylation of histone Hf2b or poly (Glu^4, Tyr^1) induced by GTP-γ-S. Our data suggest that there are at least two GTP-binding proteins associated with the insulin receptor, one that regulates insulin binding and is modulated by $MgCl_2$ and one that regulates substrate phosphorylation and/or receptor-substrate coupling and is not altered by $MgCl_2$.

There is substantial evidence that GTP-binding proteins are involved in some of insulin's actions. Insulin has been reported to phosphorylate the GDP-bound alpha subunits of Go, Gi and transducin (1-3), $p21^{ras}$ (4) and an endogenous 60 kDa protein that binds to GDP-agarose (5). Insulin also inhibits pertussis toxin-induced ADP-ribosylation of Gi (6). On the other hand, pertussis toxin, when added to intact cells, inhibits cAMP phosphodiesterase (7) and hexose transport (8) as well as many other insulin-stimulated events.

GTP-γ-S (a slowly hydrolyzable GTP-analog) has been shown to inhibit reconstituted insulin-stimulated glucose transporter activity in both plasma membranes and low density microsomes (9) and to inhibit the insulin-induced phosphorylation of an endogenous 160 kDa protein in electropermeabilized L6 muscle cells (10). We have recently reported that insulin binding and the insulin-receptor dependent phosphorylation of calmodulin, histone and poly

Abbreviations

$MgCl_2$, magnesium chloride; GTP, guanosine-5'-triphosphate; GTP-γ-S, guanosine-5'-O-(3-thiotriphosphate); GDP, guanosine-5'-diphosphate; GDP-ß-S, guanosine-5'-O-(2-thiodiphosphate); EGTA, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid.

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(Glu^4, Tyr^1) are inhibited by GTP-γ-S (11). These studies were conducted in the presence of 2 mM $MgCl_2$. However, we have since discovered that different results may be obtained when higher concentrations of $MgCl_2$ are used. In this paper we describe our investigation of the role of $MgCl_2$ in the guanine nucleotide effects on insulin receptor function.

Materials and Methods

Poly-L-lysine (M_r = 30,000-70,000), wheat germ lectin sepharose 6 MB, poly (Glu^4, Tyr^1), GTP-γ-S and other nucelotides were obtained from Sigma (St. Louis, MO). Histone Hf2b was purchased from Worthington Biochemical (Freehold, NJ). Phosphocellulose (P81) filter paper was from Whatman. Unlabeled insulin was obtained from Eli Lilly (Indianapolis, IN) and ^{125}I insulin was from the Washington University Diabetes Research and Training Center (St. Louis, MO). Calcium-free porcine brain calmodulin was purchased from Ocean Biologics, Inc. (Edmonds, WA). Protosol, Aquasol II and [γ-^{32}P]-ATP were obtained from DuPont-New England Nuclear (Boston, MA).

Adipocytes were isolated from the epididymal fat pads of male Sprague Dawley rats (120-150 g, Sasco Inc., O'Fallon, MO) by the method of Rodbell (12). Insulin receptors were partially purified from adipocyte plasma membranes by wheat germ lectin chromatography (13).

Calmodulin (5 μg) was phosphorylated by the insulin receptor in the presence of poly-L-lysine as described by Wong et al. (14). Histone phosphorylations were conducted under the same condition except that calmodulin and poly-L-lysine were deleted and 1.8 mM histone Hf2b was added. Proteins were separated by polyacrylamide gel electrophoresis as described by Laemmli (15) on 12.5% 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 ^{32}P into proteins was determined by cutting the appropriate protein band from the gel, solubilizing it in water:Protosol:Aquasol II (1:2:10,v/v) and quantifying the radioactivity by liquid scintillation spectrometry. Poly (Glu^4, Tyr^1) was phosphorylated in a system similar to that used for histone phosphorylation and the samples were spotted on P-81 paper as described by Sahal and Fujita-Yamaguchi (16). Insulin binding to the partially purified adipocyte insulin receptors was quantified by using a modification of the method of Cuatrecasas (17) as described by Wong (14).

Results and Discussion

We have previously shown that insulin binding and insulin receptor kinase activity are inhibited by high concentrations (> 250 μM) of GTP-γ-S (11). We now show in Figure 1 that the effect of GTP-γ-S on insulin binding is reversed in the presence of high concentrations of $MgCl_2$ while binding in the absence of GTP-γ-S is unaltered. In the standard assay system (2 mM $MgCl_2$), 1 mM GTP-γ-S inhibits submaximal and maximal insulin binding by roughly 60%, however in the presence of 10 mM $MgCl_2$ the inhibition by GTP-γ-S is abolished. As we suggested in our previous paper (11), GTP-γ-S may reduce insulin binding by increasing dissociation of insulin from its receptor. Magnesium may restrict GTP-γ-S binding to the receptor and thereby reduce the dissociation of insulin.

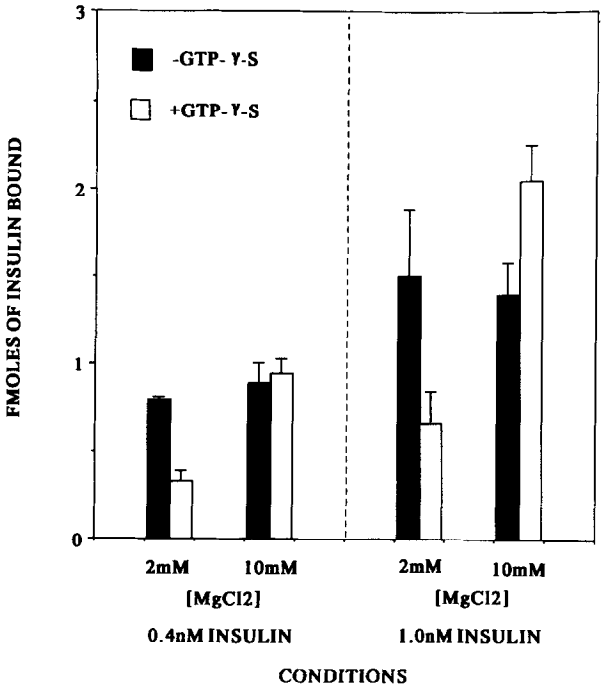

Figure 1. The effect of 1 mM GTP-γ-S on insulin binding in the presence of 2 mM or 10 mM $MgCl_2$. Insulin binding was conducted as described in "Materials and Methods" at a submaximal (0.4 nM) and a maximal (1 nM) insulin concentration. The binding was performed in a buffer containing 2 mM or 10 mM $MgCl_2$ in the presence □ or absence ■ of 1 mM GTP-γ-S. The data are presented as the mean ± SEM of 3 determinations.

Although $MgCl_2$ has no effect on insulin binding in the absence of GTP-γ-S, calmodulin phosphorylation by the insulin receptor is enhanced by increasing the $MgCl_2$ concentration to 10 mM (Figure 2). Once again the inhibition seen with GTP-γ-S (in this case 500 μM) is reduced as $MgCl_2$ is increased (Figure 2). As shown previously, the inhibition of calmodulin phosphorylation is specific for guanine nucleotides (11). Besides GTP-γ-S, GTP, GDP and GDP-β-S (1 mM each) inhibit calmodulin phosphorylation at 2 mM $MgCl_2$ (Figure 3). Again the inhibition of phosphorylation is either partially or completely reversed at 10 mM $MgCl_2$. The nonselectivity between the guanine nucleotides is somewhat surprising since GTP and GTP-γ-S should activate and GDP, and GDP-β-S should inhibit GTP binding protein activity. There are reports, however, where GDP and GDP-β-S have similar effects to those of the guanosine triphosphates (18,19). In addition, our previous results indicate that a unique GTP-binding protein which behaves very differently than the classical G proteins is likely to be involved.

In contrast to the results obtained with calmodulin phosphorylation, $MgCl_2$ has only a limited effect on the inhibition of histone Hf2b and poly

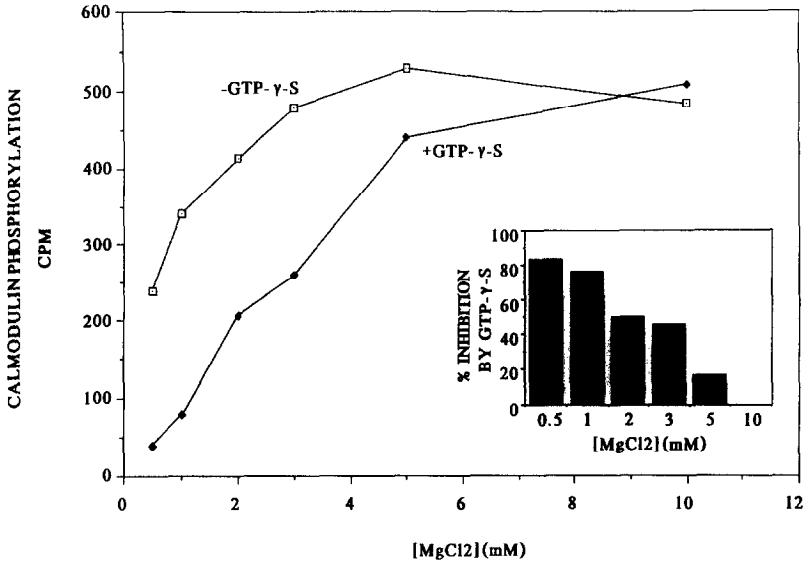

Figure 2. The effect of $MgCl_2$ concentration on the phosphorylation of calmodulin in the presence and absence of GTP-γ-S. Calmodulin phosphorylation was conducted as described under "Materials and Methods" at various $MgCl_2$ concentrations and in the presence ■ and absence □ of 500 μM GTP-γ-S. The insert shows the % inhibition of calmodulin phosphorylation at each $MgCl_2$ concentration.

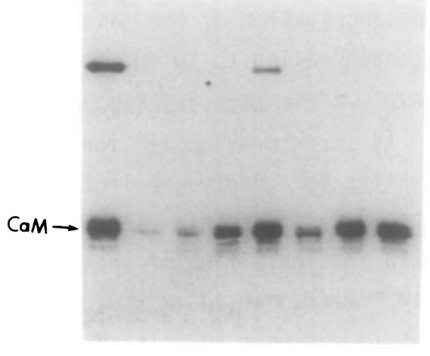

Figure 3. The effect of $MgCl_2$ concentration on the inhibition of calmodulin phosphorylation induced by various guanine nucleotides. Calmodulin was phosphorylated as described under "Materials and Methods" at 2 mM or 10 mM $MgCl_2$ in the presence or absence of 1 mM GTP, GDP or GDP-ß-S. Shown is a representative autoradiogram from one of two experiments.

TABLE 1

The Effect of [$MgCl_2$] on the GTP-γ-S Induced Changes in Insulin Binding and Polypeptide Phosphorylations

	Effect of GTP-γ-S % of Control (-GTP-γ-S)*	
	2 mM $MgCl_2$	10 mM $MgCl_2$
Insulin Binding	58.1 ± 7.5 (14)	138.1 ± 27.2 (8)
Calmodulin Phosphorylation	36.4 ± 10.0 (5)	104.6 ± 9.8 (5)
Histone Phosphorylation	30.5 ± 5.2 (2)	47.4 ± 8.2 (2)
Poly(Glu^4,Tyr^1) Phosphorylation	7.3 ± 1.6 (3)	15.9 ± 4.7 (3)

*% of Control = $\frac{\text{Value in the presence of GTP-γ-S}}{\text{Value in the absence of GTP-γ-S}}$ X 100.

The numbers in parentheses are the number of times the experiment was conducted. Assays were conducted as described under "Materials and Methods." The GTP-γ-S concentration was 1 mM for insulin binding and 500 μM for the polypeptide phosphorylations.

(Glu^4, Tyr^1) phosphorylation by GTP-γ-S. As shown in Table 1, 10 mM $MgCl_2$ reverses the effects of GTP-γ-S on insulin binding and calmodulin phosphorylation but not on histone or poly (Glu^4, Tyr^1) phosphorylation. Changes in $MgCl_2$ concentration between 2 mM and 10 mM have little effect on insulin binding or substrate phosphorylation in the absence of GTP-γ-S. The $MgCl_2$ effect on calmodulin phosphorylation occurs primarily between 0.5 mM and 2 mM (see Fig 2). The discrepancy between the GTP-γ-S effects on calmodulin phosphorylation and those on histone and poly (Glu^4, Tyr^1) phosphorylation may be due to a direct effect of Mg^{2+} on calmodulin. Magnesium has been shown to reduce calcium binding to calmodulin (20). In addition, Sacks and McDonald (21) have reported that calcium inhibits calmodulin phosphorylation by the insulin receptor. Therefore it is possible that Mg^{2+} enhances the phosphorylation of calmodulin (as shown in Fig 2) by reducing calcium binding. Magnesium may have additional effects on calmodulin that would prevent GTP-γ-S from inhibiting its phosphorylation. Since poly-L-lysine (or another poly basic protein) is required for phosphorylation of calmodulin by the insulin receptor we thought that this peptide may be partially responsible for the differences between calmodulin and the other substrates. However, when we include poly-L-lysine in the poly(Glu^4,Tyr^1) assay there are no differences in the extent of phosphorylation, the inhibition by GTP-γ-S or the effects of $MgCl_2$ (data not shown).

Our data suggest that there are at least two GTP-binding proteins involved in insulin receptor function. The first GTP-binding protein regulates insulin binding and its function can be modulated by $MgCl_2$. The second GTP-binding protein appears to act at the level of substrate-receptor coupling and is not regulated by $MgCl_2$. At this time it is not known if both of these GTP-binding proteins are unique or even if they interact with each other. This is clearly an important issue that we are currently pursuing.

Furthermore, these data indicate that the inhibition of substrate phosphorylation by GTP-γ-S is not due to the reduction of insulin binding since the events are differentially regulated by $MgCl_2$. In the presence of 10 mM $MgCl_2$ and 1 mM GTP-γ-S (or GTP) insulin can bind but histone and poly (Glu^4, Tyr^1) are poorly phosphorylated. However, insulin dependent reactions that do not require phosphorylation may be normal under these conditions. This remains to be determined.

Since the concentration of GTP in cells is normally higher than the concentration needed to inhibit insulin binding and substrate phosphorylation (22-26) there may be modulators to prevent these GTP-binding proteins from permanent activation. One of these modulators may be magnesium. Conditions that cause a reduction in magnesium concentration, such as diabetes (27) may allow at least one of these GTP-binding proteins to become active and contribute to insulin resistance.

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