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Effect of Colchicine and Taxol on Thyrotropin-Releasing Hormone Receptor Coupling to G Protein in GH_3 Cells

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The role of membrane-associated tubulin in TRH receptor-G protein coupling was investigated with the use of compounds that influence tubulin function. TRH-stimulated G protein GTPase activity in GH_3 cell membranes was used to determine receptor-G protein coupling. TRH-stimulated GTPase activity was abolished by $G_{q\alpha}$ antibody, suggesting that TRH receptor coupling to G_q results in the activation of $G_{q\alpha}$ and the subsequent hydrolysis of GTP. TRH (1 μ*M*) stimulated the enzymatic activity by up to 69 pmol/min/mg protein, and in the presence of 1 μ*M* colchicine the hormone-stimulated activity was only 26 pmol/min/mg protein. Similar inhibition of TRH receptor-G protein coupling was observed with tubulin antibodies and purified tubulin, suggesting that perturbation of membrane-associated tubulin and/or tubulin-G protein interaction by these compounds disrupts receptor-G protein interaction. Next, the events occurring at the initial stages of TRH-mediated signal transduction were correlated to prolactin (PRL) secretion in GH_3 cells. Colchicine (1 μ*M*) and taxol (1 μ*M*) inhibited the basal PRL secretion by 38 and 44%, respectively. In addition, colchicine (1 μ*M*) and taxol (1 μ*M*) significantly inhibited TRH-stimulated PRL secretion. TRH-stimulated PRL secretion in control, colchicine-, and taxol-treated cells was 13.9, 9.1, and 6 ng/mL, respectively. Furthermore, polymerized tubulin levels were decreased by colchicine and increased by taxol. These results suggest that perturbation of the steady state of tubulin-G_q interaction may disrupt the initial events in TRH-mediated signal transduction.

Key Words: G proteins; tubulin; colchicine; taxol; tubulin antibodies; GTPase activity.

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Introduction

The discovery of cAMP had a great impact on the field of signal transduction (Rall et al., 1956), finally leading to the discovery of heterotrimeric guanine nucleotide-binding regulatory proteins (G 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 heterotrimeric proteins comprised of a variable α-subunit (39–46 kDa) and β- and γ-subunits (35–36 and 8 kDa, respectively). The regulation of a certain effector is determined by the type of G protein that is coupled to a particular hormone receptor. For example, in the pituitary, corticotropin-releasing hormone receptor coupling to $G_{s\alpha}$ results in the stimulation of adenylyl cyclase. Somatostatin receptor coupling to $G_{i\alpha}$ causes the inhibition of adenylyl cyclase. Phospholipase C is stimulated as a result of thyrotropin-releasing hormone (TRH) receptor coupling to $G_{q\alpha}$ (Spiegel et al., 1992; Conklin and Bourne, 1993; Neer, 1995).

In the same year that cAMP was discovered, Peters (1956) proposed that hormones act on cells by modifying their cytoskeleton. This interesting theory was not received with much enthusiasm at that time, probably owing to the general perception of cytoskeleton as a rigid structure, merely involved in cellular movements and the maintenance of cell shape. We now know that some of the components of cytoskeleton, such as actin and tubulin, are dynamic proteins, with a number of different functions, including the ability to bind ATP or GTP and to hydrolyze them. There is a general consensus that actin and tubulin play an important role in signal transduction in a variety of systems (Jesaitis and Klotz, 1993; Popova et al., 1994). Recent work from Rodbell's laboratory has added a new dimension to the study of the role of cytoskeleton in hormone receptor-G protein interaction. Extraction of main classes of G proteins with octyl glucoside resulted in large, polydisperse structures sensitive to

disaggregation by GTP[γS]. This observation suggested that G proteins may occur in the native membrane environment as part of larger complexes, and that they interact with cytoskeletal proteins like actin and tubulin (Jahangeer and Rodbell, 1993).

An interaction of tubulin with G proteins as well as receptors has been reported. For example, several G_α subunits bind specifically to tubulin (Higashi and Ishibashi, 1985; Popova et al., 1994). Gephyrin, a 93-kDa microtubule-binding protein, has recently been proposed to link the glycine receptor to tubulin in synaptic membranes (Kirsch et al., 1993). In COS 1 cells transfected with $G_{s\alpha}$, tubulin-Gpp(NH)p complex potentiated isoproterenol-stimulated adenylyl cyclase activity (Popova et al., 1994). Compounds that are known to bind to tubulin (e.g., colchicine or taxol), influence receptor-G protein coupling in various systems, suggesting a role for tubulin in signal transduction (Ravindra and Aronstam, 1990a, 1993; Leiber et al., 1993).

Microtubule function is generally investigated by the use of colchicine and taxol. Colchicine inhibits the assembly of tubulin monomers into microtubule polymers and taxol alters the equilibrium between soluble and polymerized form of tubulin by overstabilizing them in their polymerized form (Hamel, 1990). Our recent observations that colchicine and taxol inhibited TRH stimulation of G protein GTPase activity in rat anterior pituitary cell membranes suggested that tubulin-G protein interaction might be involved in TRH action (Ravindra and Aronstam, 1993). However, of the cell types in the rat anterior pituitary gland, TRH binds only to mammotrophs and thyrotrophs. Thus, since colchicine and taxol can interact with tubulin present in all the cell types, the results obtained using the intact pituitary may not necessarily be a result of perturbation of TRH receptor-G protein coupling, and may represent widespread changes taking place in the whole pituitary. To address this issue and in an effort to further delineate the role of tubulin in signal transduction, the present experiments were conducted using in vitro cultures of GH_3 cells. These cells are a homogeneous population of clonal strains of rat pituitary tumor. There are several advantages to employing this model system. GH_3 cell secretion represents an established model for examining hormone secretion. TRH stimulates prolactin (PRL) secretion by these cells. Furthermore, the various steps in TRH-mediated signal transduction pathway in GH_3 cells are well established and are similar if not identical to those steps seen in freshly obtained normal rat pituitary cells.

In the present study, we examined the effect of compounds, known to interact with tubulin, on TRH-stimulated GTPase activity in GH_3 cell membranes, since this assay is routinely used to evaluate TRH receptor-G protein coupling. In addition, the events occurring at the initial stages of TRH-mediated signal transduction were correlated to prolactin secretion in GH_3 cells.

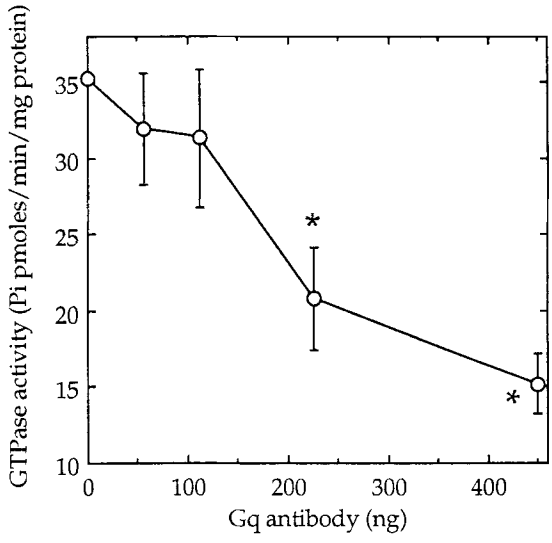

Fig. 1. Effect of $G_{q\alpha}$ antibody on basal G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with various concentrations of $G_{q\alpha}$ antibody for 2 h on ice and then incubated for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the control (labeled "0") value ($P < 0.05$).

Results

Effect of $G_{q\alpha}$ Antibody on G Protein GTPase Activity

Since there are several different G proteins present in GH_3 cell membranes (Hsieh and Martin, 1992; Wilson et al., 1994), it is important to ascertain the approximate amount of basal GTPase activity that can be attributed to $G_{q\alpha}$. Antibody to $G_{q\alpha}$ inhibited basal GTPase activity in a concentration-dependent manner. At 225 and 450 ng/100 μL, the antibody inhibited the enzymatic activity by about 40 and 60%, respectively ($P < 0.05$; Fig. 1). This observation suggested that approx 60% of the basal enzymatic activity can be owing to $G_{q\alpha}$. Similar concentrations of rabbit IgG, used as a control for $G_{q\alpha}$ antibody, did not influence the GTPase activity; GTPase activity was 58.9 ± 1.0 and 61.9 ± 1.1 pmol/min/mg protein in control and in the presence of 450 ng of rabbit IgG, respectively (mean ± SEM, $n = 3$). TRH at 1 μ*M*, maximally stimulated the GTPase activity by up to 59%; this was abolished by 450 ng of $G_{q\alpha}$ antibody (Fig. 2). These results suggest that TRH receptor-coupling to G_q results in the activation of $G_{q\alpha}$ and the subsequent hydrolysis of GTP in GH_3 cell membranes.

Effect of Colchicine and Taxol on G Protein GTPase Activity

Taxol (1 μ*M*) stimulated the basal GTPase activity by up to 37%, and colchicine (1 μ*M*) inhibited the activity by up to 36% ($P < 0.05$; Fig. 3). In addition to its effect on tubulin function, colchicine influences many other cellular func-

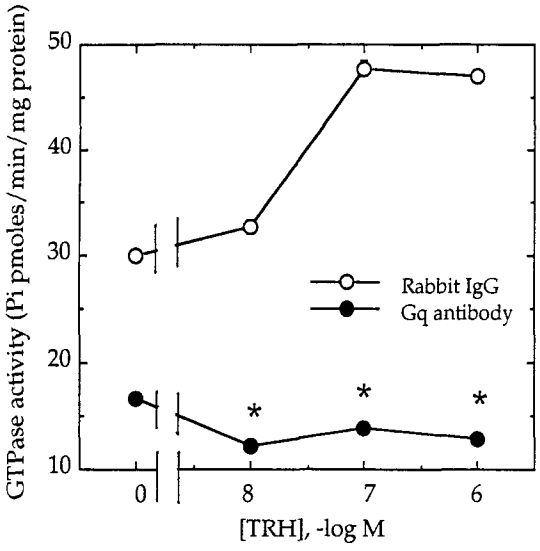

Fig. 2. Effect of $G_{q\alpha}$ antibody on TRH-stimulated G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with 450 ng $G_{q\alpha}$ antibody or 450 ng rabbit IgG (a control for the antibody) for 2 h on ice and then incubated with various concentrations of TRH for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM ($n = 3$). One of two similar experiments is shown. Asterisk (*) indicates value significantly different from the activity obtained with TRH in the presence of rabbit IgG ($P < 0.05$). Where not shown, the error bar is smaller than the symbol representing the mean.

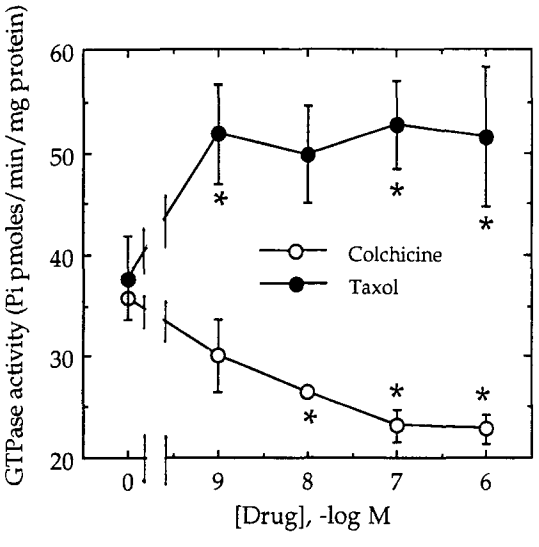

Fig. 3. Effect of colchicine and taxol on basal G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with various concentrations of colchicine or taxol for 2 h on ice and then incubated for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the control (labeled "0") value ($P < 0.05$). Where not shown, the error bar is smaller than the symbol representing the mean.

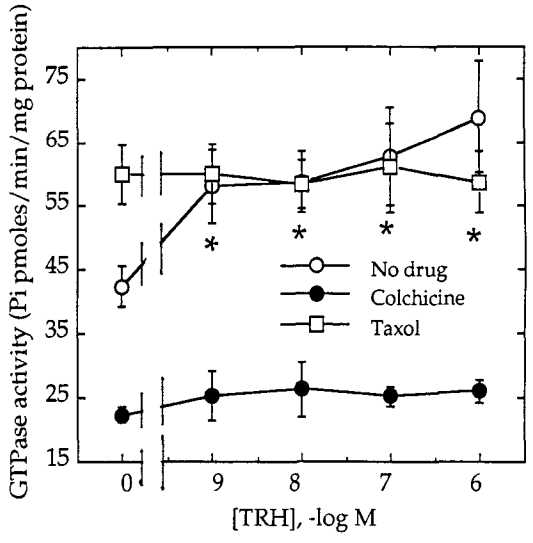

Fig. 4. Effect of colchicine and taxol on TRH-stimulated G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with 1 μ*M* colchicine or taxol for 2 h on ice and then incubated with 1 μ*M* TRH for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates that TRH stimulation is statistically significant ($P < 0.05$). Stimulation of the GTPase activity by taxol and inhibition of the activity by colchicine are statistically significant ($P < 0.05$). In the absence of TRH, values were 42.3 ± 3.2, 60 ± 4.7, and 22.3 ± 1.2 pmol/min/mg protein for control (no drug), taxol, and colchicine, respectively.

tions, including the behavior of membrane proteins. Lumicolchicine, an isomer of colchicine that does not inhibit tubulin function, is used to examine the specificity of the colchicine effect. Various concentrations of lumicolchicine, ranging from 1 n*M* to 1 μ*M*, failed to influence the GTPase activity, attesting to the specificity of colchicine action; GTPase activity was 74 ± 0.8 and 70 ± 1.6 pmol/min/mg protein in control and in the presence of 1 μ*M* lumicolchicine, respectively (mean ± SEM, $n = 3$). In the presence of colchicine, TRH-stimulated GTPase activity was inhibited ($P < 0.05$; Fig. 4). At 1 μ*M* TRH, the enzymatic activity was 69 pmol/min/mg protein, and in the presence of 1 μ*M* colchicine, TRH-stimulated activity was 26 pmol/min/mg protein ($P < 0.05$; Fig. 4). Taxol (1 μ*M*) maximally stimulated the GTPase activity by up to 60 pmol/min/mg protein, and further stimulation of the enzymatic activity by TRH was not observed in the presence of 1 μ*M* taxol (Fig. 4).

Heterotrimeric G proteins and tubulin are both "G proteins" in the sense that their activity is regulated by guanine nucleotides. Similarities between heterotrimeric G proteins and tubulin include:

1. Considerable homology and a highly conserved nature;
2. ADP ribosylation sites for both cholera and pertussis toxins; and
3. Mg^{2+}-dependent binding and hydrolysis of GTP (Burns et al., 1993).

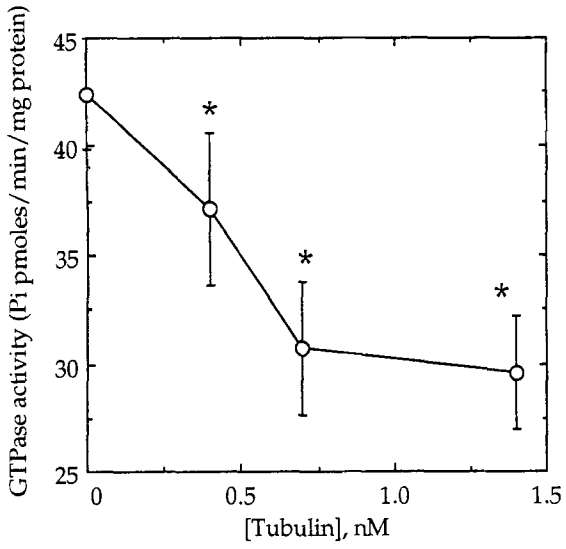

Fig. 5. Effect of purified tubulin on basal G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with various concentrations of purified tubulin for 2 h on ice and then incubated for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the control (labeled "0") value ($P < 0.05$).

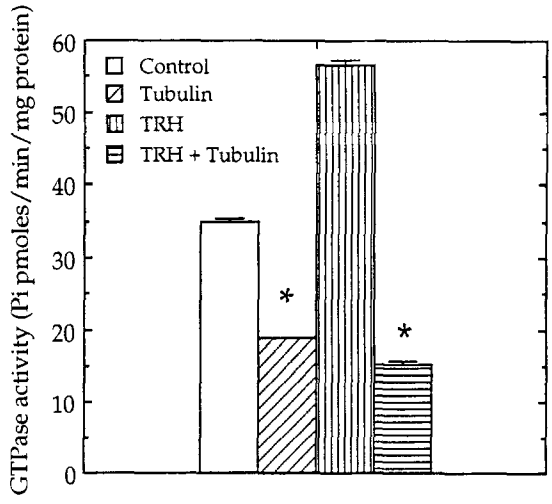

Fig. 6. Effect of purified tubulin on TRH-stimulated G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with 1.4 n*M* BSA (a control for tubulin) or purified tubulin (1.4 n*M*) for 2 h on ice and then incubated with 1 μ*M* TRH for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM ($n = 3$). One of two similar experiments is shown. Asterisk (*) indicates value significantly different from the preceding value ($P < 0.05$). TRH stimulation of the GTPase activity was statistically significant ($P < 0.05$).

Therefore, the possibility that colchicine or taxol acts directly on $G_{q\alpha}$ was considered. The drugs did not affect the GTPase activity of a mixture of partially purified bovine brain G proteins; the presence of $G_{q\alpha}$ in this mixture of proteins was determined by immunoblotting (data not shown).

Effect of Purified Tubulin on G Protein GTPase Activity

Tubulin is capable of hydrolyzing GTP under specific buffer and pH conditions, different from those used in the present study (Hamel, 1990). The behavior of tubulin in Tris-HCl buffers, used to assay G protein GTPase activity, is not known. Therefore, the ability of tubulin alone to influence GTP hydrolysis was determined under buffer conditions employed in the present study. Various concentrations of purified tubulin, ranging from 1 to 100 n*M*, did not hydrolyze GTP. In the presence of GH_3 cell membranes (25 μg protein), the enzymatic activity was 60 ± 1.4 pmol/min/mg protein; in the presence of 100 n*M* tubulin alone, the enzymatic activity was 4 ± 0.5 pmol/min/mg protein (mean ± SEM, $n = 3$). Purified tubulin (1.4 n*M*) inhibited basal GTPase activity of GH_3 membranes by about 30% ($P < 0.05$; Fig. 5). Similar concentrations of BSA, used as a control for tubulin, did not influence the GTPase activity; GTPase activity was 40.3 ± 0.2 and 37.9 ±1.0 pmol/min/mg protein in control and in the presence of 1.4 n*M* BSA, respectively (mean ± SEM, $n = 3$). In the presence of 1.4 n*M* of BSA and 1 μ*M* TRH, the enzymatic activity was 56.6 pmol/min/mg protein. However, in the presence of 1.4 n*M* purified tubulin and 1 μ*M* TRH, the activity was 15.2 pmol/min/mg protein ($P < 0.05$; Fig. 6).

Effect of Tubulin Antibodies on G Protein GTPase Activity

In view of the similarities between tubulin and G proteins (Burns et al., 1993), the tubulin antibodies used in this study may crossreact with heterotrimeric G proteins present in GH_3 cell membranes (Hsieh and Martin, 1992; Wilson et al., 1994). This possibility was investigated by immunoblotting purified bovine brain G proteins with tubulin antibodies. The tubulin antibodies used in this study did not crossreact with G proteins (data not shown).

Antibodies to α and β tubulin subunits influenced basal GTPase activity in a concentration-dependent manner. Fifty and 125 ng/100 μL of α antibodies stimulated the GTPase activity by up to 27 and 45%, respectively ($P < 0.05$; Fig. 7). Fifty and 125 ng of β antibodies inhibited the GTPase activity by up to 30 and 40%, respectively ($P < 0.05$; Fig. 7). Similar concentrations of mouse IgG, used as a control for tubulin antibodies, did not influence the GTPase activity; GTPase activity was 60.9 ± 0.6 and 59.5 ± 1.3 pmol/min/mg protein in control and in the presence of 125 ng mouse IgG, respectively (mean ± SEM, $n = 3$). In the presence of either of these antibodies, TRH-stimulated GTPase activity was inhibited ($P < 0.05$; Fig. 8). In the presence of 125 ng of mouse IgG (a control for the anti-

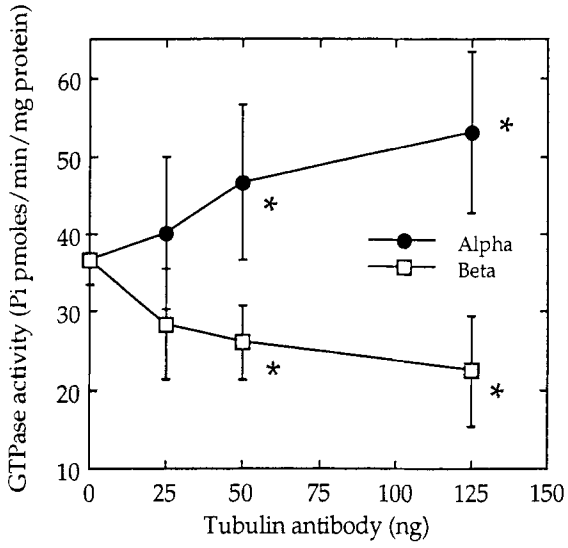

Fig. 7. Effect of tubulin antibodies on basal G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with various concentrations of anti-α (Alpha)- or anti-β (Beta)-tubulin antibody for 2 h on ice and then incubated for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the control (labeled "0") value ($P < 0.05$).

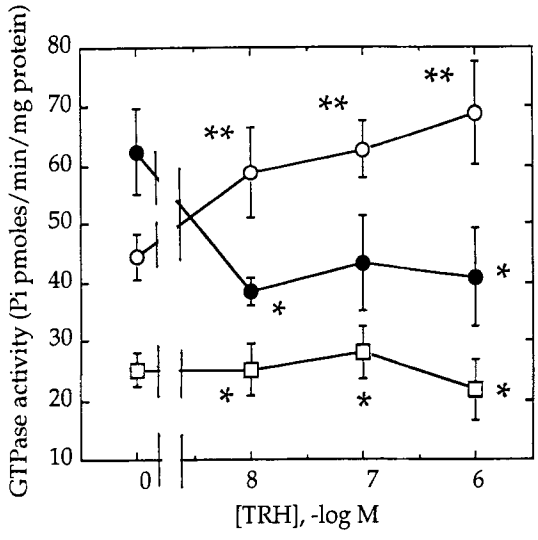

Fig. 8. Effect of tubulin antibodies on TRH-stimulated G protein GTPase activity. Cell membranes (25 μg protein) were preincubated with 125 ng of mouse IgG (a control for the antibodies), 125 ng of α (●) or 125 ng β (□) tubulin antibodies for 2 h on ice and then incubated with various concentrations of TRH for 10 min at 37°C and the enzymatic activity determined at 1 μ*M* GTP; total volume of the reaction mixture was 100 μL. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the activity obtained with TRH in the presence of mouse IgG ($P < 0.05$). Double asterisks (**) indicate that TRH stimulation is statistically significant ($P < 0.05$). Stimulation of the GTPase activity by α antibody and inhibition of the activity by β antibody are statistically significant ($P < 0.05$).

bodies) and 1 μ*M* TRH the enzymatic activity was 69 pmol/min/mg protein, whereas, in the presence of 125 ng of α antibody and 1 μ*M* TRH, the activity was 40.7 pmol/min/mg protein; similar results were obtained with β-antibody ($P < 0.05$; Fig. 8).

Effect of $G_{q\alpha}$ Antibody, Colchicine, Taxol, Tubulin, and Tubulin Antibodies on [^{3}H]TRH Binding to GH_3 Cell Membranes

We examined the possibility that $G_{q\alpha}$ antibodies, colchicine, taxol, purified tubulin, and tubulin antibodies inhibited TRH-stimulated GTPase activity by inhibiting the binding of the hormone to its receptor.

Nonspecific binding determined in the presence of 10 μ*M* methyl TRH was 3.4 ± 0.3% (mean ± SD of four experiments, each experiment was conducted in triplicate) of the total amount of [^{3}H]methyl TRH added to the reaction mixture. Specific binding was 6.5 ± 1% (mean ± SD of four experiments; each experiment was conducted in triplicate) of the total amount of [^{3}H]methyl TRH added to the reaction mixture.

[^{3}H]TRH binding to GH_3 cell membranes in control, colchicine (1 μ*M*), taxol (1 μ*M*), α-tubulin antibody (125 ng), β-tubulin antibody (125 ng), purified tubulin (1.4 n*M*), and $G_{q\alpha}$ antibody (450 ng) was 32.6 ± 0.7, 30.2 ± 0.6, 25.6 ± 0.7, 30.2 ± 0.5, 27.9 ± 0.4, 24 ± 0.2, and 25.9 ± 0.4 cpm/μg protein, respectively (mean ± SD of six determinations from two independent experiments). Colchicine, taxol, α-tubulin antibody, β-tubulin antibody, purified tubulin, and $G_{q\alpha}$ antibody modestly inhibited [^{3}H]TRH binding by 7, 23, 7, 14, 26, and 21%, respectively.

Effect of Colchicine and Taxol on PRL Secretion

PRL secretion was inhibited in GH_3 cells incubated for 24 h with 1 μ*M* colchicine or taxol. PRL secretion in control, colchicine-, and taxol-treated cells was 301, 188, and 199 ng/mL, respectively (Fig. 9; $P < 0.05$). In an effort to determine whether incubation with the drugs influenced the ability of the cells to respond to TRH, GH_3 cells were exposed to the drugs for 24 h, medium removed, cells washed with serum-free medium, and challenged with TRH for 10 min, and PRL secretion was determined. TRH-stimulated PRL secretion in control, colchicine-, and taxol-treated cells was 13.9, 9.1, and 6.0 ng/mL, respectively ($P < 0.05$; Fig. 10).

One million GH_3 cells were incubated with 1 μ*M* colchicine or taxol for 24 h, and the cell viability was tested by trypan blue exclusion. Trypan blue exclusion was 99.8% in control and drug-treated cells.

Effect of Colchicine and Taxol on Polymerized Tubulin Levels in GH_3 Cells

It is well established that tubulin polymerization is inhibited by colchicine and promoted by taxol (Hamel, 1990;

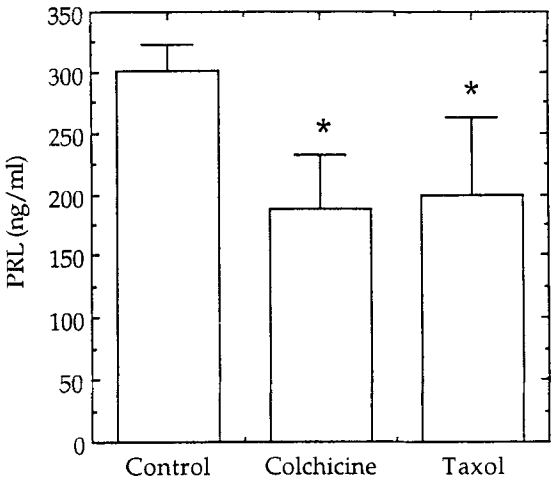

Fig. 9. Effect of colchicine and taxol on basal PRL secretion. GH_3 cells were incubated with colchicine (1 μM) or taxol (1 μM) in serum-free medium for 24 h, and PRL in medium was determined by radioimmunoassay. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the control value ($P < 0.05$).

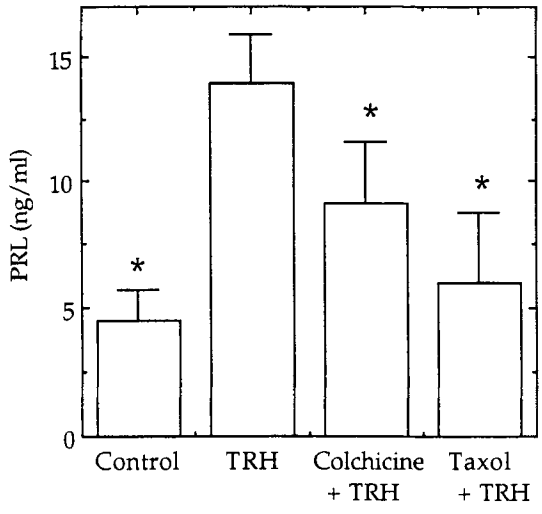

Fig. 10. Effect of colchicine and taxol on TRH-stimulated PRL secretion. GH_3 cells were incubated with colchicine (1 μM) or taxol (1 μM) in serum-free medium for 24 h. Cells were then washed twice with serum-free medium, and challenged with 100 nM TRH for 10 min. PRL in medium was determined by radioimmunoassay. Each point represents the mean ± SEM of three experiments. Each experiment was conducted in triplicate. Asterisk (*) indicates value significantly different from the TRH value ($P < 0.05$).

Wilson and Jordan, 1994). In an attempt to correlate the effects of these drugs on microtubule function to their influence on PRL secretion, polymerized tubulin (i.e., microtubules) levels were determined in GH_3 cells incubated for 24 h with colchicine or taxol. Polymerized tubulin levels were decreased by colchicine and increased by taxol (Fig. 11).

Discussion

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 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. Using GDP release, GTP binding, and GTPase activity, we have recently demonstrated that the TRH receptor in the rat anterior pituitary is coupled to a G protein (Ravindra and Aronstam, 1990b, 1992). Subsequently, it was reported that antibodies to $G_{q\alpha}$ specifically inhibited TRH-stimulated phospholipase C activity in GH_3 cell membranes, suggesting that the TRH receptor is coupled to $G_{q\alpha}$ in GH_3 membranes (Hsieh and Martin, 1992; Aragay et al., 1992). In the present study, the observation that antibodies to $G_{q\alpha}$ inhibited TRH-stimulated GTPase activity confirm and extend the previous reports.

With the use of $G_{q\alpha}$ antibody, we established that about 60% of basal GTPase activity can be attributed to $G_{q\alpha}$. Therefore, at least 40% of the GTPase activity can be owing to several other G proteins present in GH_3 cells (Hsieh and Martin, 1992; Wilson et al., 1994). Colchicine, tubulin and β-tubulin antibody inhibited basal enzymatic activity by 30–40%, and taxol and α-tubulin antibody stimulated the activity by 37–45%. Since none of these compounds influenced the basal enzymatic activity by more than 45%, it is not possible to conclude whether they affect $G_{q\alpha}$ or other G proteins. On the other hand, the observation that $G_{q\alpha}$ antibody abolished TRH-stimulated GTPase activity suggests that $G_{q\alpha}$ antibody disrupts TRH receptor coupling to $G_{q\alpha}$. Therefore, it can be concluded that any compound that inhibits TRH-stimulated GTPase activity also disrupts TRH receptor coupling to $G_{q\alpha}$.

It was previously reported that colchicine enhanced (by two-to three-fold) isoproterenol-stimulated cAMP production in S 49 cells (Leiber et al., 1993). In contrast, our observations with TRH receptor in GH_3 cells (present study) and in the rat pituitary (Ravindra and Aronstam, 1993) indicated that colchicine inhibited receptor-G protein interaction. This difference in the drug effect might reflect tissue or G protein specificity; β-adrenergic and TRH receptors are coupled to G_s and G_q, respectively.

In the presence of 1 μM taxol, TRH was unable to stimulate the GTPase activity any further; this may be owing to the fact that the enzymatic activity was maximally stimulated by taxol (Fig. 4). The effect of drugs or other compounds (e.g., tubulin antibodies) on basal GTPase activity appears to be all or none; a dose-dependent effect was not seen with either colchicine or taxol (Fig. 3). Because of the lack of clear-cut dose-response with the drugs, the effect of TRH in the presence of submaximal concentrations of taxol was not investigated.

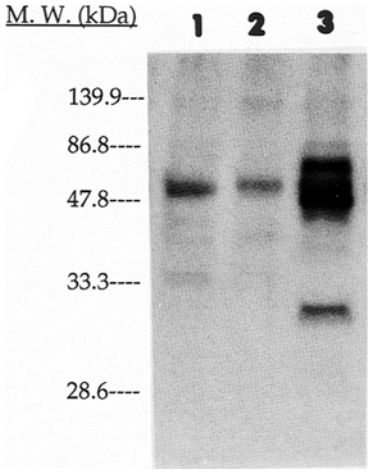

Fig. 11. Immunoblot of tubulin in GH_3 cells. GH_3 cells were incubated with colchicine (1 μ*M*) or taxol (1 μ*M*) at 37°C for 24 h. After the incubation, polymerized tubulin fraction was prepared. Protein from control or drug-treated polymerized tubulin fractions (10 μg) were separated on 12% SDS-PAGE and the proteins were transferred to PVDF membranes. PVDF membranes were incubated with polyclonal antitubulin antibodies followed by incubation with [^{125}I]labeled second antibody. After washing, the membranes were exposed to X-ray films at –70°C for 24 h. Lane 1, control; Lane 2, colchicine; Lane 3, taxol.

Colchicine, purified tubulin, tubulin antibodies and $G_{q\alpha}$ antibody only modestly inhibited [^{3}H]TRH binding to GH_3 cell membranes; the inhibition ranged from 7–26%. This partial inhibition of [^{3}H]TRH binding does not explain the complete suppression of TRH-stimulated GTPase activity by these compounds. These results suggest that these compounds act at a site beyond hormone-receptor interaction to bring about their complete inhibition of TRH-stimulated GTPase activity. However, an inhibitory effect of the compounds on receptor activation after TRH binding cannot be ruled out.

In vitro tubulin assembly into microtubules is inhibited by colchicine (Hamel, 1990; Wilson and Jordan, 1994) and tubulin antibodies (Pepper and Brinkley, 1979; Arevalo et al., 1990), and promoted by taxol (Hamel, 1990; Wilson and Jordan, 1994). However, irrespective of their effect on tubulin function, all these compounds adversely influenced TRH receptor-G protein coupling. Before any conclusions are drawn from these observations, it is important to understand the behavior of tubulin in a membrane environment. Tubulin consists of two closely related and tightly linked polypeptides called α-tubulin and β-tubulin. The tubulin heterodimers can polymerize into linear protofilaments which are roughly 2 nm in diameter and assemble into a complete microtubule, which is a hollow cylinder made up of 13 protofilaments (Engelborghs, 1990). Since the diameter of a microtubule is 25 nm and the plasma membrane is 9–11 nm thick, it is not possible for the membrane-associated tubulin to assemble into a complete microtubule in the membrane environment. However, tubulin heterodimers may be able to function in the membrane environment as individual heterodimers or as polymers arranged into short protofilaments. Visualization of heterodimeric or protofilamentous forms of tubulin in association with membranes may prove to be difficult using electron microscopy. Using immunofluorescence and two-dimensional gel electrophoresis, the presence of membrane-associated tubulin in GH_3 cells was recently demonstrated (Ravindra et al., 1994).

We recently reported that antitubulin antibodies potentiated acetylcholine-stimulated GTPase activity in rat striatal membranes (Ravindra and Aronstam, 1990a). In contrast, in the present study, tubulin antibodies inhibited TRH-stimulated GTPase activity. Viewed in the light of previous observations that tubulin binds to G proteins (Higashi and Ishibashi, 1985; Popova et al., 1994), these apparently contradictory observations might be interpreted. Tubulin-G protein interaction might be perturbed in the presence of tubulin antibodies, leading to a destabilization of hormone receptor-G protein interaction. In some tissues, such as the rat striatum, this destabilization can cause excessive activation of G protein(s) by an agonist (Ravindra and Aronstam, 1990a), and in other systems, such as the GH_3 cells, it can depress the activation of G protein(s) by the hormone.

Data presented in Fig. 11 show the crossreactivity of polyclonal antibodies with a 50-kDa protein (presumably tubulin) present in GH_3 cell cytosol; similar results were obtained with the use of α or β tubulin antibody, indicating that these monoclonal antibodies crossreact with tubulin in the cytosol. However, in GH_3 cell membranes, it is not clear whether the α antibody crossreacts with α-tubulin only and β antibody crossreacts with β-tubulin alone. Using two-dimensional gel electrophoresis, we have demonstrated that the amount of tubulin associated with the membrane fraction in GH_3 cells is very low (Ravindra et al., 1994). Owing to the extremely low amounts of the tubulin subunits present, it was difficult to demonstrate the crossreactivity of α or β tubulin antibody with the tubulin subunits present in the two-dimensional gels obtained with GH_3 cell membranes.

The reasons for the antagonistic action of TRH on α-tubulin antibody-stimulated GTPase activity are not clear. One hundred twenty-five ng of α-tubulin antibody alone stimulated the enzymatic activity by up to 40%; when 10 n*M* TRH were added to the reaction mixture, the stimulation of the GTPase activity by α-tubulin antibody was abolished. It appears that the presence of both TRH and α-tubulin antibody at the membrane level might reduce the affinity of GTP to G proteins, finally leading to reduced GTP hydrolysis.

Since tubulin antibodies inhibited TRH receptor-G protein coupling, one might have expected purified tubulin to have an opposite effect. In fact, purified tubulin also inhibited hormone receptor-G protein interaction. Addition of

purified tubulin to GH_3 cell membranes may alter the dynamics of an interaction between G_q and endogenous membrane-associated tubulin. Whether tubulin-induced inhibition of TRH-stimulated GTPase activity results from an indirect effect of tubulin interaction with other protein(s) in the membrane remains to be investigated. It appears that, in GH_3 cells, an excess of tubulin in the membrane environment may cause the system to be refractory to hormone stimulation. Interestingly, tubulin-Gpp(NH)p was observed to potentiate β-adrenergic receptor activation of adenylyl cyclase in COS 1 cells (Popova et al., 1994). Popova et al. (1994) proposed that tubulin transfers Gpp(NH)p to $G_{s\alpha}$, resulting in a change in it's conformation; this in turn causes the dissociation of $G_{s\alpha}$ from the βγ subunits and the association of $G_{s\alpha}$ with adenylyl cyclase. Thus, in COS 1 cells as well as in GH_3 cells, addition of exogenous tubulin appears to influence receptor-G protein coupling. However, in view of the fact that tubulin used in our experiments is not complexed with Gpp(NH)p, a different mechanism of tubulin-G protein interaction in GH_3 cells might be considered.

We propose that under normal conditions tubulin protofilaments are associated with the cytoplasmic face of the cell membrane. An interaction of tubulin protofilaments with a G protein modulates hormone receptor-G protein interaction. Any alteration in the length of the protofilaments might influence the equilibrium between tubulin protofilaments and G protein(s). Thus, the length of tubulin protofilaments can be reduced by colchicine or tubulin antibody, and increased by tubulin. This might, in turn, impair the ability of G protein(s) to interact with the hormone receptor (Fig. 12). We recognize that the data presented here are not sufficient to support this working model and that further work should be conducted to prove this model. We hope that our model will stimulate new and exciting studies to delineate tubulin-G protein interaction.

It is interesting that colchicine and taxol did not totally abolish either basal or TRH-stimulated PRL secretion. Taxol and colchicine suppressed basal PRL secretion by about 38 and 34%, respectively. TRH-stimulated PRL secretion was inhibited by colchicine and taxol by 35 and 57%, respectively. This suggests that a component responsible for hormone secretion does not respond to the drugs, and microtubules are only partially involved in PRL secretion. The present results indicate that the effects of colchicine and taxol on PRL secretion might be owing to their action on cytosolic tubulin. This is supported by the observation that, after 24 h incubation with the drugs, the decrease in PRL secretion was accompanied by a change in the amount of polymerized tubulin in GH_3 cells. Polymerized tubulin levels decreased with colchicine and increased with taxol. Interestingly, a smaller molecular weight immunogenic band appeared in the samples from taxol-treated cells. It is not clear whether this is a breakdown product of tubulin.

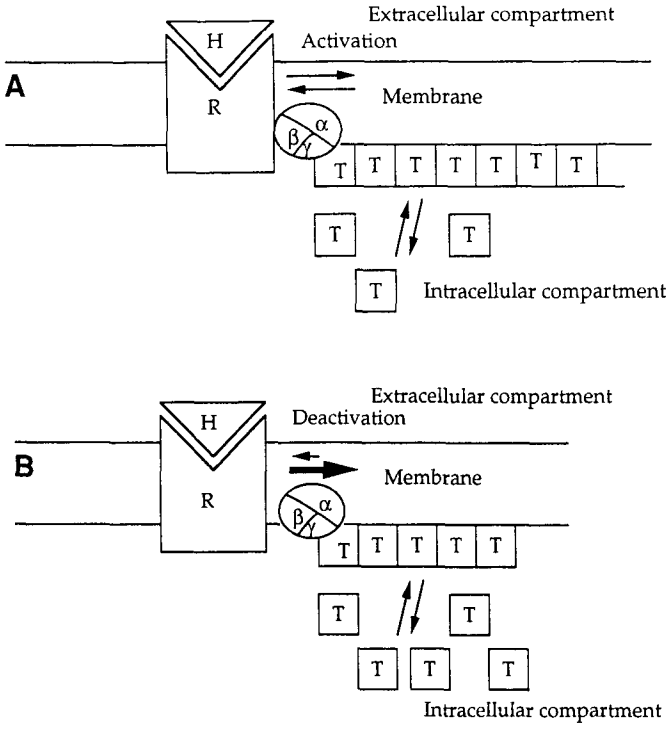

Fig. 12. Model depicting proposed the role of tubulin in hormone receptor-G protein coupling. **(A)** Under normal conditions tubulin protofilaments (T) are associated with the cytoplasmic face of the cell membrane. The binding of tubulin protofilaments to a heterotrimeric G protein (comprised of α-, β-, and γ-subunits) modulates hormone receptor-G protein interaction. **(B)** Any alteration in the length of the protofilaments might influence the equilibrium between tubulin protofilaments and G protein. Thus, the length of tubulin protofilaments can be reduced by colchicine or tubulin antibody, and increased by taxol or tubulin heterodimer. This might, in turn, impair the ability of G protein to interact with the hormone receptor. H, hormone; R, receptor. It should be pointed out that an effect of tubulin on hormone-induced receptor activation *per se* cannot be ruled out.

Taxol is currently used in clinical trials to treat various types of cancer, including breast, ovarian, and gastrointestinal cancer, and acute myeloid leukemia. However, it is not being used to treat prolactinomas or other pituitary tumors (Horwitz, 1994). In view of our observations that taxol inhibits PRL secretion by a rat pituitary tumor cell line, the efficacy of taxol in diminishing prolactin-secreting pituitary tumors might be worthy of consideration.

In conclusion, colchicine, tubulin, and tubulin antibodies suppressed TRH-stimulated GTPase activity in GH_3 cell membranes. These results suggest that tubulin is involved in TRH receptor coupling to a G protein, possibly $G_{q\alpha}$. In addition to disrupting the initial events in TRH-mediated signal transduction, colchicine also inhibited the ultimate outcome of TRH receptor-G protein coupling, which is PRL secretion. Thus, perturbation of the steady state of membrane-associated tubulin-G_q interaction may disrupt TRH receptor-G protein coupling. Further experiments should be conducted to validate this theory.

Materials and Methods

Materials

Thyrotropin releasing hormone (TRH; catalog #P-2161), methyl TRH (catalog #P-5173), colchicine (catalog #C-9754), β-lumicolchicine (catalog #L-0635), monoclonal anti-β-antibodies (catalog #T-4026, clone TUB 2.1), monoclonal anti-α-antibodies (catalog #T-9026, clone DM 1A), and protein A-Sepharose (catalog #P-9424) were purchased from Sigma (St. Louis, MO). Taxol was a kind gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Radiolabeled guanosine-5'-triphosphate ([γ-^{32}P]GTP; 1194 Ci/mmol), TRH ([^{3}H]methyl TRH; 62.8 Ci/mmol), [^{125}I]-goat antirabbit IgG F (ab')$_2$ fragment (8.2 μCi/μg), and [^{125}I]PRL (35.2 μCi/μg) were purchased from DuPont-NEN (Wilmington, DE). All other chemicals used in the present study were purchased from Sigma.

The specificities of α- and β-tubulin antibodies were confirmed by Western blots, solid phase radioimmunoassay, and immunofluorescence. Clone DM 1A (anti-α-tubulin antibody) recognizes α-tubulin; it exhibited a weak crossreactivity with β-tubulin, approx 10% of the activity observed with α-tubulin. Clone TUB 2.1 (anti-β-tubulin antibody), highly specific for β-tubulin, also had a weak secondary activity against α-tubulin (Gozes and Barnstable, 1982; Blose et al., 1984).

Crude IgG fraction of polyclonal antibody generated against C terminal peptides of G_q and G_{11} was generously provided by T. F. J. Martin (University of Wisconsin, Madison, WI). These antibodies were demonstrated to be highly specific to the α-subunits of G_q and G_{11} (Hsieh and Martin, 1992). The IgG fraction was further purified in our laboratory by chromatography on protein A-Sepharose as described previously (Harlow and Lane, 1988). Highly purified tubulin was a kind gift from E. Hamel (NIH, Bethesda, MD; Hamel and Lin, 1984).

Preparation of Membranes

GH_3 cells were maintained at 37°C at a density of 0.17 × 10^5/cm^2 in T-150 flasks containing Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 5% CO_2.

Monolayers of GH_3 cells from 15–20 flasks were rinsed twice with homogenizing TED buffer (5 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, 0.5 mg/mL leupeptine, and 0.5 mg/mL aprotinin), and scraped into 5 mL of TED buffer. Cells were allowed to swell for 10 min on ice and then were homogenized using a motor-driven Teflon-glass tissue grinder. Cell homogenates were centrifuged at 800*g* for 10 min. The supernatant was then centrifuged at 100,000*g* for 60 min. Membrane pellets were resuspended in TED buffer at a concentration of 2–3 mg/mL, and aliquots were kept at –70°C until further use. Protein content was estimated using bovine serum albumin (BSA) as the standard (Bradford, 1976). Membranes were used within 10 d of preparation.

G Protein GTPase Assay

G protein GTPase activity was determined in triplicate as described previously (Ravindra and Aronstam, 1990a). The reaction mixture (100 μL) contained 75 m*M* Tris-HCl (pH 7.4), [γ-^{32}P]GTP (70–100,000 cpm; 40–50 n*M*), 1 μ*M* unlabeled GTP, 2 m*M* $MgCl_2$, 0.5 m*M* ATP, 0.5 m*M* adenylylimidodiphosphate, 5 m*M* phosphocreatine, creatine phosphokinase (50 U/mL), BSA (50 μg), 0.1 m*M* EDTA, 0.2 m*M* EGTA, 1 m*M* cAMP, 100 m*M* NaCl, and 25 μg protein. All of these components were added to 12 × 75 mm tubes on ice, and the reaction was initiated by immersing the tubes in a 37°C water bath. After 10 min, the reaction was stopped by transferring the tubes to an ice bath followed by the addition of 5% activated charcoal (catalog # C 5260, Sigma) in 20 m*M* phosphoric acid (pH 2.5). Samples were kept on ice for 10 min and centrifuged at 800*g* for 10 min. An aliquot (100 μL) from the supernatant was mixed with 5 mL of Scintiverse BD (Fisher Scientific, Pittsburgh, PA) and the radioactivity content determined using a Beckman (Palo Alto, CA) liquid scintillation counter. Low K_m GTPase activity (EC 3.6.1.-) was routinely calculated by subtracting activity measured in the presence of 100 μ*M* unlabeled GTP from total activity (Ravindra and Aronstam, 1993).

The G protein GTPase assay was conducted in an ATP-regenerating system and manipulation of assay conditions such that only 10–20% of added GTP was hydrolyzed. The ATP-regenerating system inhibits GTP hydrolysis by nonspecific nucleosidases and the transfer of phosphate from GTP to ATP. Under these experimental conditions, GTP is hydrolyzed predominantly at the γ position and since GTP is labeled with [^{32}P] at the γ position, the radioactivity that is counted in an aliquot from the supernatant, after charcoal treatment, is a reflection of this hydrolysis. This assay has been validated in our laboratory and it was observed that GTP hydrolysis increased as a function of membrane protein concentration and linear with time between 2 and 10 min.

[^{3}H]TRH Binding to GH_3 Cell Membranes

The binding of [^{3}H]methyl TRH to GH_3 cell membranes was determined using a rapid filtration procedure. Cell membranes (50–100 μg protein) were incubated in a buffer containing 20 m*M* Tris-HCl, 2 m*M* $MgCl_2$ (pH 7.4), and 30,000 cpm of [^{3}H]methyl TRH for 4 h at 4°C. Samples were diluted with ice-cold buffer (20 m*M* Tris-HCl, 2 m*M* $MgCl_2$, 100 m*M* NaCl) and vacuum filtered through glass fiber filters (#32, Schleicher and Schuell, Keene, NH). The filters were placed in a scintillation counting vial, mixed with 5 mL of Scintiverse BD, and the radioactivity content determined using a Beckman liquid scintillation counter.

Incubation of GH_3 Cells with Colchicine and Taxol

GH_3 cells were grown in 6-well, flat bottom plates (Falcon, Catalog # 3046, Beckton Dickinson, Lincoln Park, NJ) at a density of 10^6/well. Just before the addition of colchicine or taxol, the cells were washed twice with serum-free

medium containing 0.25% BSA, and incubated with the same medium containing drugs. After the incubation, medium was collected and kept frozen at –70°C until further analysis. The cells were washed twice with serum-free medium containing 0.25% BSA, challenged with 100 n*M* TRH, and medium collected and kept frozen at –70°C until further analysis.

PRL levels were estimated in the medium collected from individual wells. The concentration of prolactin (PRL) in the medium was determined by a double antibody radioimmunoassay using National Hormone and Pituitary Program reagents and procedures. Determinations were made in duplicate at two dilutions and all samples from an experiment were measured in one assay. The antibody to prolactin was NIDDK-rPRL-I-6 and NIDDK-rPRL-RP-3 served as the reference standard. The intra- and interassay coefficients were 8 and 12%, respectively.

Isolation of Polymerized Tubulin Fractions

Cells were homogenized at room temperature in MES buffer (100 m*M* morpholinoethane sulfonic acid, 2 m*M* $MgCl_2$, 50 m*M* KCl, and 4 m*M* EGTA, pH 6.8) with a motor-driven Teflon pestle (Kontes, Vineland, NJ). The homogenate was spun at 100,000*g* for 60 min at 27°C. The supernatant contained monomeric tubulin and was designated the "soluble tubulin fraction," and stored at –70°C until further use. The pellet was resuspended in MES buffer (pH 6.8), and kept at 4°C for 60 min. Exposure to cold temperatures results in the depolymerization of microtubules present in the pellet. The suspension was then spun at 100,000*g* for 60 min at 4°C, and the supernatant was designated the "polymerized tubulin fraction," and stored at –70°C until further use (Ravindra and Grosvenor, 1988). Protein content was estimated in both fractions using bovine serum albumin (BSA) as the standard (Bradford, 1976).

Immunoblot Analysis of Tubulin Levels

The proteins in the polymerized tubulin fraction were separated by electrophoresis using 12% SDS-PAGE gels (Laemmli, 1970), and proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) using a Trans-blot apparatus (Bio-Rad). The membranes were incubated with polyclonal antitubulin antibodies (Chemicon, CA) followed by incubation with [^{125}I]labeled second antibody. After washing, the membranes were exposed to x-ray films and the autoradiographs were developed.

Data Analysis

Statistical differences among the groups were determined by analysis of variance and Fisher tests using the Statview II program on a Macintosh IIci computer.

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