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Thyrotropin-releasing hormone stimulates GTP hydrolysis by membranes from GH_4C_1 rat pituitary tumor cells

(peptide hormone/GTPase/receptor/lactotroph/hypothalamic releasing factor)

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ABSTRACT **Thyrotropin-releasing hormone (TRH) stimulates prolactin production by GH_4C_1 rat pituitary tumor cells, which possess high-affinity membrane receptors for the peptide. TRH caused up to a 50% increase in the activity of a low-K_m GTPase in membranes from GH_4C_1 cells. The TRH stimulatory effect was maximal at GTP concentrations of 1 μM or lower. TRH caused an increase in GTPase activity of between 0.2 and 20 pmol of GTP hydrolyzed per mg of protein per min, depending on GTP concentration, while TRH binding was 0.3 pmol/mg of protein. TRH did not stimulate GTPase activity in membranes from GH_12C_1, or GH-Y cells, two pituitary lines lacking TRH receptors. Stimulation of GTPase depended on occupancy of the TRH receptor; half-maximal increases in GTPase activity required 46 nM TRH and 25 nM [N^3-methyl-His]TRH, but the TRH free acid was inactive. The apparent K_ds of these peptides for receptors were similar when measured under the same conditions. The fact that TRH binding to receptors is regulated by guanyl nucleotides, together with the demonstration of TRH stimulation of low-K_m GTPase activity, suggests that the TRH receptor is associated with a guanyl nucleotide regulatory protein in the lactotroph membrane.**

The hypothalamic tripeptide thyrotropin-releasing hormone (TRH) stimulates the secretion and synthesis of thyrotropin and prolactin by the anterior pituitary gland (1, 2). Clonal lines of rat pituitary tumor mammotrophs, collectively termed GH cells, have been used extensively as model systems for investigating the mechanism of action of TRH (3). TRH initiates its actions on GH cells by binding to specific membrane receptors, which have been extensively characterized with both intact cells and isolated membranes (3–5). When intact cells are exposed to TRH, there is a rapid increase in the rate of metabolism of phosphoinositides (6, 7) and an increase in cytosolic free calcium ion concentration (8, 9). These effects result from receptor occupancy and are thought to be important in generating the biological response in these cells, stimulation of prolactin release and synthesis. TRH can also increase intracellular cyclic AMP concentrations under some conditions, but it has not been established that this effect precedes TRH-induced hormone release (10, 11). Treatment of intact cells with TRH causes changes in the pattern of protein phosphorylation that are clearly different from those observed when GH cells are exposed to hormones and drugs known to increase cyclic AMP (12, 13). Under different experimental conditions TRH either has no effect upon (6, 14) or increases (15) adenylate cyclase activity of pituitary cell membranes. Gautvik *et al.* (15) recently reported that TRH can stimulate GH-cell adenylate cyclase; however, they found that stimulation required higher concentrations of TRH and related peptides than receptor binding, and higher calcium concentrations than those found in the cytosol of GH cells (9). It has not, therefore, been established that cyclic AMP plays a central role in TRH action. Despite the progress that has been made in defining the events that follow TRH binding in the intact cell, it has been difficult to demonstrate any responses to this tripeptide in a broken cell system.

In isolated membranes, TRH binding is regulated by guanyl nucleotides and sodium ion (16, 17). GTP and the nonhydrolyzable analog 5′-guanylyl imidodiphosphate inhibit TRH binding up to 70% in membranes prepared from GH_4C_1 tumor cells with IC_{50} values of 0.3 μM (17). Guanyl nucleotides decrease the affinity of TRH for its receptor by a factor of 3 without altering the number of binding sites. GTP accelerates dissociation of TRH from membrane receptors but has no effect on the rate of dissociation of the peptide from detergent-solubilized receptors. These results raise the possibility that the TRH receptor is coupled to a GTP-binding regulatory protein in the membrane of the pituitary mammotroph.

Guanyl nucleotides regulate the binding of numerous hormones and neurotransmitters to their respective receptors (18–20). In the case of receptors coupled to adenylate cyclase, these guanyl nucleotide effects result from interaction of the receptor with GTP binding components of adenylate cyclase that transmit the signal from the receptor to the catalytic moiety. Receptors for hormones that stimulate adenylate cyclase are associated with a GTP-binding protein termed N_s, while receptors for hormones that inhibit adenylate cyclase are associated with a GTP-binding protein termed N_i. As shown by Cassel and Selinger for β-adrenergic agents, ligand binding may stimulate GTP hydrolysis (21). Hormonal activation of receptors coupled to both N_s [(e.g., β-adrenergic agents (21, 22), glucagon (23), and pancreozymins (24)] and to N_i [(e.g., α_2-adrenergic agents (25) and opiates (26)] stimulate GTP hydrolysis and stimulate the release of bound GDP or 5′-guanylyl imidodiphosphate.

Because of the data suggesting that the TRH receptor may be coupled to a GTP-binding protein, we investigated the effect of this hypothalamic releasing factor on GTPase activity in GH_4C_1 cells, a clonal rat pituitary line that secretes growth hormone and prolactin and responds to TRH with an increase in prolactin synthesis and release (3, 5, 6). In this report we demonstrate that TRH stimulates GTP hydrolysis by membranes prepared from GH_4C_1 cells.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture media were obtained from GIBCO, sera from Microbiological Associates, and tissue culture plasticware from Costar, Cambridge, MA. [α-^{32}P]- and [γ-^{32}P]GTP (13–30 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham, and [^{3}H]TRH (100 Ci/mmol) and

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Abbreviation: TRH, thyrotropin-releasing hormone.

[^{3}H][N^3-methyl-His]TRH (76 Ci/mmol) from New England Nuclear. Creatine phosphate, creatine kinase (rabbit muscle type I), ouabain, and unlabeled nucleotides were from Sigma. TRH (which is an amide), [N^3-methyl-His]TRH, and the TRH free acid were gifts from Abbott. The GH_4C_1 and GH_12C_1 cell lines were kindly provided by Armen H. Tashjian, Jr. (Harvard Medical School and Harvard School of Public Health), and the GH-Y line was provided by Priscilla S. Dannies (Yale University School of Medicine).

Methods. Cells were grown in monolayer culture as previously described (27). Dishes (100 mm) were rinsed twice with 0.15 M NaCl and the cells in each dish were scraped into 5 ml of 20 mM Tris·HCl/2 mM $MgCl_2$, pH 7.6 (Tris/Mg buffer), at 0°C. The cells were allowed to swell for at least 10 min and then lysed by Dounce homogenization and centrifuged for 10 min at 7500 × *g*. The pellet was resuspended in Tris/Mg buffer at a protein concentration of 0.5–2 mg/ml.

GTPase activity was determined by a modification (26) of the procedure described by Cassel and Selinger (21). Reaction mixtures contained, in a final volume of 100 μl: 25 mM Tris·HCl, 6 mM $MgCl_2$, 1 mM ouabain, 10 mM creatine phosphate, 5 units of creatine kinase, 0.1 or 1 mM 5′-adenylyl imidodiphosphate, 1 mM ATP, 2 mM dithiothreitol, 0.1 mM EDTA, and [γ-^{32}P]GTP, unlabeled GTP, and membrane protein as indicated in the text, at pH 7.6. Reactions were initiated by the addition of cell membranes and proceeded for 10–20 min at 20–37°C. The reaction was terminated by placing the tubes on ice and adding 0.9 ml of 5% charcoal (Norit) in 6.7 mM phosphoric acid, pH 2.3. Tubes were allowed to sit at 0°C for 5–10 min and then centrifuged at 1500 × *g* for 5 min. A 500-μl aliquot of the supernatant fluid containing released $^{32}P_i$ was removed and radioactivity was determined by liquid scintillation counting. All experiments included a blank lacking cell membranes; the supernatant fluid of the blank contained 0.15–1% of the added radioactivity, which was at most 10% of the amount of $^{32}P_i$ released in the presence of membranes. In most experiments parallel reactions were run in the presence of [γ-^{32}P]GTP with and without 100 μM unlabeled GTP.

The purpose of the ATP, 5-adenylyl imidodiphosphate, and ATP-regenerating system is to minimize the hydrolysis of [γ-^{32}P]GTP by nonspecific nucleoside triphosphatases and to inhibit the exchange of ^{32}P from GTP to ATP. The system effectively minimized nucleoside triphosphatase activity; in the absence of the above ingredients, the rate of [γ-^{32}P]GTP hydrolysis was increased 10-fold and 5-fold at total GTP concentrations of 0.5 and 50 μM, respectively. In subsequent experiments we have found that [γ-^{32}P]GTP hydrolysis can be reduced just as effectively by either 1 mM ATP or 1 mM 5-adenylyl imidodiphosphate as by the systems used for experiments described here. To determine whether there was any exchange of ^{32}P from GTP to ATP, the products of reactions in mixtures containing 0.05 and 5 μM [γ-^{32}P]GTP with and without 100 nM TRH were analyzed by thin-layer chromatography on poly(ethyleneimine) plates in a solvent system containing a 1:1 mixture of 2 M LiCl and 2 M formic acid (28). There was no detectable incorporation of ^{32}P into ATP under any of the conditions tested. The products of reactions that took place under standard conditions with [α-^{32}P]GTP as the label were also analyzed chromatographically. At least 85% of the reaction product migrated with marker GDP, and the remaining radioactivity migrated with GMP. In reaction mixtures containing TRH and [α-^{32}P]GTP, 85% of the product was radioactive GDP, as in control reactions.

To measure the affinity of peptides for TRH receptors, membranes were incubated with [^{3}H]TRH or [^{3}H][N^3-methyl-His]TRH. The reaction mixtures were diluted to 2 ml with Tris/Mg buffer at 0°C and passed through a 25-mm Millipore filter (0.45-μm pore diameter). The filter was rinsed three times with 2 ml of cold Tris/Mg buffer to trap receptor-bound [^{3}H]TRH (4). Filters were dried and their radioactivities were measured in toluene-based scintillation fluid.

Values shown in the text and in figures are the mean and SEM of triplicate determinations. Statistical evaluations were performed with Student's *t* test. Each result has been obtained in a minimum of three experiments. Proteins were determined by the method of Lowry *et al.* (29), using bovine serum albumin as standard.

RESULTS

Extracts of GH_4C_1 cells contain both low-K_m and high-K_m GTPase activities. The rate of hydrolysis of 0.1 μM [γ-^{32}P]GTP was determined at three concentrations of GH_4C_1 membranes ranging from 40 to 360 μg/ml (Fig. 1, solid lines). Under these conditions both low-K_m and high-K_m GTPases contribute to GTP hydrolysis. To measure the contribution of high-K_m enzymes to the total rate, parallel reactions were performed in the presence of 0.1 μM [γ-^{32}P]GTP and 100 μM unlabeled GTP (Fig. 1, broken lines). High-K_m GTPases accounted for 40% of the total [γ-^{32}P]GTP hydrolysis; when the unlabeled GTP concentration was raised to 1–2 mM, [γ-^{32}P]GTP hydrolysis was reduced by 90% (data not shown). Low-K_m GTPase is defined as the difference between the rates of [γ-^{32}P]GTP hydrolysis in the presence and absence of 100 μM unlabeled GTP. The initial rates of [γ-^{32}P]GTP hydrolysis were linearly related to the protein concentration over the range tested, as shown in the *Inset* to Fig. 1. All experiments were performed with protein concentrations and reaction times selected to ensure that [γ-^{32}P]GTP hydrolysis was in the linear portion of the curve—i.e., substrate utilization of less than 25%.

TRH caused a 40% increase in low-K_m GTPase activity in GH_4C_1 cell membranes (Fig. 2). In this experiment TRH was added simultaneously with [γ-^{32}P]GTP; thus, there was no appreciable lag in the hormonal stimulation of GTP hydrolysis. TRH has no effect upon or decreases slightly (5% or less) high-K_m GTPase activity. The extent of TRH stimulation of low-K_m GTPase activity varied from as little as 10% to a maximum of 50% in different experiments. Analysis of the reaction products confirmed that TRH stimulated the hy-

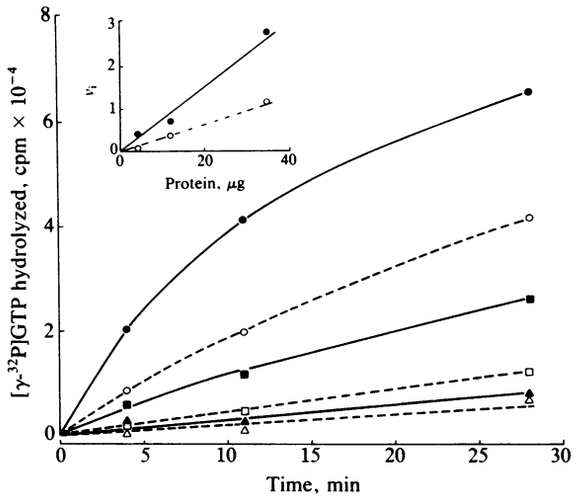

FIG. 1. Rate of [γ-^{32}P]GTP hydrolysis. GTPase activity was measured at 22°C in reaction mixtures containing 4 μg (△, ▲), 12 μg (□, ■), or 36 μg (○, ●) of GH_4C_1 membrane protein. The filled symbols and solid curves show the rate of hydrolysis of 0.1 μM [γ-^{32}P]GTP (18,500 cpm/pmol), or total GTPase activity. The open symbols and broken lines show the rate of hydrolysis of [γ-^{32}P]GTP in reaction mixtures that included 100 μM unlabeled GTP, representing high-K_m GTPase. (*Inset*) Initial velocity, v_i, as cpm × 10^{-4}/5 min, versus protein concentration.

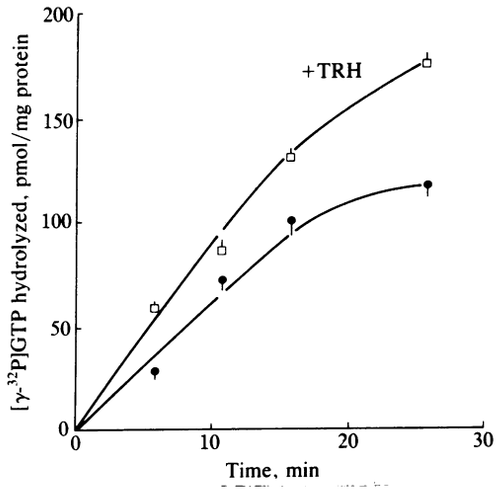

FIG. 2. Effect of TRH on low-K_m GTPase activity. The rate of hydrolysis of 0.9 μM [γ-^{32}P]GTP was measured at 22°C in reaction mixtures containing 11.4 μg of protein to give total GTPase activity. Either buffer alone (●) or 100 nM TRH (□) was added at zero time. High-K_m GTPase activity was determined in a parallel set of reaction mixtures that contained 200 μM unlabeled GTP. High-K_m activity was approximately 50% of the total under all conditions and has been subtracted to yield low-K_m activity. Values shown are the mean ± SEM of triplicate determinations. Low-K_m GTPase activity in the tubes containing TRH was significantly greater than control ($P < 0.02$) at all times except 11 min.

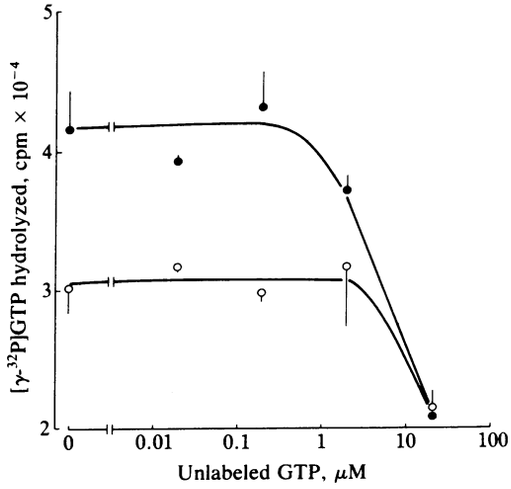

FIG. 3. Effect of GTP concentration on TRH stimulation. The rate of hydrolysis of 0.05 μM [γ-^{32}P]GTP (6500 cpm/pmol) was determined in a 15-min incubation at 22°C. Reaction mixtures contained the indicated concentrations of unlabeled GTP and either no additions (○) or 100 nM TRH (●). Hydrolysis of [γ-^{32}P]GTP was decreased to 2000 cpm in reaction mixtures containing 2 mM unlabeled GTP. Values shown are the mean ± SEM of triplicate determinations. GTPase activity was significantly greater than control in reactions in the presence of TRH ($P < 0.02$) in all reaction mixtures containing less than 2 μM unlabeled GTP.

drolysis of GTP to GDP. The effect of TRH in stimulating GTP hydrolysis was limited to the particulate fraction of broken cell preparations. When [γ-^{32}P]GTP (0.01 μM) was added to intact GH_4C_1 cells, the rates of GTP hydrolysis (0.005 pmol/mg·min low-K_m activity, 0.03 pmol/mg·min total activity) were much less than those in membranes, and [γ-^{32}P]GTP hydrolysis was not stimulated by TRH. Since GTP is not expected to penetrate the plasma membrane, this result confirms that the TRH-responsive GTPase is not on the external surface of the pituitary cell.

In other systems in which hormones activate low-K_m GTPase, the relative hormonal effect can be augmented by adding sodium ion, increasing the magnesium concentration, or treating membranes with *N*-ethylmaleimide (21, 26). The TRH effect on low-K_m GTPase was not enhanced by varying NaCl from 0 to 100 mM or $MgCl_2$ from 1 to 10 mM or by preincubating membranes with 10 mM *N*-ethylmalemide for 30 min at 0°C. We did find, however, that TRH sometimes caused a larger increase in low-K_m GTPase activity when the membrane-containing fractions sat on ice before enzyme assay. For example, in an experiment in which TRH stimulated low-K_m GTPase 12% initially, the peptide increased activity 35% at 4 hr and 56% at 24 hr. This increase in the TRH effect was not prevented by protease inhibitors. All of the experiments described here were performed with membranes prepared within a few hours of the assay.

The ability of TRH to increase the hydrolysis of 0.05 μM [γ-^{32}P]GTP was determined in reaction mixtures containing increasing concentrations of unlabeled GTP (Fig. 3). The TRH effect was greatest at GTP concentrations below 1 μM, and a half-maximal stimulation was obtained at 1.8 μM unlabeled GTP. When the concentration of [γ-^{32}P]GTP was varied, half-maximal TRH stimulation occurred at 0.3 μM [γ-^{32}P]GTP. The "apparent K_m" for TRH-stimulated GTPase has varied in four independent experiments from 0.3 to 1.8 μM.

The dependence of low-K_m GTPase activity on the concentration of TRH and structural analogs of the tripeptide is shown in Fig. 4. TRH, (<Glu-His-Pro-NH_2) caused a half-maximal increase in [γ-^{32}P]GTP hydrolysis at approximately 46 nM, and the potent agonist [N^3-methyl-His]TRH exhibited an ED_{50} of 25 nM. The TRH free acid was ineffective. Because temperature and guanyl nucleotides alter TRH binding in GH_4C_1 membranes (4, 17), the affinity of the peptides for receptors was measured in a competition displacement experiment performed under the conditions of the GTPase assays (Fig. 5). Binding of 5 nM [^{3}H][N^3-methyl-His]TRH reached equilibrium within 5 min under these conditions, and 50% receptor occupancy required 1 min (data not shown). TRH and [N^3-methyl-His]TRH caused 50% displacement of bound [^{3}H]TRH at concentrations of 86 and 19 nM, respectively, and the TRH free acid did not bind signifi-

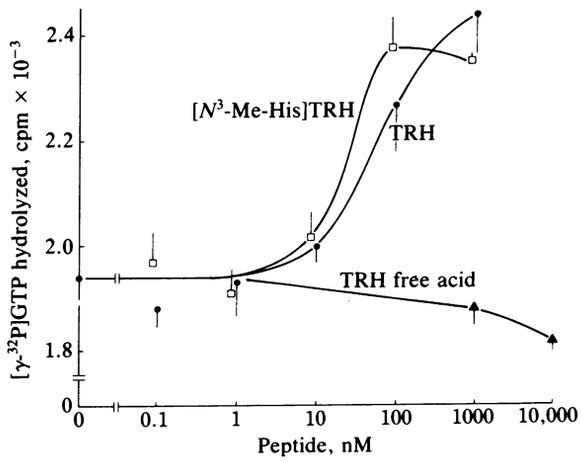

FIG. 4. Dependence of low-K_m GTPase activity on peptide concentration. Hydrolysis of 0.2 μM [γ-^{32}P]GTP (6500 cpm/pmol) was determined in a 20-min incubation at 22°C in reaction mixtures containing the indicated concentration of TRH (●), [N^3-methyl-His]TRH (□), or TRH free acid (<Glu-His-Pro) (▲). High-K_m GTPase activity, determined in the presence of 100 μM unlabeled GTP, averaged 600 cpm and has been subtracted. Values shown are the mean ± SEM of three to six determinations. Similar dose–response curves have been obtained in three independent experiments.

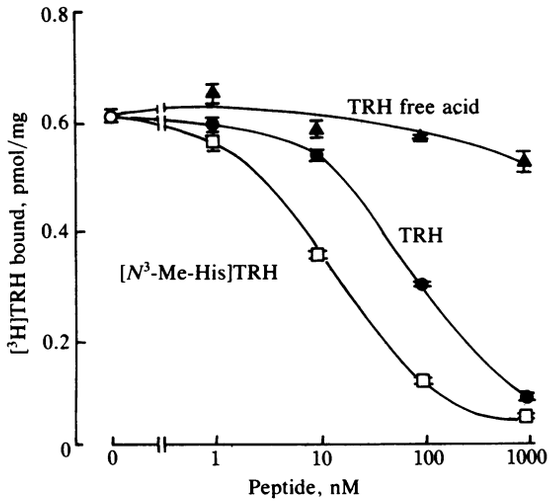

FIG. 5. Binding of peptides under GTPase assay conditions. GH_4C_1 membranes (53 μg/100-μl assay) were incubated for 20 min at 22°C with 20 nM [^{3}H]TRH and the indicated concentrations of TRH (●), [N^3-methyl-His]TRH (□), or TRH free acid (▲) under the conditions of the GTPase assay in Fig. 4 before the determination of receptor-bound [^{3}H]TRH. Values shown are the mean ± SEM of triplicate determinations.

cantly to receptors. The apparent affinities of peptides for receptors are lower in the GTPase assay mixture than those obtained in Tris/Mg buffers at the same temperature (4).

To determine whether TRH stimulation of GTPase is specific, we studied two pituitary cell lines that lack TRH receptors. GH_12C_1 cells were obtained from an earlier passage of the same tumor that gave rise to the GH_4C_1 line. The GH_12C_1 line secretes growth hormone but does not bind or respond to TRH (30). GH-Y cells arose spontaneously from GH_4C_1 cells; they secrete growth hormone and prolactin but lack TRH receptors and responses (31). As shown in Table 1, membranes prepared from GH_4C_1 and GH-Y cells exhibit similar low-K_m GTPase activities but TRH stimulates activity only in GH_4C_1 membranes. [^{3}H]TRH binding experiments confirmed that the GH_12C_1 and GH-Y lines lack specific TRH receptors.

DISCUSSION

The results presented here demonstrate that TRH stimulates a low-K_m GTPase in membranes prepared from GH_4C_1 rat pituitary tumor cells. Stimulation of GTP hydrolysis apparently results from the interaction of TRH with its specific receptor. The parent TRH molecule and the potent agonist [N^3-methyl-His]TRH caused half-maximal stimulation of low-K_m GTPase at concentrations of 46 and 25 nM, and under identical conditions they caused 50% displacement of bound [^{3}H]TRH at concentrations of 86 and 19 nM, respectively; the TRH free acid neither stimulated GTPase nor bound to receptors. These results suggest that stimulation of GTP hydrolysis is coupled tightly to receptor occupancy. This conclusion is supported further by the observation that TRH did not stimulate GTPase from two cell lines lacking TRH receptors.

It is likely that guanyl nucleotide effects on TRH binding and the effect of TRH to stimulate low-K_m GTPase both result from the interaction of the TRH receptor with the same guanyl nucleotide-binding moiety in the lactotroph membrane. TRH stimulation of GTPase occurred at GTP concentrations similar to those inhibiting [^{3}H]TRH binding (17). Half-maximal TRH stimulation of low-K_m GTPase occurred at 0.3–1.8 μM GTP, while half-maximal inhibition of binding required 0.3 μM GTP in the presence of a GTP-regenerating system. The increase in low-K_m GTPase caused by TRH is small, at most 50%. This is anticipated because the TRH receptor is presumably coupled to only a small fraction of membrane GTPase molecules. The increase in GTP hydrolysis caused by TRH ranges from less than 1 to 20 pmol/mg·min, depending on the GTP concentration, corresponding to 1 to 50 molecules of GTP hydrolyzed per min per receptor occupied.

In other systems in which activation of a receptor leads to stimulation of GTP hydrolysis, the function of the guanyl nucleotide binding protein is to transduce a signal from the receptor to an enzyme: for example, β-adrenergic agents stimulate adenylate cyclase via N_s; α_2-adrenergic agents inhibit adenylate cyclase via N_i; and light activation of rhodopsin stimulates cyclic GMP phosphodiesterase via transducin (18–20, 32). The GTP-binding proteins N_s, N_i, and transducin are composed of α, β, and γ subunits, and there is striking structural homology or perhaps identity in the β subunits of all of these guanyl nucleotide regulatory proteins (33, 34).

The present data do not establish whether the TRH receptor is associated with N_s, N_i, or perhaps a different guanyl nucleotide regulatory protein. As discussed above, both calcium ion and cyclic AMP have been proposed as intracellular mediators of TRH action. The involvement of guanyl nucleotide binding proteins in the hormonal regulation of adenylate cyclase is well established. Recently, it has been suggested that calcium mobilization may also involve a GTP-binding protein (35). Additional data are necessary to establish the nature of the TRH receptor–GTP protein interaction and to determine its physiological significance. The findings that guanyl nucleotides regulate TRH binding and that TRH stimulates a low-K_m GTPase in isolated membranes should provide a basis for subsequent efforts to study the biochemical mechanism of TRH action in cell-free systems.

Table 1. Specificity of TRH stimulation of GTPase

Cell line	Low-K_m GTPase, pmol/mg·min			[^{3}H][N^3-methyl-His]TRH bound, pmol/mg
	No TRH	With TRH	Difference	
GH_4C_1	0.893 ± 0.015	1.123 ± 0.021	0.230 ± 0.026 ($P < 0.01$)	0.278 ± 0.003
GH_12C_1	1.828 ± 0.036	1.742 ± 0.028	−0.086 ± 0.046 (NS)	0.005 ± 0.003
GH-Y	0.985 ± 0.014	0.956 ± 0.008	−0.029 ± 0.016 (NS)	0.004 ± 0.004

Membranes were prepared from GH_4C_1, GH_12C_1, and GH-Y cells. For measurement of total GTPase activity, membranes (19–24 μg of protein) were incubated for 30 min with 0.05 μM [γ-^{32}P]GTP with or without 1 μM TRH. High-K_m GTPase was measured in parallel reaction mixtures containing 100 μM unlabeled GTP. High-K_m GTPase was subtracted to give low-K_m GTPase; high-K_m activity was 45, 57, or 23% of total, respectively for GH_4C_1, GH_12C_1, and GH-Y cells. Results are mean ± SEM of four determinations. NS, not significant. The concentration of TRH receptors was determined by incubating membranes (56–285 μg per reaction) with a saturating concentration of [^{3}H][N^3-methyl-His]TRH, 10 nM, for 30 min. Nonspecific binding was determined in parallel reaction mixtures containing 1 μM unlabeled TRH; it was between 120 and 160 cpm, 8% of the total for GH_4C_1 cells. Results are mean ± SEM of triplicates.

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