Thyroliberin action in pituitary cells is not inhibited by pertussis toxin

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The effects of pertussis toxin on the responses of rat pituitary-tumour (GH) cells to thyrotropin-releasing hormone (thyroliberin, TRH) were examined. Treatment of cells with pertussis toxin did not alter the affinity or concentration of TRH receptors, or the sensitivity of the TRH receptor to inhibition by guanine nucleotides. TRH caused an increase in low- K_m GTPase activity in membrane-containing fractions from both control and pertussis-toxin-treated cells. TRH stimulation of inositol phosphate formation was insensitive to pertussis toxin. TRH caused a biphasic increase in the concentrations of cytosolic free Ca^{2+} as monitored by intracellularly trapped Quin 2, and this increase was the same in control and toxin-treated cultures. The toxin did not alter the increase in prolactin and growth-hormone (somatotropin) release stimulated by TRH or shift the TRH dose-response curve, and it did not affect the TRH-induced rise in prolactin synthesis measured over 24 h. However, pertussis toxin did block the ability of somatostatin and muscarinic agonists to inhibit prolactin and growth-hormone secretion stimulated by vasoactive intestinal peptide when analysed under the same conditions as those in which the TRH system was unaffected. These data indicate that the guanine nucleotide effects on TRH binding and activity are not mediated by N_i , but possibly by another member of the family of guanine-nucleotide-dependent regulatory proteins.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) stimulates the release and synthesis *de novo* of prolactin from the clonal lines of rat pituitary cells (GH cells). The tripeptide binds to specific high-affinity receptors on target cells (Hinkle & Tashjian, 1973) and within seconds causes an increase in the metabolism of phosphoinositides and a rise in $[Ca^{2+}]_i$ (reviewed by Gershengorn, 1986). These are thought to be key events in the stimulation of hormone release and, perhaps, synthesis; a likely consequence of increased phosphoinositide turnover is the activation of protein kinase C (Nishizuka, 1984). Guanine nucleotides decrease the affinity of membrane receptors for TRH by as much as 70%, and GTP and the non-hydrolysable GTP analogue p[NH]ppG exert half-maximal effects at sub-micromolar concentrations (Hinkle & Kinsella, 1984). TRH binding to GH-cell membranes is inhibited by Na^+ and Li^+ , but not substantially by K^+ (Hinkle & Kinsella, 1984). TRH stimulates the hydrolysis of GTP by a low- K_m GTPase only in membranes from TRH-responsive clones (Hinkle & Phillips, 1984). These data strongly suggest that the TRH receptor is coupled to a GTP-binding protein in the membrane. Lastly, GTP and TRH act synergistically to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate in plasma membranes isolated from GH cells (Lucas *et al.*, 1985; Straub & Gershengorn, 1986).

A number of guanine-nucleotide-binding regulatory membrane proteins have been described: N_s , N_i , N_o and transducin. These proteins are structurally related, and comprise similar or identical β subunits but distinct α and

γ subunits (Sternweis & Robishaw, 1984; Smigel *et al.*, 1985). Receptors coupled to adenylate cyclase transmit their signals to the enzyme via either N_s (stimulatory agonists) or N_i (inhibitory agonists), whereas in the retina light-activated rhodopsin activates cyclic GMP phosphodiesterase via transducin. The function of N_o , which is found in high quantity in brain, has not been established. It is not known whether the TRH receptor is coupled to one of these proteins.

A bacterial toxin from *Bordetella pertussis* (pertussis toxin or islet-activating protein) has provided valuable insight into the function of N_i (reviewed by Hewlett *et al.*, 1984; Jakobs *et al.*, 1984). Pertussis toxin catalyses the ADP-ribosylation of the α subunit of N_i , N_o and transducin (Hewlett *et al.*, 1984; Jakobs *et al.*, 1984; Sternweis & Robishaw, 1984; Smigel *et al.*, 1985). Pertussis toxin functionally inactivates receptors that inhibit adenylate cyclase, such as α_2 -adrenergic and muscarinic agents, opiates, dopamine, adenosine and somatostatin (SRIF) (Hewlett *et al.*, 1984; Jakobs *et al.*, 1984), consistent with the role of N_i in signal transduction for inhibitory agents. It has also been shown that pertussis toxin prevents agonist activation of Ca^{2+} fluxes and polyphosphoinositide turnover in neutrophils and mast cells (Okajima & Ui, 1984; Molski *et al.*, 1984; Bokoch & Gilman, 1984; Nakamura & Ui, 1984, 1985; Okajima *et al.*, 1985; Volpi *et al.*, 1985; Lad *et al.*, 1985). In contrast, pertussis toxin does not prevent the stimulation of phosphoinositide turnover elicited by muscarinic agonists in heart cells (Masters *et al.*, 1985), or regulation of muscarinic receptors by guanine nucleotides in neural cells (Evans *et al.*, 1985). These data

Abbreviations used: TRH, thyrotropin-releasing hormone (thyroliberin); p[NH]ppG, guanosine 5'-[β -imidio]triphosphate; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; VIP, vasoactive intestinal peptide.

suggest that N_1 may function as a transducing mechanism in ligand-induced activation of lipid breakdown by some agonists in some tissues.

To determine whether the TRH receptor is functionally coupled to N_1 , we have compared the actions of TRH in control and pertussis-toxin-treated cells, and we demonstrate that, although the effects of somatostatin and muscarinic agonists are blocked, TRH action in GH cells is unaffected.

EXPERIMENTAL

Materials

Tissue-culture media and horse serum were obtained from Grand Island Biological Co., and fetal calf serum was from Flow Laboratories. p[NH]ppG and GTP were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and TRH was a gift from Abbott Laboratories (N. Chicago, IL, U.S.A.). [^3H][N^3 -methyl-His 2]TRH ([^3H]methyl-TRH; 70 Ci/mmol) and [^{32}P]P $_i$ (carrier-free) were from New England Nuclear Corp. [γ - ^{32}P]GTP (30 Ci/mmol) was from Amersham Corp. Quin 2 and Quin 2/AM (its tetra-acetoxymethyl ester) were from Calbiochem-Behring (La Jolla, CA, U.S.A.). Pertussis toxin from *Bordetella pertussis* strain 165 was prepared as described previously (Cronin *et al.*, 1984).

Methods

GH cells were grown in monolayer culture in F10 medium containing 15% (v/v) horse serum and 2.5% (v/v) fetal-calf serum (growth medium) at 37 °C in a humidified atmosphere of air/CO $_2$ (19:1). Secretion experiments were performed on replicate 35 mm dishes inoculated with equal portions from a single donor culture. Specific binding of [^3H]methyl-TRH was measured as described by Hinkle & Tashjian (1973). Membrane-containing fractions were incubated with radiolabelled TRH as described, and receptor-bound hormone was determined in reactions containing a 200-fold or greater molar excess of unlabelled TRH; this averaged less than 10% of total, and has been subtracted from all values.

Low- K_m GTPase was determined by modifications of the procedure of Cassel & Selinger (1976), as previously applied to GH cells (Hinkle & Phillips, 1984). The hydrolysis of 0.05 μM -[γ - ^{32}P]GTP was measured at 25 °C in 11 min reactions containing no added unlabelled nucleotide (total GTPase activity) or 100 μM unlabelled GTP (high- K_m GTPase activity), and low- K_m GTPase was defined as the difference between total and high- K_m GTPase.

Prolactin and growth-hormone (somatotropin) concentrations were measured by specific radioimmunoassay using materials generously provided by the Hormone Distribution Program of NIH. Intracellular hormone was measured by extracting cells with 0.1% Triton X-100; standard curves contained equivalent concentrations of detergent. Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Statistical comparisons were performed by Student's *t* test.

To determine the effect of pertussis toxin on TRH-induced changes in phosphoinositide metabolism, cells labelled with [^3H]inositol to isotopic steady state (Rebecchi *et al.*, 1984) were harvested with 0.02% EDTA, washed and incubated in balanced salt solution

(135 mM-NaCl, 4.5 mM-KCl, 0.5 mM-MgCl $_2$, 1.5 mM-CaCl $_2$, 5.6 mM-glucose and 10 mM-Hepes, pH 7.4) containing 500 ng of pertussis toxin/ml, at 5×10^6 cells/0.5 ml, at room temperature. After 3 h 50 min, 10 mM-LiCl was added to inhibit inositol monophosphatase (Berridge *et al.*, 1982), the enzyme that converts inositol monophosphate into inositol, and thereby allow the changes in the contents of inositol phosphates and phosphoinositides caused by TRH to be measured more readily (Drummond *et al.*, 1984). After 4 h, 1 μM -TRH was added and the incubation was terminated 20 min later by adding 2 ml of chloroform/methanol/conc. HCl (100:100:1, by vol.), followed by 0.1 ml of 10 mM-EDTA. The lower and upper phases were separated. The lower phases were washed once with pre-equilibrated upper phase. Portions of the washed lower phase were counted for radioactivity, as total [^3H]phosphoinositides. ^3H -labelled inositol phosphates and inositol were analysed by anion-exchange column chromatography by the method of Downes & Michell (1981) with minor modification (Rebecchi *et al.*, 1984).

[Ca $^{2+}$] $_i$ was measured as described previously (Gershengorn & Thaw, 1983), with intracellularly trapped Quin 2 (Tsien *et al.*, 1982). In brief, cells were loaded with Quin 2 by incubation in balanced salts solution containing 0.015 mM-Quin 2/AM at room temperature. After 20 min, the cells were centrifuged at 1200 *g* for 1 min and resuspended in fresh balanced salts solution (8×10^6 – 10×10^6 cells/ml). Cells incubated under these conditions accumulate approx. 1.5 mM-Quin 2 in the cytoplasm after hydrolysis by non-specific esterases (Gershengorn & Thaw, 1983). The cell suspension was placed in a cuvette, stirred continuously with a micro-stirrer (Lawrence RDK Scientific, London, Canada) at room temperature, and the fluorescence intensity was monitored continuously in a Perkin-Elmer model LS-5 fluorescence spectrophotometer interfaced with a model 3600 Data Station; excitation wavelength was 340 nm, emission wavelength was 493 nm and slit widths were 5 and 10 nm for excitation and emission respectively. At the end of the analysis, 0.1% Triton X-100 was added to measure maximum fluorescence (F_{max}) and then excess of alkaline (pH > 8.5) EGTA was added to measure minimum fluorescence (F_{min}); Quin 2 fluorescence intensity was corrected for the autofluorescence of the cells (< 10% of that in unstimulated cells), which was not increased by TRH, and for the fluorescence of Triton X-100 where appropriate. [Ca $^{2+}$] $_i$ was calculated by using the equation:

$$[\text{Ca}^{2+}]_i = K_d \times (F - F_{\text{min}}) / (F_{\text{max}} - F)$$

A value of 115 nM was used for K_d of Quin 2, as reported by Gershengorn & Thaw (1983). TRH was added at the time indicated. The effect of TRH was quantified as the peak height of the first- and second-phase responses. As previously reported (Gershengorn & Thaw, 1983), neither basal nor TRH-stimulated prolactin secretion was affected by loading cells with Quin 2.

Measurements of Ca $^{2+}$ concentrations and phosphoinositide breakdown were performed with the GH $_3$ cell line, whereas measurements of TRH receptors, TRH stimulation of GTPase and hormone release were performed with the GH $_4$ C $_1$ line. GH $_4$ C $_1$ is a subclone of GH $_3$ cells which was originally characterized by high prolactin and low growth-hormone secretion, but now secretes both hormones in similar amounts. We have also found lack

Table 1. Inhibition of TRH receptors by guanine nucleotides

Cells were incubated in serum-free medium for 4 h with the concentrations of pertussis toxin shown. Membrane-containing fractions were prepared and incubated with 5 nM-[³H]methyl-TRH with or without 10 μ M-p[NH]ppG for 30 min at 30 °C before determination of specific binding. Values shown are means $\pm$ S.E.M. of triplicate measurements.

Pertussis toxin (ng/ml)	[³ H]Me-TRH bound specifically (fmol/mg)		Inhibition by p[NH]ppG (%)
	Control	+ p[NH]ppG	
0	89.8 $\pm$ 1.8	43.6 $\pm$ 1.5	51
0.8	115.8 $\pm$ 9.5	57.3 $\pm$ 10.9	51
4	123.1 $\pm$ 9.2	53.9 $\pm$ 3.4	56
20	101.9 $\pm$ 2.8	58.5 $\pm$ 1.2	43
100	94.4 $\pm$ 0.1	49.7 $\pm$ 5.1	47
500	95.3 $\pm$ 13.2	49.8 $\pm$ 4.6	48

of effect of pertussis toxin on TRH-stimulated hormone release with the GH₃ line. For each of the experiments shown, a parallel experiment was performed in which cells from the same passage were incubated identically with pertussis toxin and then challenged with VIP and VIP plus somatostatin or somatostatin and a muscarinic agonist (carbachol or oxotremorine). In each case, the inhibitory response to somatostatin or somatostatin plus muscarinic agent was prevented by pertussis toxin.

RESULTS

TRH receptors in pertussis-toxin-treated cells

In order to determine if changes in TRH binding induced by guanine nucleotides were mediated by N_i, we prepared membrane-containing fractions from cells treated with different concentrations of the toxin and measured the ability of p[NH]ppG to inhibit [³H]methyl-TRH binding (Table 1). Pertussis toxin did not significantly alter the amount of [³H]methyl-TRH bound, the inhibition by p[NH]ppG (43–56%), or the amount of p[NH]ppG giving 50% inhibition (0.1 μ M in control cells, 0.2 μ M in cells incubated for 4 h with 350 ng of toxin/ml, a maximal dose). Furthermore, pertussis-toxin treatment did not change TRH binding measured in intact cells (Fig. 1).

TRH stimulation of GTPase activity

As with other agonists with receptors interacting with a guanine-nucleotide-binding protein, TRH stimulated a low- K_m GTPase activity in GH-cell membranes (Hinkle & Phillips, 1984). TRH increased the total amount of [γ -³²P]GTP hydrolysis from 12704 $\pm$ 642 to 18082 $\pm$ 190 c.p.m. (control) and 14865 $\pm$ 1158 to 18583 $\pm$ 502 c.p.m. (cultures treated 4 h with 350 ng of pertussis toxin/ml). High- K_m GTPase was unaffected by TRH or pertussis toxin.

TRH stimulation of phosphoinositide hydrolysis

In GH cells labelled to isotopic steady state with [³H]inositol, the [³H]phosphoinositides comprise [³H]phosphatidylinositol (88%), [³H]lysophosphatidylinositol (6.5%), [³H]phosphatidylinositol 4-monophosphate (2.5%) and [³H]phosphatidylinositol 4,5-bisphos-

Fig. 1. TRH binding in control (●) and pertussis-toxin-treated (□) cells

GH cells were incubated without or with pertussis toxin (100 ng/ml) for 48 h in F10 medium containing 5% horse serum and 1% fetal-calf serum. The cells were then washed twice with serum-free medium and incubated for 1.5 h at 37 °C with 1 nM-[³H]methyl-TRH and the indicated concentrations of unlabelled TRH. Values are means $\pm$ S.E.M. or range for two or three dishes.

phate (2.5%) (Rebecchi *et al.*, 1984). In these experiments (Table 2), cells were incubated with 10 mM-LiCl for 10 min and then for 20 min with TRH. There were marked effects of TRH to decrease [³H]phosphoinositides and to increase [³H]inositol tris-, bis- and monophosphate; there was a small decrease in [³H]inositol. TRH effects were not altered by prior treatment of cells with pertussis toxin. In contrast, neutrophils treated with the same preparation of pertussis toxin manifest the previously described effect (Okajima & Ui, 1984; Molski *et al.*, 1984; Bokoch & Gilman, 1984; Volpi *et al.*, 1985; Okajima *et al.*, 1985), namely total abolition of fMet-Leu-Phe-stimulated oxidative activity (results not shown).

TRH stimulation of an increase in [Ca²⁺]_i

TRH causes a biphasic increase in [Ca²⁺]_i in GH cells, which is presumed to be mediated in part by phosphatidylinositol 4,5-bisphosphate hydrolysis (Gershengorn, 1986). This TRH response was also unaffected by prior treatment of the cells by pertussis toxin (Fig. 2). The peak first- and second-phase TRH responses were 376 $\pm$ 6 and 259 $\pm$ 30 (control) and 366 $\pm$ 47 and 216 $\pm$ 66 (toxin-treated) nM.

Secretory responses of GH cells

The effect of treatment with 100 or 1000 ng of pertussis toxin/ml on hormone release is shown in Table 3. TRH elicited a typical increase in the rate of growth-hormone and prolactin release which was unaffected by prior incubation with toxin. In the same experiment, pertussis toxin blocked inhibition of VIP-stimulated secretion by somatostatin and oxotremorine, a muscarinic agonist. The effects of somatostatin and muscarinic agents were measured in VIP-stimulated cells, since these are larger than the effects on basal secretion.

Table 2. TRH stimulation of lipid metabolism

Cells prelabelled with [^3H]inositol were incubated in balanced salts solution without (control) or with 500 ng of pertussis toxin/ml at room temperature. After 3 h 50 min, 10 mM-LiCl was added; after 4 h, 1 μM -TRH was added and the incubation was continued for an additional 20 min. Results (c.p.m.) are means $\pm$ S.D. of triplicate determinations from one of three similar experiments. [^3H]Phosphoinositide radioactivity (c.p.m.) in control cells was $81\,900 \pm 9900$, in control cells stimulated by TRH $56\,400 \pm 7750$, in pertussis-toxin-treated cells $76\,600 \pm 4650$, and in pertussis-toxin-treated cells stimulated by TRH $51\,900 \pm 1000$.

	Untreated		Pertussis-toxin-treated	
	Control	+ TRH	Control	+ TRH
Inositol trisphosphate	328 ± 67	791 ± 64	332 ± 34	737 ± 133
Inositol bisphosphate	438 ± 109	781 ± 160	414 ± 89	725 ± 124
Inositol phosphate	3080 ± 351	$30\,000 \pm 1\,510$	3050 ± 103	$29\,800 \pm 2\,420$
Inositol	$12\,600 \pm 977$	$8\,940 \pm 171$	$13\,200 \pm 268$	$8\,860 \pm 458$

Fig. 2. TRH stimulation of elevation of $[\text{Ca}^{2+}]_i$ in control and pertussis-toxin-treated cells

Suspensions of GH cells were incubated in growth medium without (control) or with pertussis toxin (200 ng/ml) for 16 h and then incubated in balanced salts solution containing 0.015 mM-Quin 2/AM at room temperature. After 20 min, cells were resuspended in fresh medium without Quin 2/AM and TRH was added at the time indicated. Fluorescence tracings of control and pertussis-toxin-treated cells are shown.

To assess the effect of pertussis toxin on long-term TRH responses, GH cells were first incubated with the toxin for 24 h, and then treated for an additional 24 h with toxin with or without TRH. TRH stimulated prolactin synthesis *de novo* as effectively in pertussis-toxin-treated cells. Prolactin synthesis was $179 \pm 20\%$ and $300 \pm 41\%$ of that in cultures without TRH for control and toxin-treated cells respectively. Because of the possibility that pertussis toxin shifts the dose-response curve for TRH, we measured the ability of different concentrations of methyl-TRH to stimulate hormone release. The toxin did not alter the concentrations of the tripeptide necessary to elicit responses (Fig. 3).

DISCUSSION

Several observations of the interaction of TRH with its receptor in membranes isolated from GH cells indicate that the TRH receptor is coupled to a guanine-nucleotide-binding protein. These include: (1) the affinity of the TRH receptor is controlled by GTP (Hinkle & Kinsella, 1984); (2) receptor occupancy by TRH stimulates GTP hydrolysis (Hinkle & Phillips, 1984); and (3) GTP enhances TRH stimulation of inositol phosphate formation (Straub & Gershengorn, 1986). The GTP-

Table 3. Pertussis-toxin effects on hormone release

GH cells in monolayer culture were incubated for 24 h at 37 °C in complete F10 medium containing 0, 100 or 1000 ng of pertussis toxin/ml. The cultures were washed twice with serum-free F10 medium, and then incubated for 60 min with F10 medium containing: no additions (control), 100 nM-TRH, 100 nM-VIP, or 100 nM-VIP + 100 nM-somatostatin + 10 μM -oxotremorine (OXO). Total prolactin and growth hormone in the cells plus medium was the same in all treatment groups. Values are means $\pm$ S.E.M. of triplicate determinations of secreted hormones.

Hormone	Addition	Toxin...	Hormone release (ng/ml)		
			None	100 ng/ml	1000 ng/ μl
Growth hormone	Control		109 ± 10	117 ± 15	117 ± 19
	TRH		188 ± 2	200 ± 8	201 ± 23
	VIP		229 ± 11	250 ± 7	278 ± 7
	VIP + somatostatin + OXO		113 ± 12	180 ± 9	190 ± 10
Prolactin	Control		71 ± 4	84 ± 21	89 ± 15
	TRH		152 ± 18	134 ± 9	128 ± 10
	VIP		183 ± 33	188 ± 14	173 ± 3
	VIP + somatostatin + OXO		75 ± 3	133 ± 4	173 ± 19

Fig. 3. Dose-response curve for methyl-TRH in control and pertussis-toxin-treated cells

GH cells were incubated for 48 h without or with pertussis toxin (100 ng/ml) and then washed and incubated for 30 min with different concentrations of methyl-TRH. ●, ■, Control; ○, □, pertussis-toxin-treated.

binding protein involved is probably not N_s , since TRH does not increase cyclic AMP at early times (Brostrom *et al.*, 1983), TRH effects on hormone secretion and synthesis are partially additive with those of phosphodiesterase inhibitors (Dannies *et al.*, 1976), and cholera toxin treatment does not affect TRH stimulation of phosphoinositide hydrolysis in intact cells (Imai & Gershengorn, 1985). Cholera toxin also has no effect on TRH or GTP stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in membranes isolated from toxin-treated cells (I. Winocov & M. C. Gershengorn, unpublished work).

The results described herein also demonstrate that the TRH system is insensitive to pertussis toxin. Pertussis-toxin treatment did not alter TRH receptor number or affinity, and did not inhibit immediate post-receptor events such as stimulation of inositol phosphate formation and elevation of $[Ca^{2+}]_i$. The acute and chronic effects of TRH on hormone secretion and synthesis were likewise insensitive to toxin. The insensitivity to pertussis toxin suggests that the GTP-binding protein involved in TRH action is not N_i , since ADP-ribosylation of N_i leads to its partial or complete inactivation (for reviews, see Smigel *et al.*, 1985; Hewlett *et al.*, 1984; Jakobs *et al.*, 1984). N_o is also a substrate for ADP-ribosylation by pertussis toxin (Sternweis & Robishaw, 1984), although it may react less completely than N_i under the same conditions (Neer *et al.*, 1985). Since the function of N_o remains unknown, it is not clear if ADP-ribosylation of N_o would inactivate it. However, initial experiments by Wojcikiewicz *et al.* (1984) suggest that GH cells do not have N_o since only one protein with properties expected for N_i was ADP-ribosylated by pertussis toxin. Therefore N_o is an unlikely candidate for the transducer of TRH action, and it is probable that an as yet unidentified GTP-binding protein functions in this capacity.

Because pertussis toxin did not affect TRH action in GH cells, it is important to have an independent measure

of toxin action. The poisoning of the target cells was demonstrated by the loss of inhibitory responses to muscarinic agents and somatostatin. This result is in agreement with published reports that pertussis toxin catalyses ADP-ribosylation of the α subunit of N_i in GH₃ cells (Wojcikiewicz *et al.*, 1984) and blocks inhibitory responses to both muscarinic agonists and somatostatin (Brown *et al.*, 1984; Koch *et al.*, 1986). We have found that pertussis toxin did not affect TRH responses in cells treated with up to 5 μ g of pertussis toxin/ml, a concentration vastly in excess of that needed to block somatostatin responses. It seems unlikely, therefore, that the failure of the toxin to block TRH responses resulted from incomplete ADP-ribosylation of N_i . These results further support the conclusion that the TRH receptor is not functionally coupled to N_i .

In addition to receptors that interact with adenylate cyclase, there is a growing list of receptors that activate phosphoinositide turnover, Ca^{2+} mobilization and arachidonic acid release, and interact with GTP-binding proteins. Gomperts (1983) was the first to provide evidence that guanine-nucleotide-binding proteins may be involved in signal transduction of receptors of this type in mast cells. Activation of phosphoinositide hydrolysis by GTP and GTP analogues has been demonstrated in permeabilized platelets (Haslam & Davidson, 1984) and in membranes from neutrophils (Cockcroft & Gomperts, 1985; Smith *et al.*, 1985), blowfly salivary gland (Litosch *et al.*, 1985) and GH cells (Straub & Gershengorn, 1986; Lucas *et al.*, 1985). Moreover, in blowfly salivary-gland membranes, Litosch *et al.* (1985) showed that receptor-mediated stimulation by 5-hydroxytryptamine of inositol phosphate formation, which defines the effect to be via activation of a phosphoinositide-hydrolysing phospholipase C (or phosphodiesterase), was enhanced by guanine nucleotides. Similar observations have been made for TRH stimulation of inositol phosphate formation in membranes from GH cells (Straub & Gershengorn, 1986; Lucas *et al.*, 1985). Receptors that act via Ca^{2+} appear to fall into two groups, pertussis-toxin-sensitive and toxin-insensitive. Chemotactic-peptide stimulation of phosphoinositide turnover (Okajima & Ui, 1984; Volpi *et al.*, 1985), Ca^{2+} influx (Okajima & Ui, 1984; Molski *et al.*, 1984), arachidonic acid release (Okajima & Ui, 1984; Bokoch & Gilman, 1984; Okajima *et al.*, 1985) and secretion (Okajima & Ui, 1984; Bokoch & Gilman, 1984; Lad *et al.*, 1985) in neutrophils, and stimulation by compound 48/80 of phosphoinositide hydrolysis, arachidonic acid release and histamine secretion in mast cells (Nakamura & Ui, 1984, 1985) are inhibited by pertussis toxin. At present, therefore, the toxin-sensitive cells appear to be bone-marrow-derived cells, in which receptor occupancy causes rapid increases in arachidonic acid. It remains controversial whether the proximal step in these systems is the activation of a phospholipase A_2 causing arachidonic acid release (Bormann *et al.*, 1984) or the activation of a phospholipase C causing inositol phosphate release, or whether these enzymes are activated simultaneously. Nevertheless, coupling in these cases appears to involve N_i or a ' N_i -like' protein which is a pertussis-toxin substrate. In contrast, pertussis toxin does not block the effects of muscarinic agonists that act via Ca^{2+} in heart cells (Masters *et al.*, 1985), nor does it block guanine nucleotide modulation of muscarinic receptors in astocytoma cells (Evans *et al.*, 1985). These examples are

similar to the present results for TRH action in GH cells. In these pertussis-toxin-insensitive systems, receptor occupancy clearly stimulates phospholipase C activity, but arachidonic acid release is not observed. Coupling in these cases apparently involves a guanine-nucleotide regulatory protein which is neither N_s nor N_i . Very recently, Murayama & Ui (1985) showed that pertussis-toxin treatment of 3T3 fibroblasts inhibits stimulation of arachidonic acid release by thrombin, but does not affect stimulation of inositol release by thrombin. Their findings suggest that receptor coupling to a phospholipase C may occur via one GTP-binding protein, which is neither N_i nor ' N_i -like', and coupling to a phospholipase A_2 may occur through a different member of the family of guanine-nucleotide regulatory proteins, which is N_i or ' N_i -like'. A corollary to this hypothesis would be that the ability of a given stimulus-receptor interaction to activate either or both of these enzymes is dependent on the presence of one or both of these GTP-binding proteins. This would explain our previous observations that TRH causes phospholipase C-mediated hydrolysis of phosphoinositides and arachidonic acid release in thyrotropic tumour cells (Kolesnick *et al.*, 1984) and only phosphoinositide hydrolysis, but not arachidonic acid release, in GH cells (Rebecchi *et al.*, 1984), if GH cells were deficient in the pertussis-toxin-sensitive, i.e. ' N_i -like', GTP-binding protein that would couple TRH-receptor complex to the phospholipase A_2 enzyme. The protein that may be deficient in GH cells is not N_i because a functional N_i is present in these cells.

In conclusion, available evidence supports a role for a GTP-binding protein in signal transduction by TRH. The insensitivity of the TRH system to pertussis toxin indicates that N_i is not involved and raises the possibility that a new member of the family of GTP-regulatory proteins couples the TRH receptor to a phospholipase C. Identification of the guanine-nucleotide-binding protein involved in TRH action should provide important information about the mechanism of action of this peptide hormone.

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REFERENCES

- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
- Bokoch, G. M. & Gilman, A. G. (1984) *Cell* **39**, 301–308
- Bormann, B. J., Huang, C.-K., Mackin, W. M. & Becker, E. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 767–771
- Brostrom, M. A., Brostrom, C. O., Brotman, L. A. & Green, S. S. (1983) *Mol. Pharmacol.* **23**, 399–408
- Brown, B. L., Wojcikiewicz, R. J. H., Dobson, P. R. M., Robinson, A. & Irons, L. I. (1984) *Biochem. J.* **223**, 145–149
- Cassel, D. & Selinger, Z. (1976) *Biochim. Biophys. Acta* **452**, 538–551
- Cockcroft, S. & Gomperts, B. D. (1985) *Nature (London)* **314**, 534–536
- Cronin, M. J., Hewlett, E. L., Evans, W. S., Thorner, M. O. & Rogol, A. D. (1984) *Endocrinology (Baltimore)* **114**, 904–913
- Dannies, P. S., Gautvik, K. M. & Tashjian, A. H., Jr. (1976) *Endocrinology (Baltimore)* **98**, 1147–1159
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
- Drummond, A. H., Bushfield, M. & Macphee, C. A. (1984) *Mol. Pharmacol.* **25**, 201–208
- Evans, T., Martin, M. W., Hughes, A. R. & Harden, T. K. (1985) *Mol. Pharmacol.* **27**, 32–37
- Gershengorn, M. C. (1986) *Annu. Rev. Physiol.* **48**, 515–526
- Gershengorn, M. C. & Thaw, C. (1983) *Endocrinology (Baltimore)* **113**, 1522–1524
- Gomperts, B. D. (1983) *Nature (London)* **306**, 64–66
- Haslam, R. J. & Davidson, M. M. L. (1984) *J. Recept. Res.* **4**, 605–629
- Hewlett, E. L., Cronin, M. L., Moss, J., Anderson, H., Myers, G. A. & Pearson, R. D. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 173–182
- Hinkle, P. M. & Kinsella, P. A. (1984) *J. Biol. Chem.* **259**, 3445–3449
- Hinkle, P. M. & Phillips, W. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6183–6187
- Hinkle, P. M. & Tashjian, A. H., Jr. (1973) *J. Biol. Chem.* **248**, 6180–6186
- Imai, A. & Gershengorn, M. C. (1985) *J. Biol. Chem.* **260**, 10536–10540
- Jakobs, K. H., Aktories, K. & Schultz, G. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**, 135–143
- Koch, B. D., Dorfinger, L. J. & Schonbrunn, A. (1986) *J. Biol. Chem.* **260**, 13138–13145
- Kolesnick, R. N., Musacchio, I., Thaw, C. & Gershengorn, M. C. (1984) *Endocrinology (Baltimore)* **114**, 671–676
- Lad, P. M., Olson, C. V. & Smiley, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 869–873
- Litosch, I., Wallis, C. & Fain, J. N. (1985) *J. Biol. Chem.* **260**, 5464–5471
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lucas, D. O., Bajjalieh, S. M., Kowalchuk, J. A. & Martin, T. F. J. (1985) *Biochem. Biophys. Res. Commun.* **132**, 721–728
- Masters, S. B., Martin, M. W., Harden, T. K. & Brown, J. H. (1985) *Biochem. J.* **227**, 933–937
- Molski, T. F. P., Naccache, P. H., Marsh, M. L., Kermode, J., Becker, E. L. & Sha'afi, R. I. (1984) *Biochem. Biophys. Res. Commun.* **124**, 644–650
- Murayama, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 7226–7233
- Nakamura, T. & Ui, M. (1984) *FEBS Lett.* **173**, 414–418
- Nakamura, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 3584–3593
- Neer, E. J., Lok, J. M. & Wolf, L. G. (1985) *J. Biol. Chem.* **259**, 14222–14229
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Okajima, F. & Ui, M. (1984) *J. Biol. Chem.* **259**, 13863–13871
- Okajima, F., Katada, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 6761–6768
- Rebecchi, M. J., Kolesnick, R. N. & Gershengorn, M. C. (1984) *J. Biol. Chem.* **258**, 227–234
- Smigel, M. D., Ferguson, K. M. & Gilman, A. G. (1985) *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **19**, 103–111
- Smith, C. D., Lane, B. C., Kusaka, I., Verghese, M. W. & Snyderman, R. (1985) *J. Biol. Chem.* **260**, 5875–5878
- Sternweis, P. C. & Robishaw, J. D. (1984) *J. Biol. Chem.* **259**, 13806–13813
- Straub, R. E. & Gershengorn, M. C. (1986) *J. Biol. Chem.* **261**, 2712–2717
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
- Volpi, M., Naccache, P. H., Molski, T. F. P., Shefchyk, J., Huang, C.-K., Marsh, M. L., Munoz, J., Becker, E. L. & Sha'afi, R. I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2708–2712
- Wojcikiewicz, R. J. H., Dobson, P. R. M., Irons, L. I., Robinson, A. & Brown, B. L. (1984) *Biochem. J.* **224**, 339–342