

# The human thyrotropin receptor: A heptahelical receptor capable of stimulating members of all four G protein families

(thyroid-stimulating hormone/glycoprotein hormone receptors/photoaffinity labeling/immunoprecipitation)

KARL-LUDWIG LAUGWITZ\*<sup>†</sup>, ANOUK ALLGEIER<sup>†‡</sup>, STEFAN OFFERMANN<sup>\*§</sup>, KARSTEN SPICHER<sup>\*¶</sup>,  
JACQUELINE VAN SANDE<sup>‡</sup>, JACQUES E. DUMONT<sup>‡</sup>, AND GÜNTER SCHULTZ<sup>\*||</sup>

\*Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69-73, D-14195 Berlin, Germany; and <sup>‡</sup>Institut de Recherche Interdisciplinaire, Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium

Communicated by Henry R. Bourne, University of California, San Francisco, CA, August 28, 1995 (received for review February 14, 1995)

**ABSTRACT** Thyrotropin is the primary hormone that, via one heptahelical receptor, regulates thyroid cell functions such as secretion, specific gene expression, and growth. In human thyroid, thyrotropin receptor activation leads to stimulation of the adenylyl cyclase and phospholipase C cascades. However, the G proteins involved in thyrotropin receptor action have been only partially defined. In membranes of human thyroid gland, we immunologically identified  $\alpha$  subunits of the G proteins  $G_{s \text{ short}}$ ,  $G_{s \text{ long}}$ ,  $G_{i1}$ ,  $G_{i2}$ ,  $G_{i3}$ ,  $G_o$  ( $G_{o2}$  and another form of  $G_o$ , presumably  $G_{o1}$ ),  $G_q$ ,  $G_{i1}$ ,  $G_{i2}$ , and  $G_{i3}$ . Activation of the thyrotropin (TSH) receptor by bovine TSH led to increased incorporation of the photoreactive GTP analogue [ $\alpha$ -<sup>32</sup>P]GTP azidoanilide into immunoprecipitated  $\alpha$  subunits of all G proteins detected in thyroid membranes. This effect was receptor-dependent and not due to direct G protein stimulation because it was mimicked by TSH receptor-stimulating antibodies of patients suffering from Grave disease and was abolished by a receptor-blocking antiserum from a patient with autoimmune hypothyroidism. The TSH-induced activation of individual G proteins occurred with EC<sub>50</sub> values of 5–50 milliunits/ml, indicating that the activated TSH receptor coupled with similar potency to different G proteins. When human thyroid slices were pretreated with pertussis toxin, the TSH receptor-mediated accumulation of cAMP increased by  $\approx 35\%$  with TSH at 1 milliunits/ml, indicating that the TSH receptor coupled to  $G_s$  and  $G_i$ . Taken together, these findings show that, at least in human thyroid membranes, in which the protein is expressed at its physiological levels, the TSH receptor resembles a naturally occurring example of a general G protein-activating receptor.

A large group of receptors regulates cellular functions by coupling to and activating heterotrimeric GTP-binding proteins (G proteins) (1–3). G proteins are defined by their  $\alpha$  subunits, which exhibit a considerable structural and functional diversity and can be assigned to four families,  $G_s$ ,  $G_i$ ,  $G_q$ , and  $G_{12}$ , according to sequence homologies of the  $\alpha$  subunits (2). As many receptors have been shown to interact with several G proteins to elicit their cellular effects (4, 5), it is assumed that accurate signal transduction requires activated receptors to interact specifically with a certain pattern of G proteins. Indeed, reconstitution, cotransfection, and biochemical *in situ* experiments have shown that there is a certain degree of specificity in the receptor–G protein interaction. With these different methodological approaches, coupling of receptors to G proteins of  $G_i$  and  $G_o$  subtypes (6, 7), to  $G_i$  and  $G_q$  family members (8), to  $G_i$  and  $G_s$  (9), to  $G_i$ ,  $G_q$ , and  $G_{12}$  family members (10), or to  $G_i$ ,  $G_q$ , and  $G_s$  (11, 12) has been demonstrated.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Thyrotropin (TSH), the main physiological regulator of the thyroid gland, exerts its cellular effects by binding to a membranous receptor that belongs to the group of G protein-coupled receptors (13). By immunoprecipitating receptor-dependently photolabeled G protein  $\alpha$  subunits, we recently showed that the human TSH receptor can interact with  $G_s$  and  $G_{q/11}$  (14), leading to the activation of adenylyl cyclase and phospholipase C, respectively (15). Thus we extended our examinations and made the unexpected observation that the human TSH receptor studied in human thyroid membranes not only activates  $G_s$  and  $G_{q/11}$  but also members of the  $G_i$  and  $G_{12}$  families, indicating a more complex effector modulation than a dual regulation of downstream enzymatic effectors.

## MATERIALS AND METHODS

**Materials.** Bovine TSH was from Sigma, and [ $\alpha$ -<sup>32</sup>P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq) was from DuPont/New England Nuclear. TSH receptor-stimulating antibodies were IgG preparations from four patients suffering from Grave disease, and TSH receptor-blocking antibodies were from a patient suffering from autoimmune hypothyroidism (diagnosed by standard clinical and laboratory tests). Sources of other materials have been described (16, 17).

**Membrane Preparation and Cell Culture.** Human thyroid membranes were prepared from normal glands (partly removed during the resection of laryngeal cancer) or from paranodular tissue obtained from patients undergoing thyroidectomy because of solitary nodules. Dog thyroid membranes were prepared from resected normal glands. Membranes were prepared as described (14). FRTL-5 cells were grown as monolayers in Coon's modified Ham's F-12 medium/5% (vol/vol) fetal calf serum containing TSH (1 milliunit/ml), transferrin (5  $\mu$ g/ml), insulin (10  $\mu$ g/ml), somatostatin (10 ng/ml), cortisone (10 nM), and the tripeptide glycyl-L-histidyl-L-lysine acetate (10 ng/ml) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Membranes were prepared by nitrogen cavitation (6).

**Photolabeling of Membrane Proteins.** [ $\alpha$ -<sup>32</sup>P]GTP azidoanilide was synthesized and purified as described (16). Cell membranes were incubated at 30°C in a buffer containing 0.1 mM EDTA, 10 mM MgCl<sub>2</sub>, 30 mM NaCl, 1 mM benzamidine, and 50 mM Hepes (pH 7.4). After 5 min of preincubation with or without receptor agonist, samples were incubated for the indicated periods with 10 nM [ $\alpha$ -<sup>32</sup>P]GTP azidoanilide (130 kBq per tube) in a final volume of 120  $\mu$ l. The reaction was

Abbreviation: TSH, thyrotropin or thyroid-stimulating hormone.

<sup>†</sup>Both authors contributed equally to this work.

<sup>§</sup>Present address: California Institute of Technology, Division of Biology 147-75, Pasadena, CA 91125.

<sup>¶</sup>Present address: University of California, Department of Anesthesiology, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90024-1778.

<sup>||</sup>To whom reprint requests should be addressed.

stopped by placing the samples on ice. All subsequent procedures were done as described (16).

**Immunoprecipitation.** Immunoprecipitation was done in a precipitating buffer containing 1% (wt/vol) Nonidet P-40, 1% (wt/vol) deoxycholate, 0.5% (wt/vol) SDS, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, aprotinin at 10  $\mu$ g/ml, and 10 mM Tris-HCl (pH 7.4) (17). Fifty microliters of 10% (wt/vol) protein A-Sepharose was added for preclearing the lysates. The subsequent immunoprecipitation steps were done as described (17).

**SDS/PAGE, Autoradiography, Immunoblotting, and Antibodies.** If not stated otherwise, SDS/PAGE was performed on separating gels containing 9% (wt/vol) acrylamide and 6 M urea or on 13% acrylamide gels, which were run until the prestained 30-kDa standard protein reached the gel bottom. Photolabeled proteins were visualized by autoradiography of dried gels with Kodak X-Omat AR-5 films. Blotting of membrane proteins separated by SDS/PAGE, processing of immunoblots, and detection of immunoreactive proteins by a chemiluminescence procedure have been described (6). Antisera were raised against peptides corresponding to specific regions of G protein  $\alpha$  subunits, and specificity of antisera was controlled by testing their selectivity for  $\alpha$  subunits expressed in *Escherichia coli*. Antisera AS 8 ( $\alpha_{\text{common}}$ ), AS 6 ( $\alpha_{\text{o common}}$ ), AS 266 ( $\alpha_{\text{i common}}$ ), AS 190 ( $\alpha_{\text{i1}}$ ), AS 269 ( $\alpha_{\text{i2}}$ ), AS 105 ( $\alpha_{\text{i3}}$ ), AS 348 ( $\alpha_{\text{s}}$ ), AS 368 ( $\alpha_{\text{q/11/14}}$ ), AS 255 ( $\alpha_{\text{i1}}$ ), AS 232 ( $\alpha_{\text{i2}}$ ), AS 233 ( $\alpha_{\text{i2}}$ ), and AS 343 ( $\alpha_{\text{i3}}$ ) have been described (6, 14, 17, 18). Antiserum AS 252 ( $\alpha_{\text{q}}$ ) was raised against the peptide sequence CVAFENPYVDAIK. Antiserum AS 86 was raised against the C-terminal 10 amino acids of  $\alpha_{\text{i3}}$  (19). Antiserum AS 371 was raised against the C-terminal sequence of  $\alpha_{\text{o2}}$ ; it showed crossreactivity with the three  $\alpha_{\text{i}}$  subtypes at a dilution of 1:1000.

**Human Thyroid Slice Incubation and cAMP Determination.** Thyroid tissues were sliced at room temperature with a Stadie-Riggs microtome (Arthur Thomas). They were first incubated for 18 hr at 37°C under an atmosphere of 95% O<sub>2</sub>/5% CO<sub>2</sub> (vol/vol) in 2 ml of Krebs-Ringer bicarbonate buffer (130 mM NaCl/5 mM KCl/1.25 mM KH<sub>2</sub>PO<sub>4</sub>/20 mM NaHCO<sub>3</sub>) supplemented with 8 mM glucose/bovine serum albumin (0.5 g/liter)/streptomycin/penicillin (100  $\mu$ g/ml) containing, where indicated, pertussis toxin (200 ng/ml). Slices were then transferred to fresh incubation medium supplemented with 25  $\mu$ M rolipram and the agents to be tested. After 1 hr of incubation, the slices were dropped into boiling water for 5 min, homogenized, and centrifuged; the supernatant was dried in a vacuum concentrator. cAMP measurements were performed by RIA according to the method of Brooker *et al.* (20).

**Reproducibility.** The photolabeling experiments shown are representative of at least three independently performed experiments using different membrane preparations. cAMP measurements were performed twice in triplicates, and the two-tailed Student's *t* test was used to calculate significance of the data between control conditions and pertussis toxin-treated slices.

## RESULTS

To determine the G protein  $\alpha$ -subunit expression in human thyroid tissue, immunoblotting experiments with subtype-specific antibodies raised against specific sequences of different  $\alpha$  subunits were performed (Fig. 1). An  $\alpha_{\text{common}}$  antiserum, recognizing at least the  $\alpha$  subunits of G<sub>i1</sub>, G<sub>i2</sub>, G<sub>i3</sub>, and G<sub>o</sub>, reacted with several proteins in the range of 39–41.5 kDa. The  $\alpha_{\text{i common}}$  antiserum specific for the three  $\alpha$  subunits of G<sub>i1</sub>, G<sub>i2</sub>, and G<sub>i3</sub> detected proteins with molecular masses of 40–41.5 kDa. Proteins with molecular masses of 41.5 kDa were also recognized by antisera selective for  $\alpha_{\text{i1}}$  and  $\alpha_{\text{i3}}$ , whereas the 40-kDa protein was identified by an antiserum specific for the G<sub>i2</sub>  $\alpha$  subunit. An antiserum that is specific for G<sub>o</sub>  $\alpha$  subunits detected proteins of 39 and 40 kDa. An antiserum directed

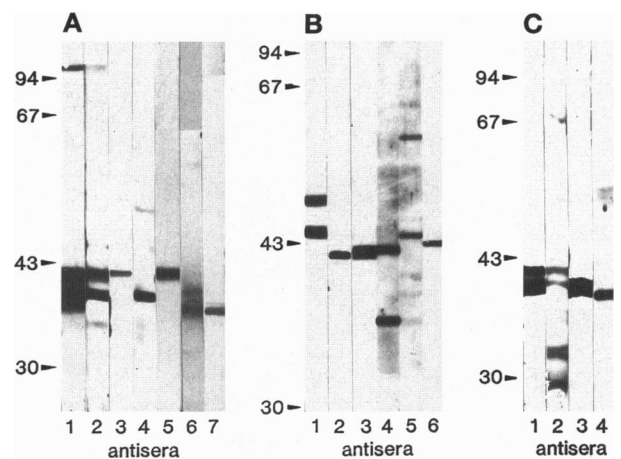
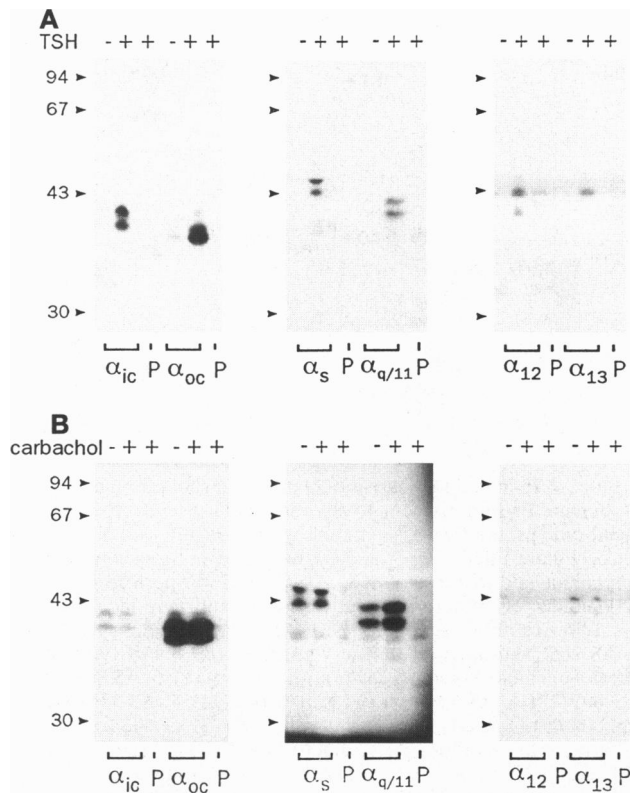


FIG. 1. Immunoblot analysis of G protein  $\alpha$  subunits in membranes of human thyroid tissue (A, B) and FRTL-5 cells (C). Thyroid membrane proteins were separated by SDS/PAGE and blotted onto nitrocellulose filters. For A and C, the separating gels contained 9% acrylamide and 6 M urea; for B, SDS/PAGE was performed on 13% acrylamide gels. Filters were cut into strips and were incubated with the following antisera. (A) Lanes: 1, AS 8 (anti- $\alpha_{\text{common}}$ , 1:150) (21); 2, AS 266 (anti- $\alpha_{\text{i common}}$ , affinity purified) (8); 3, AS 190 (anti- $\alpha_{\text{i1}}$ , affinity purified) (8); 4, AS 269 (anti- $\alpha_{\text{i2}}$ , 1:150) (8); 5, AS 86 (anti- $\alpha_{\text{i3}}$ , 1:5000) (22); 6, AS 6 (anti- $\alpha_{\text{o common}}$ , 1:100) (21); 7, AS 371 (anti- $\alpha_{\text{o2}}$ , 1:5000). (B) Lanes: 1, AS 348 (anti- $\alpha_{\text{s}}$ , 1:150) (21); 2, AS 252 (anti- $\alpha_{\text{q}}$ , 1:150); 3, AS 368 (anti- $\alpha_{\text{q/11/14}}$ , 1:500) (23); 4, AS 255 (anti- $\alpha_{\text{i1}}$ , 1:150) (19); 5, AS 232 (anti- $\alpha_{\text{i2}}$ , 1:150) (23); 6, AS 343 (anti- $\alpha_{\text{i3}}$ , 1:150) (23). (C) Lanes: 1, AS 8 (anti- $\alpha_{\text{common}}$ , 1:150); 2, AS 266 (anti- $\alpha_{\text{i common}}$ , 1:150); 3, AS 6 (anti- $\alpha_{\text{o common}}$ , 1:150); 4, AS 371 (anti- $\alpha_{\text{o2}}$ , 1:5000). Bound antibodies were visualized as described. Numbers at left indicate molecular masses (kDa) of marker proteins.

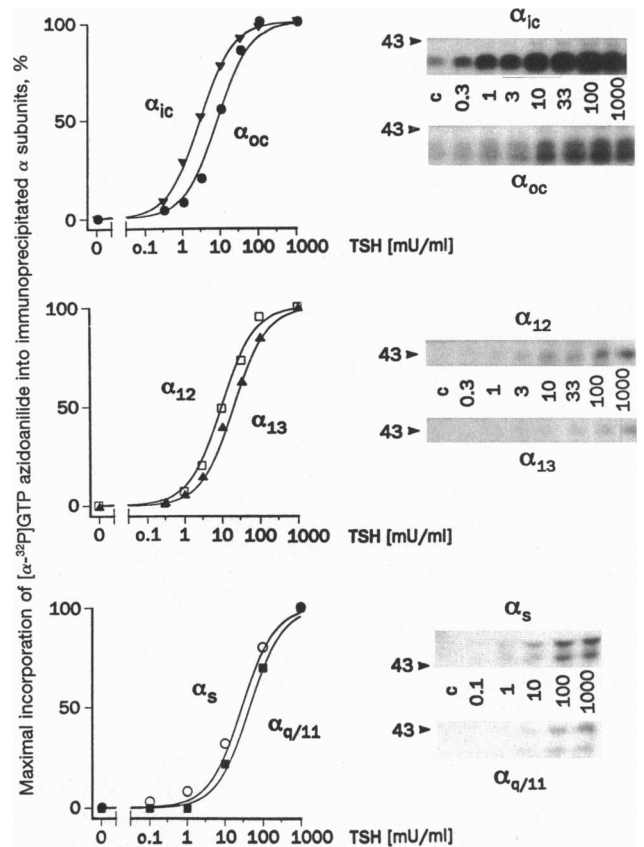
against the C terminus of  $\alpha_{\text{o2}}$  selectively detected a protein at 39 kDa (AS 371 at a dilution of 1:5000). A photolabeled 40-kDa protein comigrated with another  $\alpha_{\text{o}}$  subunit, likely to be  $\alpha_{\text{o1}}$  (Figs. 2 and 3). Additional immunoblotting experiments showed the expression of two forms of  $\alpha_{\text{s}}$ ,  $\alpha_{\text{s short}}$  at 43.5 kDa and  $\alpha_{\text{s long}}$  at 45 kDa, as well as of  $\alpha_{\text{q}}$  and  $\alpha_{\text{i1}}$ , exhibiting molecular masses of 42 and 43 kDa, respectively. Finally, G<sub>i2</sub> and G<sub>i3</sub>  $\alpha$  subunits were detected, migrating at 43.5 and 43 kDa, respectively. Expression of  $\alpha_{\text{z}}$  and  $\alpha_{\text{i4}}$  could not be demonstrated in immunoblotting experiments. In membranes of FRTL-5 cells, the  $\alpha_{\text{common}}$ ,  $\alpha_{\text{i common}}$ , and  $\alpha_{\text{o common}}$  antisera immunohistochemically recognized the same protein pattern as in membranes of human thyroid tissue (see Fig. 1). These data show that the two  $\alpha_{\text{o}}$  subunits immunologically detected are G proteins endogenously expressed in the thyrocyte-derived cell line.

To investigate the possible interaction of the activated TSH receptor with the different G proteins detected in human thyrocytes, membranes of thyroid glands were photolabeled with the GTP analogue [ $\alpha$ -<sup>32</sup>P]GTP azidoanilide in the absence or presence of TSH at 100 milliunits/ml (Fig. 2A). Immunoprecipitation of the 40- to 41.5-kDa  $\alpha$  subunits of G<sub>i1</sub>, G<sub>i2</sub>, and G<sub>i3</sub> and the 39- to 40-kDa  $\alpha$  subunits of G<sub>o</sub> from solubilized membranes revealed an increased incorporation of the GTP analogue into these G protein  $\alpha$  subunits in response to TSH. In addition, immunoprecipitation of the 43.5- and 45-kDa  $\alpha$  subunits of G<sub>s short</sub> and G<sub>s long</sub> and of the 42- and 43-kDa  $\alpha$  subunits of G<sub>q</sub> and G<sub>i1</sub>, as well as of the 43.5- and 43-kDa  $\alpha$  subunits of G<sub>i2</sub> and G<sub>i3</sub>, showed an increased photolabeling in response to TSH (see Fig. 2A). Subtype-specific immunoprecipitation for  $\alpha_{\text{i1}}$  (AS 190),  $\alpha_{\text{i2}}$  (AS 269), and  $\alpha_{\text{i3}}$  (AS 105) revealed an increased photolabeling after TSH receptor activation for the three G<sub>i</sub> subtypes (data not shown). To test whether this broad G protein activation via the stimulated TSH receptor could be due to an artifact of our membrane preparation, we tried to find a receptor selectively activating G proteins of only one family in the same system. As ATP and bradykinin induce inositol trisphosphate formation in



**FIG. 2.** Immunoprecipitation of thyroid membrane G proteins photolabeled in the presence of TSH and carbachol. (A) Human membranes (150  $\mu\text{g}/\text{tube}$ ) were photolabeled in the absence (-) or presence of TSH at 100 milliunits/ml (+). (B) Dog membranes were photolabeled in the absence (-) or presence (+) of 100  $\mu\text{M}$  carbachol with [ $\alpha$ - $^{32}\text{P}$ ]GTP azidoanilide. Thereafter, membranes were solubilized and incubated with the indicated antisera [ $\alpha_{ic}$ ,  $\alpha_i$  common antiserum (AS 266);  $\alpha_{oc}$ ,  $\alpha_o$  common antiserum (AS 6);  $\alpha_s$ ,  $\alpha_s$  antiserum (AS 348);  $\alpha_{q/11}$  antiserum (AS 368);  $\alpha_{12}$ ,  $\alpha_{12}$  antiserum (AS 233);  $\alpha_{13}$ ,  $\alpha_{13}$  antiserum (AS 343)]. Control samples (P) were incubated with the antiserum preincubated in the presence of the peptide against which it was generated. Peptides were applied at 5  $\mu\text{g}/\text{ml}$  ( $\alpha_{ic}$  and  $\alpha_{oc}$  antisera) or 10  $\mu\text{g}/\text{ml}$  ( $\alpha_s$ ,  $\alpha_{q/11}$ ,  $\alpha_{12}$ , and  $\alpha_{13}$  antisera). Precipitated proteins were subjected to SDS/PAGE; autoradiograms of SDS gels are shown with molecular masses (kDa) of marker proteins at left.

human thyrocytes (24), we tried to compare the effects of these agonists as adequate controls. Unfortunately, neither one reproducibly stimulated [ $\alpha$ - $^{32}\text{P}$ ]GTP azidoanilide binding to  $\alpha$  subunits of  $G_q$  and  $G_{11}$  in human thyroid membranes. Because of the weakness of these effects and because of the scarcity of the human material, we photolabeled dog thyroid membranes with and without 100  $\mu\text{M}$  carbachol (Fig. 2B). Muscarinic receptor activation showed an increased incorporation of the GTP analogue selectively into 42-kDa and 43-kDa  $\alpha$  subunits of  $G_q$  and  $G_{11}$ , which is in accordance with reported phospholipase C stimulation by carbachol (25). Carbachol was without any stimulatory effect on G protein  $\alpha$  subunits of the  $G_i$ ,  $G_s$ , and  $G_{12}$  families (see Fig. 2B). Thus, the antibodies directed against  $\alpha$  subunits of  $G_s$ ,  $G_q$ ,  $G_i$ , and  $G_{12}$  family members specifically precipitated photolabeled proteins with molecular masses corresponding to those found in immunoblotting experiments, and the radioactivity incorporated into all immunoprecipitated  $\alpha$  subunits was increased in response to TSH, suggesting that the activated TSH receptor coupled to two forms of  $G_s$  and  $G_o$ , to  $G_{11}$ ,  $G_{12}$ ,  $G_{13}$ ,  $G_q$ ,  $G_{11}$ , as well as to  $G_{12}$  and  $G_{13}$ . Fig. 3 shows the effect of TSH at increased concentrations on binding of the photoreactive GTP analogue to G protein  $\alpha$  subunits immunoprecipitated with the  $\alpha_i$  common,  $\alpha_o$  common,  $\alpha_{12}$ ,  $\alpha_{13}$ ,  $\alpha_s$ , and  $\alpha_{q/11}$  antisera. Maximally effective concentrations of TSH led to a stimulation of basal [ $\alpha$ - $^{32}\text{P}$ ]GTP azidoanilide incorporation into  $\alpha_i$  and  $\alpha_o$ ,



**FIG. 3.** Effect of TSH at increased concentrations on incorporation of [ $\alpha$ - $^{32}\text{P}$ ]GTP azidoanilide into different G proteins. (Right) Membranes (150  $\mu\text{g}$ ) were incubated with or without increased TSH concentrations, and photolabeled  $\alpha$  subunits were immunoprecipitated with the indicated antisera as described. Immunoprecipitates were separated by SDS/PAGE, and incorporation of [ $\alpha$ - $^{32}\text{P}$ ]GTP azidoanilide into  $\alpha_i$  (AS 266),  $\alpha_o$  (AS 6),  $\alpha_{12}$  (AS 233),  $\alpha_{13}$  (AS 343),  $\alpha_s$  (AS 348),  $\alpha_{q/11}$  (AS 368) subunits was quantified by laser densitometry of autoradiograms from dried gels. (Left) The stimulation of photolabeling of  $\alpha_i$  ( $\blacktriangledown$ ),  $\alpha_o$  ( $\bullet$ ),  $\alpha_{12}$  ( $\square$ ),  $\alpha_{13}$  ( $\blacktriangle$ ),  $\alpha_s$  (short/long) ( $\circ$ ), and  $\alpha_{q/11}$  ( $\blacksquare$ ) is shown as a percentage of maximal stimulation; mean values of five independent experiments are shown.

subtypes by 280% and 192%, into  $\alpha_{12}$  and  $\alpha_{13}$  by 123% and 95%, and into  $\alpha_s$  and  $\alpha_{q/11}$  by 320% and 183%, respectively. The  $EC_{50}$  values of TSH-induced photolabeling of G protein  $\alpha$  subunits were in the range of 5–50 milliunits/ml.

To test whether the effect of TSH on photolabeling of different G protein  $\alpha$  subunits was receptor-mediated, TSH receptor-blocking (TB-Ab) as well as TSH receptor-stimulating (TS-Ab) antibodies were applied (Fig. 4). Whereas incubation of membranes with receptor-blocking antibodies completely abolished the effect of TSH on photolabeling of immunoprecipitated  $\alpha_i$ ,  $\alpha_o$ ,  $\alpha_s$ ,  $\alpha_{q/11}$ , and  $\alpha_{12}$ , receptor-stimulating antibodies increased the incorporation of [ $\alpha$ - $^{32}\text{P}$ ]GTP azidoanilide into these  $\alpha$  subunits, thus mimicking the effect of TSH. A control serum from healthy volunteers failed to stimulate photolabeling of G protein  $\alpha$  subunits. Moreover, TSH at 100 milliunits/ml did not affect photolabeling of G protein  $\alpha$  subunits in membranes of other tissues—e.g., liver and brain, which do not express TSH receptors (data not shown). These data show that TSH-induced photolabeling of different G protein  $\alpha$  subunits in thyroid membranes occurred by a receptor-mediated mechanism and is neither unspecific nor due to a direct TSH effect on G proteins.

To further elucidate the coupling of the TSH receptor to  $G_i$ , we determined intracellular cAMP concentrations after TSH or forskolin stimulation in human thyroid slices pretreated

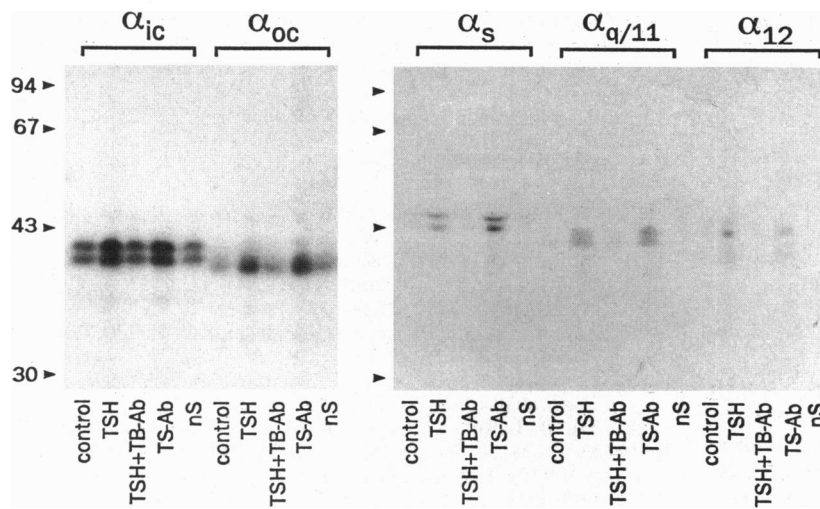


FIG. 4. Effect of receptor-binding antibodies on photolabeling of G protein  $\alpha$  subunits. Thyroid membranes were photolabeled with [ $\alpha$ - $^{32}$ P]GTP azidoanilide in the absence (control) or presence of TSH alone (TSH), TSH and a TSH receptor-blocking antiserum (T+TB-Ab), a TSH receptor-stimulating antibody (TS-Ab), or a control serum from healthy volunteers (nS). After solubilization, membrane proteins were immunoprecipitated with the antibodies indicated at top [ $\alpha_{ic}$ ,  $\alpha_{ic}$  antiserum (AS 266);  $\alpha_{oc}$ ,  $\alpha_o$  common antiserum (AS 6);  $\alpha_s$ ,  $\alpha_s$  antiserum (AS 348);  $\alpha_{q/11}$ ,  $\alpha_{q/11}$  antiserum (AS 368); and  $\alpha_{12}$ ,  $\alpha_{12}$  antiserum (AS 233)]. Autoradiograms of dried gels are shown. TSH and the antibodies were applied at the following concentrations: TSH, 1 milliunit/ml (precipitation of  $\alpha_i$  and  $\alpha_o$ ) or 10 milliunit/ml (precipitation of  $\alpha_s$ ,  $\alpha_{q/11}$ , and  $\alpha_{12}$ ); TB-Ab, 2 mg/ml (precipitation of  $\alpha_i$  and  $\alpha_o$ ) or 3 mg/ml (precipitation of  $\alpha_s$ ,  $\alpha_{q/11}$ , and  $\alpha_{12}$ ); TS-Ab, 2 mg/ml; normal human control serum, 2 mg/ml.

with or without pertussis toxin (Table 1). In pertussis toxin-pretreated slices, the TSH-induced increase in cAMP accumulation was significantly higher compared with control slices; on average, a 32% larger increase in the cAMP concentration was seen at various TSH concentrations. In contrast, forskolin-dependent cAMP accumulation was unaffected by the pertussis toxin pretreatment. These experiments strengthen the photolabeling results and demonstrate coupling of the human TSH receptor to  $G_s$  and  $G_i$  in intact cells.

## DISCUSSION

In the present study, we identified the G proteins activated by bovine TSH via the human TSH receptor in membranes of thyroid glands. We showed that the activated TSH receptor coupled not only to  $G_s$  and  $G_{q/11}$  but also to several subtypes of  $G_i$  and  $G_o$ , as well as to  $G_{12}$  and  $G_{13}$ . No pronounced difference in the  $EC_{50}$  values of TSH to activate G proteins was observed (Fig. 3), suggesting that the activated human TSH receptor coupled with similar potencies to different G proteins. Because this study was done with thyroid membranes under *in situ* conditions that presumably maintain the physiological membrane organization, an artificial receptor-G protein interaction appears unlikely; the method has been demonstrated to reliably detect specific receptor G protein interactions in a variety of systems (6, 10, 21, 23). We observed selective activation of  $G_q$  and  $G_{11}$  in response to carbachol by

the same method in dog thyroid membranes prepared in the same way as the human membranes; although this system is not quite an adequate control system, the finding suggests that our results obtained for the human TSH receptor are not an artifact of our membrane preparations (Fig. 2B).

Our results suggest that the activated human TSH receptor studied in thyroid membranes can couple to at least 10 different G proteins—i.e.,  $G_{s\text{ short}}$ ,  $G_{s\text{ long}}$ ,  $G_q$ ,  $G_{11}$ ,  $G_{12}$ ,  $G_{13}$ ,  $G_o$ ,  $G_{12}$ , and  $G_{13}$ . Such a high level of unspecificity or promiscuity in receptor-G protein interaction is without precedent so far, and the question arises whether this effect is mediated by a single receptor or by different TSH receptors. Alternative splicing of the receptor mRNA has been shown to result in different isoforms—for example, the pituitary adenylyl cyclase-activating peptide type I receptor. The resulting isoforms of this receptor couple to different G proteins (22). However, no subtypes of the TSH receptor have been described, and the carboxyl-terminal portion of the receptor including the seven transmembrane segments and the cytoplasmic surface is encoded by one single exon (26). Moreover, receptor-stimulating and receptor-blocking antibodies affected the photolabeling of all G protein  $\alpha$  subunits activated by TSH (see Fig. 4). Therefore, it is most likely that a single species of TSH receptors mediated the described effects. Despite these supporting data with antibodies against human TSH receptors, it needs to be seen whether a similar receptor G protein coupling pattern is observed in cotransfection or

Table 1. Influence of pertussis toxin on TSH-stimulated cAMP accumulation in human thyroid slices

Exp.	PTX	pmol of cAMP/100 mg of protein				
		Control	TSH			Forskolin
			0.05 mU/ml	0.1 mU/ml	1 mU/ml	
a	–	15.2 ± 3.9	62 ± 14.4	145 ± 34	630 ± 21	1131 ± 158
	+	15.1 ± 2.5	79 ± 6.4	184 ± 4.9	900 ± 112	1122 ± 265
b	–	13.7 ± 1.1	35 ± 6.1	103 ± 11.3	724 ± 34	1009 ± 57
	+	13.5 ± 2.8	43 ± 3.7	124 ± 7.3	901 ± 108	1105 ± 8.3
c	–	21 ± 3.9	150 ± 21.7		508 ± 54.3	724 ± 58.9
	+	22.5 ± 4	235 ± 38.5		610 ± 29	774 ± 17.4
d	–	24 ± 4.2	29 ± 7.8		245 ± 47	1118 ± 124
	+	22.3 ± 4.8	35.2 ± 7.7		370 ± 21	1100 ± 254

Human thyroid slices were pretreated for 18 hr with pertussis toxin (PTX) at 200 ng/ml. Thereafter, thyroid cells were incubated with TSH at the indicated concentrations and 10  $\mu$ M forskolin in a buffer containing 25  $\mu$ M rolipram for 1 hr. Cells were disrupted, and cAMP accumulation was determined as described. Results shown are means ( $\pm$  SEMs) of triplicates. Results were confirmed in four independent experiments (a–d). The increase in TSH-stimulated intracellular cAMP concentrations between the pertussis toxin-pretreated cells and the control cells was significant with  $P < 0.05$  for TSH at 0.05 and 0.1 milliunit/ml (mU) and with  $P < 0.0025$  for TSH at 1 milliunit/ml.

reconstitution systems. That a single, although artificial, receptor is principally able to activate a wide variety of G proteins has recently been shown by constructing a chimeric muscarinic M<sub>1</sub>/M<sub>2</sub> receptor in which the third intracellular loop or its N-terminal sequence was replaced by the corresponding part of the  $\beta_1$  adrenergic receptor. These chimeric receptors were able to couple to G<sub>i</sub>, G<sub>o</sub>, G<sub>z</sub>, G<sub>q</sub>, and G<sub>s</sub> with very similar potencies (27).

Multiple evidence shows that G<sub>s</sub>-mediated signal transduction resulting in an elevated intracellular cAMP concentration is the predominant signaling pathway induced by TSH in human thyroid (13). Therefore, coupling of the activated TSH receptor to G<sub>i</sub> subtypes (see Figs. 2–4) appears to be surprising because activation of a G<sub>i</sub>-dependent pathway may lead to a decrease in adenylyl cyclase activity. The increase in TSH-stimulated cAMP accumulation in human thyrocytes after pertussis toxin treatment, which leads to uncoupling of G<sub>i</sub> family members from the receptor, clearly underlines dual activation of G proteins of the G<sub>s</sub> and G<sub>i</sub> families via the human TSH receptor (Table 1). When TSH-stimulated adenylyl cyclase activity is measured, G<sub>i</sub> activation is functionally masked by the dominant G<sub>s</sub>-mediated stimulatory effect; a similar phenomenon was recently described for the parathyroid receptor (28).

Coupling of the activated TSH receptor to subtypes of the pertussis toxin-sensitive G proteins G<sub>i</sub> and G<sub>o</sub>, which usually represent the major portion of cellular heterotrimeric G proteins, may be functionally relevant by the release of  $\beta\gamma$ -complexes.  $\beta\gamma$ -heterodimers not only regulate the activity of different enzymes—e.g., adenylyl cyclase and phospholipase C  $\beta$  isoforms (29, 30) or phosphatidylinositol 3-kinase (31)—but also have recently been implicated in the regulation of the ras mitogen-activated protein kinase/extracellularly regulated kinase kinase (MEK)/mitogen-activated kinase (MAP) kinase pathway (32). The mitogenic response of human thyroid epithelial cells to serum factors has been reported to be reduced by pretreatment of cells with pertussis toxin (33), indicating that G<sub>i</sub> (or G<sub>o</sub>) can mediate mitogenic effects in human thyroid cells. A TSH-induced pertussis toxin-insensitive and cAMP-independent MAP kinase activation has been described in primary cultured human thyroid follicles (34).

In neuronal and neuroendocrine tissues, inhibition of voltage-gated Ca<sup>2+</sup> channels is mediated by G<sub>o</sub> (35). Up to now, expression of Ca<sup>2+</sup> channels has not been demonstrated in human thyroid tissue either by functional or by protein or mRNA demonstration. Despite this fact, TSH-stimulated activation of G<sub>o</sub> proteins may play a functional role in cellular secretion processes of thyrocytes.

Knowledge of the physiological function of G<sub>12</sub> and G<sub>13</sub> is still sparse. Both G proteins have oncogenic potential in mouse NIH 3T3 fibroblasts and are predicted to be involved in processes of differentiation and organogenesis (36, 37).  $\alpha$  subunits of G<sub>13</sub> and G<sub>12</sub> showed the ability to stimulate Na<sup>+</sup>/H<sup>+</sup> exchangers (38). Our data suggest that the TSH-induced G<sub>12</sub> and G<sub>13</sub> activation may elicit cellular processes related to either growth or differentiation of thyrocytes.

Although the functional implications of the TSH-induced stimulation of multiple signaling pathways is not clear, the fact that a naturally occurring receptor can activate members of all known G protein families in thyroid membranes is interesting. This finding is in conflict with the previous concept of selective receptor–G protein interaction and points to a highly complex multifunctional signaling by the human TSH receptor.

We thank Evelyn Bombien for expert technical assistance, Drs. M. Ludgate and S. Costagliola for providing the high-activity TSH receptor-stimulating and TSH receptor-blocking immunoglobulins, and Drs. Th. Gudermann and B. Nürnberg for critical discussion of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, the Belgian Program on Interuniversity Poles of Attraction initiated by the Belgian State,

Prime Minister's Office, Federal Service of Science, Technology and Culture, the Fonds National de la Recherche Scientifique (F.N.R.S.-Televie), and the Foundation Rose et Jean Hoguet.

- Bourne, H. R., Sanders, D. A. & McCormick, F. (1991) *Nature (London)* **349**, 117–127.
- Simon, M. I., Strathmann, M. P. & Gautam, N. (1991) *Science* **252**, 802–808.
- Hepler, J. R. & Gilman, A. G. (1992) *Trends Biochem. Sci.* **17**, 383–387.
- Milligan, G. (1993) *Trends Pharmacol. Sci.* **14**, 239–244.
- Offermanns, S. & Schultz, G. (1994) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **350**, 329–338.
- Laugwitz, K.-L., Offermanns, S., Spicher, K. & Schultz, G. (1993) *Neuron* **10**, 233–242.
- Law, S. F., Yasuda, K., Bell, G. I. & Reisine, T. (1993) *J. Biol. Chem.* **268**, 10721–10727.
- Wu, D., LaRosa, G. J. & Simon, M. I. (1993) *Science* **261**, 101–103.
- Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R. & Liggett, S. B. (1992) *J. Biol. Chem.* **267**, 15795–15801.
- Offermanns, S., Laugwitz, K.-L., Spicher, K. & Schultz, G. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 504–508.
- Chabre, O., Conklin, B. R., Brandon, S., Bourne, H. R. & Limbird, L. E. (1994) *J. Biol. Chem.* **269**, 5730–5734.
- Aramori, I. & Nakanishi, S. (1992) *Neuron* **8**, 757–765.
- Vassart, G. & Dumont, J. E. (1992) *Endocr. Rev.* **13**, 596–611.
- Allgeier, A., Offermanns, S., Van Sande, J., Spicher, K., Schultz, G. & Dumont, J. (1994) *J. Biol. Chem.* **269**, 13733–13735.
- Van Sande, J., Raspe, E., Perret, J., Lejeune, C., Maenhaut, C., Vassart, G. & Dumont, J. E. (1990) *Mol. Cell. Endocrinol.* **71**, R1–R6.
- Offermanns, S., Schultz, G. & Rosenthal, W. (1991) *Methods Enzymol.* **195**, 286–301.
- Laugwitz, K.-L., Spicher, K., Schultz, G. & Offermanns, S. (1994) *Methods Enzymol.* **235**, 283–294.
- Spicher, K., Kalkbrenner, F., Zobel, A., Harhammer, R., Nürnberg, B., Söling, A. & Schultz, G. (1994) *Biochem. Biophys. Res. Commun.* **198**, 906–914.
- Nürnberg, B., Spicher, K., Harhammer, R., Bosserhoff, A., Frank, R., Hiltz, H. & Schultz, G. (1994) *Biochem. J.* **300**, 387–394.
- Brooker, G., Harper, J. F., Terasaki, W. L. & Moylan, R. D. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 1–33.
- Offermanns, S., Wieland, T., Hamann, D., Sandmann, J., Bombien, E., Spicher, K., Schultz, G. & Jakobs, K. H. (1994) *Mol. Pharmacol.* **45**, 890–898.
- Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H. & Journot, L. (1993) *Nature (London)* **365**, 170–176.
- Prather, P. L., McGinn, T. M., Erickson, L. J., Evans, C. J., Loh, H. H. & Law, P.-Y. (1994) *J. Biol. Chem.* **269**, 21293–21302.
- Raspé, E., Laurent, E., Andry, G. & Dumont, J. E. (1991) *Mol. Cell. Endocrinol.* **81**, 175–183.
- Laurent, E., Mockel, J., Takazawa, K., Erneux, G. & Dumont, J. E. (1989) *Biochem. J.* **263**, 795–801.
- Gross, B., Mirrahi, M., Sar, S. & Milgrom, E. (1991) *Biochem. Biophys. Res. Commun.* **177**, 679–687.
- Wong, S. K. F. & Ross, E. M. (1994) *J. Biol. Chem.* **269**, 18968–18976.
- Akiko, I. K., Guo, J., Lin, Y. X., Jüppner, H., Potts, J. T., Kronenberg, H. M., Bringham, F. R., Abou-Samra, A. B. & Segre, G. V. (1995) *J. Biol. Chem.* **270**, 8458–8465.
- Clapham, D. E. & Neer, E. J. (1993) *Nature (London)* **365**, 403–406.
- Sternweis, P. C. (1994) *Curr. Opin. Cell Biol.* **6**, 198–203.
- Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C. & Hawkins, P. T. (1994) *Cell* **77**, 83–93.
- Faure, M., Voyno-Yasenetskaya, T. A. & Bourne, H. R. (1994) *J. Biol. Chem.* **269**, 7851–7854.
- Selzer, E., Wilfing, A., Schiferer, A., Herrmann, M., Grubeck-Lobenstein, B. & Freissmuth, M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1609–1613.
- Saunier, B., Tournier, C., Jaquemin, C. & Pierre, M. (1995) *J. Biol. Chem.* **270**, 3693–3697.
- Hescheler, J. & Schultz, G. (1993) *Curr. Opin. Neurobiol.* **3**, 360–367.
- Xu, N., Bradley, L., Ambudkar, I. & Gutkind, S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6741–6745.
- Parks, S. & Wieschaus, E. (1991) *Cell* **64**, 447–458.
- Dhanasekaran, N., Vara Prasad, M., Wadsworth, S. J., Dermott, J. M. & van Rossum, G. (1994) *J. Biol. Chem.* **269**, 11802–11806.