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Thyrotropin, like Luteinizing hormone (LH) and Chorionic Gonadotropin (CG), Increases cAMP and Inositol Phosphate Levels in Cells with Recombinant Human LH/CG Receptor

Akinari Hidaka, Takashi Minegishi* and Leonard D. Kohn

Section on Cell Regulation, Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

*Department of Obstetrics and Gynecology, Gunma University School of Medicine, Maebashi 371, Japan

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Glycoprotein hormones and their receptors are each structurally related; thus, ligand-receptor cross reactivity may exist in pathologic situations, i.e., high human chorionic gonadotropin (hCG) levels in patients has been suggested to activate the thyrotropin receptor (TSHR). Studies with Cos-7 cells transfected with human CG and TSH receptor cDNAs suggest the converse may be more likely. Thus, in cells with TSHR, about $3x10^{-11}$ and $3x10^{-10}$M TSH cause half maximal increases in cAMP and inositol phosphate (IP) levels, respectively, whereas 10^{-6}M hCG has no effect on either. In cells with CGR, about 10^{-11} and 10^{-9}M CG or lutropin (LH) significantly increase cAMP and IP levels. Surprisingly, however, 10^{-11} and 10^{-9}M TSH are similarly effective in the two assays, respectively, and TSH increases cAMP and IP levels to the same extent as CG and LH. LH contamination of TSH is unlikely given similar results with highly purified TSH preparations from different sources, including recombinant TSH, and the specificity of simultaneously measured binding data. Thus, TSH binds with high affinity ($K_d=7x10^{-11}$M) to the human TSHR; hCG (up to 10^{-7}M) does not displace TSH binding. Similarly, hCG binds with high affinity ($K_d=5x10^{-10}$M) to the hCGR and TSH is only a weak inhibitor ($K_i=1x10^{-8}$M). Stimulating TSHR autoantibodies, with no epitopes on the CGR, do not duplicate TSH action. The unusual agonist action of TSH with recombinant CGR is consistent with TSHR models describing separate agonist and antagonist determinants; it may be a factor in the precocious puberty of juvenile hypothyroidism with high TSH levels.

Thyrotropin (TSH), lutropin (LH), folliotropin (FSH) and chorionic gonadotropin (CG) are structurally homologous hormones (1). Similarly, their respective receptors constitute a subgroup of structurally homologous, 7 transmembrane domain, G-protein-coupled receptors having a large extracellular domain with an important role in hormone recognition (1-4). The TSHR has an additional 64 residues which result in two regions in the N- and C-terminus of the extracellular domain which are nonhomologous with gonadotropin receptors (2-4). The

Abbreviations: TSH, thyrotropin; LH, lutropin; FSH, folliotropin; CG, chorionic gonadotropin; TSHRAb, thyrotropin receptor autoantibody; PIP_2, phosphatidylinositol bisphosphate.

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former contains determinants important for the agonist activity of TSHR autoantibodies (TSHRAbs) in patients with Graves' disease; the latter contains an immunodominant epitope related to the development of TSHRAbs as well as determinants important for the antagonist activity of blocking TSHRAbs in patients with hypothyroidism and idiopathic myxedema (4-13). Despite this difference, the TSHR and LH/CGR are both examples of single receptors which can couple to the cAMP as well as the phosphatidylinositol bisphosphate (PIP_2) signal cascades (3, 4, 11, 12, 14-18).

Although the hormone-receptor interaction is usually highly specific, cross-reactivity between glycoprotein hormones and their receptors has been considered because of the structural similarities of each (2, 19). For example, CG has been suggested to interact with the TSHR and cause hyperthyroidism in patients with hydatidiform mole or choriocarcinoma (20). Thyroid tissue appears to contain small amounts of LH/CGR (21) and, conversely, gonadal tissue contains small amounts of TSHR (22). The present report was thus aimed at evaluating cross-reactivity using recombinant human (h) TSHR and LH/CGR. Surprisingly we find that bovine TSH (bTSH) increased cAMP and inositol phosphate (IP) levels in cells transfected with hLH/CGR almost as well CG or LH, whereas the converse was not true. Further, this was evident despite the fact we could detect only low affinity TSH binding. We additionally report two findings. First, stimulatory IgGs from Graves' patients did not increase the two signals in cells with LH/CGR, supporting conclusions of mutagenesis studies which indicate they react with TSHR-specific sequences (4-12). Second hLH and hCG increased IP and cAMP levels in cells with human LH/CGR, as reported with the murine receptor (16).

MATERIALS AND METHODS

Materials - Bovine TSH (NIDDK - bTSH-I-1, 30 units/mg) and hCG (CR-127, 14900 IU/mg) were obtained from the NIH hormone distribution program; hLH (11000 IU/mg) was from Calbiochem (La Jolla, CA). Bovine TSH and hCG was iodinated (specific activity: 51 and 41 μCi/μg, respectively) using lactoperoxidase (5, 8, 10, 15, 17, 18, 21). Econo-Pac column-purified normal or Graves' IgG and wild type hTSHR or hLH/CGR cDNA, each in a pSG5 plasmid, were preparations previously described (5, 7-12, 15, 17, 18). Myo-[2-^{3}H(N)]-inositol and cAMP RIA kits were from DuPont-NEN (Boston, MA).

Transfection and Assays - Transfection of Cos-7 cells with 25 μg plasmid cDNA was by electroporation (5, 8, 10, 15, 17, 18); cells transfected with pSG5 alone were negative controls. The same batch of transfected cells was plated in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum: 6-well plates ($5x10^5$ cells/well) for binding assays or 24-well plates ($1x10^5$ cells/well) for cAMP and IP assays (15, 17, 18). Medium was inositol free in the latter assays and supplemented with 2 mCi/L myo-[2-^{3}H(N)]-inositol (specific activity approximately 20 Ci/mmol). All assays were initiated simultaneously, 48 hours after transfection, after washing with assay buffer: NaCl-free, Hanks' Balanced Salt Solution containing 0.5% BSA, 222 mM sucrose, and 20 mM HEPES, pH 7.4 (15, 17, 18).

[^{125}I]TSH or [^{125}I]hCG binding was measured after incubation for 8 hours at 22° in 1 ml assay buffer containing $3x10^4$ or $1.5 x10^5$ cpm iodinated ligand, respectively, and 0 to 10^{-7}M

unlabeled TSH or hCG (15, 17, 18). Specific binding was calculated by subtracting values obtained at the same concentrations of radiolabeled and unlabeled ligand in the pSG5 control transfectants; in each case, nonspecific binding was less than 10% and was the same as hormone binding in the presence of 10^{-7}M unlabeled ligand.

Total cAMP and IP levels were measured (15, 17, 18) in the same wells after incubation for 2 h at 37° in 0.2 ml assay buffer containing 10 mM LiCl, 0.5 mM isobutyl-methylxanthine, and, as noted, 10^{-11} to 10^{-6}M hormone (bTSH, hCG, hLH) or 5.0 mg/ml IgG (Graves' or normal). After adding 1.0 ml 5% perchloric acid, samples were centrifuged to remove protein debris, neutralized with KOH, and centrifuged to remove insoluble salts. Total cAMP in aliquots of the supernatant was measured by radioimmunoassay; IP formation was determined using Dowex AG1-X8 columns (15, 17, 18, 23). Data are expressed as the fold increase above the negative controls, i.e. cells transfected with pSG5 and exposed to buffer alone.

All assays were performed in duplicate, on at least 3 separate occasions, with different batches of cells. Values in each well were corrected either for cell protein, measured using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) and a BSA standard, or for total tritiated inositol incorporated (15, 17, 18). The program LIGAND (24) and a single high affinity binding site model, which best fit all data, were used to calculate K_d and EC_{50} values (5, 8, 10, 15, 17, 18).

RESULTS

bTSH and hCG Binding in Cells with Recombinant hTSHR or hLH/CGR - Cos-7 cells transfected with hTSHR cDNA specifically bind 15% of the added [^{125}I]bTSH. Increasing concentrations of unlabelled bTSH progressively displaced [^{125}I]bTSH binding with a K_d of $7x10^{-11}$M; unlabeled hCG, up to 10^{-7}M, did not displace [^{125}I]bTSH binding (Fig. 1A). There was no specific [^{125}I]hCG binding in the absence or presence of unlabeled hCG or TSH (data not shown). Cos-7 cells transfected with hLH/CGR cDNA specifically bind 20% of the

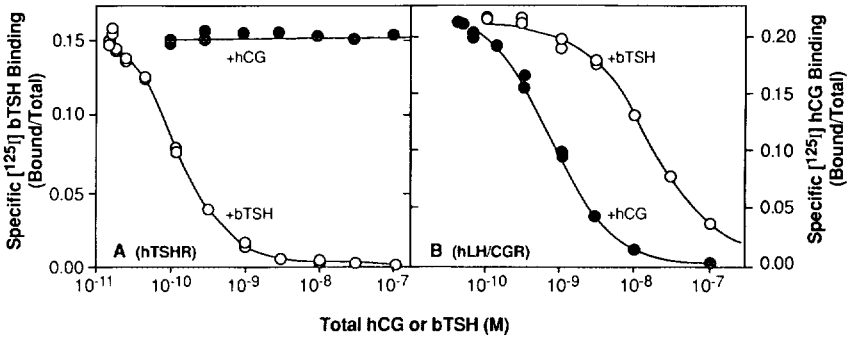

Figure 1. Displacement of [^{125}I]TSH (A) and [^{125}I]hCG binding (B) by unlabeled bTSH (○) or hCG (●) in Cos-7 cells transfected with human TSHR (A) or human LH/CGR (B) cDNA. Assays were performed as detailed in Materials and Methods. Specific binding was calculated by subtracting values obtained at the same concentrations of radiolabeled and unlabeled ligand in the pSG5 control transfectants. Values are duplicates from a representative experiment; the same TSH results were obtained on 3 different occasions whether using highly purified bTSH from the NIH distribution program or a previously characterized preparation of highly purified bTSH, neither of which have significant LH contamination (25, 26, 29).

[^{125}I]hCG added. Increasing concentrations of unlabeled hCG progressively displaced the [^{125}I]hCG binding with a K_d of $5x10^{-10}$M (Fig. 1B). bTSH was able to displace the [^{125}I]hCG binding only weakly (Fig. 1B), the K_i being $1x10^{-8}$M, and no specific high affinity binding was detected using [^{125}I]bTSH.

Hormone-induced cAMP and Inositol Phosphate Increases in Cells with Recombinant hTSHR or hLH/CGR - In Cos-7 cells transfected with hTSHR cDNA, bTSH caused concentration-dependent increases in cAMP and IP levels, half maximal activities being evident at approximately $3x10^{-11}$ and $3x10^{-10}$M TSH, respectively (Fig. 2A). Neither hCG (Fig. 2B) nor hLH (data not shown), up to 10^{-6}M, increased cAMP or IP levels in cells with hTSHR. The need for higher concentrations of TSH to stimulate the PIP_2 cascade (Fig. 2A) is well documented and has been shown to be modulated by the P_1-purinergic receptor (17, 23). Similarly, the high basal level of cAMP in the absence of TSH in Cos-7 transfectants with wild

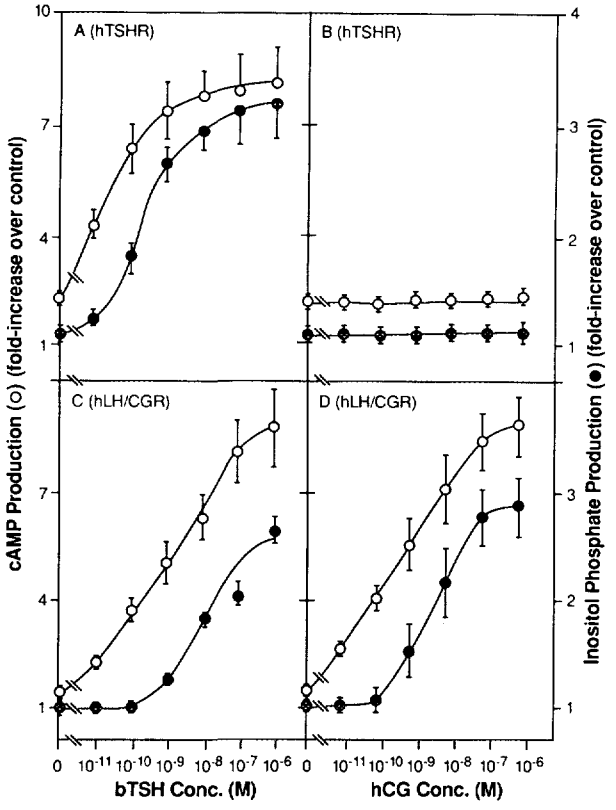

Figure 2. Ability of bTSH (A, C) or hCG (B, D) to increase cAMP (○) or IP (●) levels in Cos-7 cells transfected with human TSHR (A, B) or human LH/CGR (C, D) cDNA by comparison to Cos-7 cells transfected with the pSG5 vector alone and exposed only to buffer. Assays were performed as detailed in Materials and Methods. Values are the mean ± S.E. of duplicates from three separate experiments.

type TSHR (Fig. 2A and 2B) has been described (8, 10, 15, 18) and related to specific sequences in the 3rd cytoplasmic loop which regulate constitutive cAMP levels (15, 18).

In Cos-7 cells transfected with hLH/CGR cDNA, the basal levels of cAMP were not different from those in control cells transfected with pSG5 (Fig. 2C and 2D); this contrasts with the increased basal cAMP levels seen in cells with hTSHR (Fig. 2A and 2B). In cells transfected with hLH/CGR cDNA, hCG caused a concentration dependent increase in cAMP and IP levels (Fig. 2D); as in the case of TSH and TSHR, higher concentrations of hCG were necessary in the latter assays (Fig. 2D). The effect of hCG was duplicated by hLH (data not shown). Surprisingly, however, bTSH also increased cAMP and inositol phosphate levels in cells with hLH/CGR and the ability of bTSH to increase the two signals was similar to that of hCG and hLH, both with respect to maximal stimulation and ligand concentration, i.e. 10^{-11} and 10^{-9}M concentrations of hCG or TSH exhibited significant ($P<0.05$) and similar effects (Fig. 2C and 2D).

The bTSH activity was particularly surprising given its minimal ability to inhibit [^{125}I]hCG binding to the hLH/CGR (Fig. 1B) and the absence of detectable high affinity [^{125}I]TSH binding to the same cells. The bTSH used in the assays above was the highly purified NIH preparation. The same results were obtained using a previously described (25, 26), highly purified bTSH preparation which is homogeneous in the ultracentrifuge, has the molecular weight, amino acid, and carbohydrate composition of authentic bTSH used in sequencing studies (1), and has no spectral evidence of tryptophan residues, which are present in LH but not TSH. Finally, we used recombinant hTSH, kindly provided by Dr. B. Weintraub (NIDDK, NIH), and showed that it had no effect on hCG binding to Cos-7 cells transfected with the hLH/CGR but was bioactive in both assays, albeit less active than bTSH as a function of concentration, but proportional to bTSH in its effect on FRTL-5 thyroid cells (data not shown). These data support the conclusion that the effect of TSH on hLH/CGR transfected Cos-7 cells was not a reflection of LH contamination.

Graves' IgG-induced cAMP and Inositol Phosphate Levels in Cells with Recombinant hLH/CGR - Stimulating TSHR autoantibodies in the sera of patients with Graves' disease can increase cAMP and PIP_2 signalling in FRTL-5 cells (17, 27), in Cos-7 cells transfected with rat TSHR (15, 17, 18), and in CHO cells transfected with hTSHR (28). The epitopes for these actions are, however, specific for the TSHR (4-13). We examined the effect of Graves' IgG on hTSHR and hLH/CGR. IgG from 2 patients with Graves' disease increased cAMP and IP levels in cells with hTSHR (Fig. 3) to the same extent as bTSH, i.e. equivalent to 10^{-9}M bTSH (Fig. 2). However, neither Graves' IgG preparation increased cAMP or inositol phosphate levels in Cos-7 cells transfected with hLH/CGR (Fig. 3), whereas 10^{-9}M TSH was effective in either assay in the same cells (Figs. 2C).

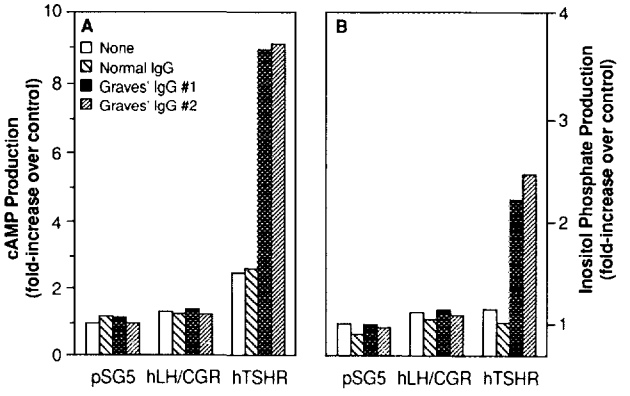

Figure 3. Ability of normal IgG or two different Graves IgGs, by comparison to buffer alone, to increase cAMP (A) or IP (B) levels in Cos-7 cells transfected with human LH/CGR or human TSHR cDNA by comparison to Cos-7 cells transfected with the pSG5 vector alone. Assays were performed as detailed in Materials and Methods. Values are the mean of duplicates from a representative experiment; the same results were obtained in three separate experiments using different batches of cells.

DISCUSSION

Amir et. al. (29) reported that highly purified bTSH bound to rat testis particulate fractions, that its [^{125}I]hCG-displacing activity exceeded that of bLH, that bTSH at higher concentrations increased cAMP levels in rat Leydig cells, and that this was not explained by LH contamination of the TSH preparations (29). The bTSH binding was localized (30) to interstitial cell membranes and was inhibited by Graves' IgG. In retrospect, it is not, however, clear whether TSH was interacting with LH/CGR or with the TSHR, since the latter may exist in gonadal tissues (21), since epitopes for Graves' IgG interactions with TSHR do not appear to exist on the LH/CGR (4-13), and since the K_d for TSH binding was the same as in thyroid membranes, but the B_{max} was markedly lower (30). In this report, we show that bTSH increases cAMP and IP levels in Cos-7 cells transfected with hLH/CGR cDNA. This is not due to LH contamination of bTSH; thus, the bTSH effect on both signals, though similar to hCG and hLH, is not associated with similar effects on hCG binding, which would be anticipated were contamination a factor.

The observations are interesting in several respects. First, they do not contradict data in initial descriptions of the LH/CGR cDNA cloning, since TSH bioactivity in neither cAMP nor IP assays was tested. Thus, this is the first recognition of the agonist activity of TSH in the recombinant LH/CGR system. The agonist activity of TSH, in the absence of measurable high affinity binding to the recombinant LH/CGR, is consistent with studies of the TSHR. Thus, we have shown that the TSHR has separate sites for high affinity hormone binding and

for agonist action (4, 5, 7-12). Considering structural homologies (1-4), this may be true of all glycoprotein hormone receptors. Preliminary studies with TSHR-LH/CGR chimeras (Abstract, Am. Thyroid Assoc. Ann. Mting., 1993; Ref. 31) indicate that the agonist action of TSH on LH/CGR can be explained by an interaction with the N-terminal part of the extracellular domain. Thus, substitution of the N-terminal part of the TSHR extracellular domain with the corresponding domain of LH/CGR results in a receptor wherein hCG increases cAMP and IP levels the same as with wild type LH/CGR, but with only low affinity hCG binding compared to wild type LH/CGR. Separate ligand-receptor interactions for binding and function in the LH/CGR have also been uncovered in studies of mutants of the 2nd transmembrane domain of LH/CGR (32). Two potentially important conclusions can be drawn: (a) partial occupation of the agonist site may induce the activated state of a glycoprotein hormone receptor and (b) caution must be used in evaluating the specificity of glycoprotein hormone-receptor interactions solely with binding studies.

Second, the inability of Graves' IgG to increase cAMP and IP levels in cells with hLH/CGR, whereas bTSH could, is consistent with disease specificity and the fact that epitopes for these antibodies do not exist in LH/CGR (4, 5, 7-12). These results are consistent with a study (33) showing that Graves' IgG-induced increases in cAMP levels were minimal in human testicular slices and overlapped data with normal IgG. Third, our data showing that CG and LH increase IP as well as cAMP levels in Cos-7 cells transfected with human LH/CGR cDNA are consistent with observations in L cells expressing murine LH/CGR (16). Recently (34), hCG was reported to increase intracellular calcium in human granulosa-lutein cells only in the presence of an α-adrenergic receptor agonist. It should be noted that P_2-purinergic agonists reciprocally modulate the TSH- or TSHRAb-induced cAMP and IP signals (17, 23); receptor cross-talk in the regulation of cAMP and IP signal generation induced by the glycoprotein hormones may, therefore, be generally important.

Finally, in vitro studies of cells transfected with receptor recombinants cannot be construed as exhibiting normal in vivo specificities; membrane determinants other than the glycoprotein component of the receptor may contribute to ligand specificity (19). Nevertheless, these data may have relevant and important in vivo correlates. Thus, neonatal hypothyroidism in rats can cause increases in adult testis and reproductive organ size, increased numbers of Sertoli cells, and increased numbers of Leydig cells, despite suppressed levels of FSH and LH throughout adulthood (35-38). Our data raise the possibility that the increased TSH resultant from the hypothyroidism might stimulate gonadal cells through the LH/CGR and be an important causative factor. Rat, human, ovine, and bovine TSH can stimulate Sertoli cells to a similar extent as FSH (39) indicating that a similar mechanism, i.e. TSH-FSHR interactions, might exist. Macroorchism is relatively common in primary hypothyroidism in boys. The

possibility that TSH stimulation of the LH/CGR might play a role in the development of precocious puberty of juvenile hypothyroidism should be considered.

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