0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(12):4570–4582

doi: 10.1210/en.2002-220670

Ghrelin and Growth Hormone (GH) Secretagogues Potentiate GH-Releasing Hormone (GHRH)-Induced Cyclic Adenosine 3′,5′-Monophosphate Production in Cells Expressing Transfected GHRH and GH Secretagogue Receptors

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GHRH stimulates GH secretion from somatotroph cells of the anterior pituitary via a pathway that involves GHRH receptor activation of adenylyl cyclase and increased cAMP production. The actions of GHRH to release GH can be augmented by the synthetic GH secretagogues (GHS), which bind to a distinct G protein-coupled receptor to activate phospholipase C and increase production of the second messengers calcium and diacylglycerol. The stomach peptide ghrelin represents an endogenous ligand for the GHS receptor, which does not activate the cAMP signaling pathway. This study investigates the effects of GHS and ghrelin on GHRH-induced cAMP production in a homogenous population of cells expressing the cloned GHRH and GHS receptors. Each epitope-tagged receptor was shown to be appropriately expressed and to functionally couple to its respective second messenger pathway in this heterologous cell system. Although activation of the GHS receptor alone had no effect on cAMP production, coactivation of the GHS and GHRH receptors produced a cAMP response approximately twice that observed after activation of the GHRH receptor alone. This potentiated response is dose dependent with respect to both GHRH and GHS, is dependent on the expression of both receptors, and was observed with a variety of peptide and nonpeptide GHS compounds as well as with ghrelin-(1–5). Pharmacological inhibition of signaling molecules associated with GHS receptor activation, including G protein $\beta\gamma$-subunits, phospholipase C, and protein kinase C, had no effect on GHS potentiation of GHRH-induced cAMP production. Importantly, the potentiation appears to be selective for the GHRH receptor. Treatment of cells with the pharmacological agent forskolin elevated cAMP levels, but these levels were not further increased by GHS receptor activation. Similarly, activation of two receptors homologous to the GHRH receptor, the vasoactive intestinal peptide and secretin receptors, increased cAMP levels, but these levels were not further increased by GHS receptor activation. Based on these findings, we speculate that direct interactions between the GHRH and GHS receptors may explain the observed effects on signal transduction. (*Endocrinology* 143: 4570–4582, 2002)

REGULATION OF the GH axis is coordinated by the hypothalamic hormones GHRH and somatostatin. GHRH stimulates GH synthesis and secretion from the somatotroph cells of the anterior pituitary, whereas somatostatin inhibits GH secretion (1). Recently, an endogenous GH secretagogue (GHS), ghrelin, was isolated from stomach (2). Although GHRH and somatostatin regulate the ultradian rhythm of GH secretion, ghrelin appears to coordinate food intake with GH secretion (3–5).

GHSs are a family of peptidergic and nonpeptidergic compounds that are potent stimulators of GH secretion. GHSs were developed based on the observation that metabolic fragments of opiate peptides stimulate GH secretion (6). The earliest GHSs, which consisted of the pentapeptides Tyr-DTrp-Gly-Phe-Met-NH_2 and Tyr-DPhe-Gly-Phe-Met-NH_2, had low potency *in vitro* and no activity *in vivo* (7, 8). The first GHS to potently stimulate GH secretion both *in vitro* and *in vivo* was the synthetic hexapeptide, GH-releasing peptide-6 (GHRP-6) (9). Subsequent generations of GHSs that include the peptidergic compounds GHRP-1 (10), GHRP-2 (11), and hexarelin (12) and the nonpeptidergic compounds L692-429 (13, 14), L-692-585 (15), and MK-0677 (16) were generated to increase the half-life and bioavailability of these compounds.

Ghrelin, an endogenous GH secretagogue, is a 28-amino-acid peptide with a unique *n*-octanoyl modification on serine residue 3 that is necessary for its biological activity (2). Ghrelin secretion rises before food intake (4), and it has been shown to stimulate food consumption (3–5) and adipogenesis (17). In addition, ghrelin stimulates the secretion of insulin and gastric acid (18–20). Structure-function analysis of ghrelin has demonstrated that the first five amino acids of this peptide, including the *n*-octanoyl modification at position 3, are capable of binding the GHS receptor to stimulate the release of intracellular calcium (21).

GHRH, ghrelin, and somatostatin act through G protein-coupled receptors (GPCRs) associated with distinct second messenger pathways. The GHRH receptor stimulates adenylyl cyclase (AC) to increase the production of the cellular second messenger cAMP. The GHS receptor, which binds ghrelin, activates phospholipase C (PLC), protein kinase C (PKC), and intracellular calcium mobilization (22). Somatostatin receptors 2 and 5, the subtypes predominantly as-

Abbreviations: AC, Adenylyl cyclase; GHRP, GH-releasing peptide; GPCR, G protein-coupled receptor; GHS, GH secretagogue; HA, hemagglutinin; PKC, protein kinase C; PLC, phospholipase C; TPA, 12-*O*-tetraphorbol 12-myristate 13-acetate; VIP, vasoactive intestinal peptide.

sociated with inhibition of GH secretion from the rat pituitary, reduce AC activity and stimulate the PLC/PKC second messenger pathway (23–25).

In vivo, ghrelin synergizes with GHRH to stimulate a potentiated GH response (26). Consistent with this finding, a potentiated GH response has been demonstrated in human studies after treatment with GHRH and GHRP-6 (8, 27). Efforts to determine the mechanism responsible for this synergistic response have been confounded by the fact that GHSs act on both the hypothalamus and pituitary. One effect of the GHSs on the hypothalamus includes stimulating GHRH release (28). Although it has been suggested that GHS-induced GHRH release is responsible for this synergism, no studies have provided convincing data to support this hypothesis. In fact, studies in cultured pituitary cells demonstrate synergistic GH and cAMP responses to GHRH and GHS treatments (13, 29, 30). These findings suggest that the actions of GHSs on the pituitary, and presumably on the somatotroph cells, are sufficient to elicit, at least in part, these synergistic responses. Pituitary cultures are a heterogeneous cell population, and the pituitary cell type-specific expression of the GHRH and GHS receptors has yet to be fully characterized. Thus, it is unclear whether the potentiated cAMP and GH responses arise from the direct actions of GHRH and GHS on the somatotroph cells. It is possible that GHSs may stimulate other pituitary cell types to release a paracrine factor that could then elicit the potentiated responses in somatotroph cells. In this study we generated a cell model to investigate the effect of GHS on GHRH-stimulated cAMP production in a homogeneous cell population. Using cells expressing both the GHRH and GHS receptors, we demonstrate an effect of GHS on GHRH-induced cAMP production and investigate signaling pathways to characterize the mechanism responsible for the synergistic cAMP response.

Materials and Methods

Reagents and plasmids

The GHS compounds L692-429, L692-428, and L692-585 were provided by Dr. Scott Feighner of the Merck & Co. (Rahway, NJ). GHRP-6 was purchased from Sigma-Aldrich (St. Louis, MO). Ghrelin-(1–5) was purchased from Peptides International (Louisville, KY). The rat vasoactive intestinal peptide (VIP) (31) and secretin receptors (32) initially provided by Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan) were subcloned into pcDNA3 downstream of the T7 promoter using the *Hin*dIII and *Xba*I sites (33). The βARK1 minigene construct in the pRK5 vector was provided by Dr. Robert Lefkowitz (Duke University, Durham, NC). The CD8-βARK construct was provided by Dr. J. Silvio Gutkind (NIH, Bethesda, MD).

Generation of the flag epitope-tagged GHS receptor

The rat GHS receptor was isolated from hypothalamic RNA by RT-PCR using oligonucleotides generated from the published sequence (GHS-R5′, GCGAATTCATGTGGAACGCGACCCC; GHS-R3′, GCTCT AGATCATGTGTTGATGCTCGAC). A flag epitope tag was added to the carboxyl terminus of the rat GHS receptor using PCR (GHS-R flag, GCTCTAGACCTTGTCATCGTCGTCCTTGTAGTCTGTGTTGATGCT-CGACTTTG). The rat GHS receptor was ligated into the pTracer-cytomegalovirus mammalian expression vector (Invitrogen, San Diego, CA) using the *Kpn*I and *Xba*I restriction sites.

Generation of hemagglutinin (HA) epitope-tagged rat GHRH receptor

The amino terminus of the rat GHRH receptor was excised from RPR-comp18c in pcDNA-1 (34) using the restriction enzymes *Bam*HI and *Apa*I, whereas the carboxyl terminus of the mouse GHRH receptor, including an HA epitope tag in pGEM5z, was excised with the restriction enzymes *Apa*I and *Xba*I. The amino and carboxyl termini were ligated into the pcDNA3.0 vector (Invitrogen) using the *Bam*HI and *Xba*I restriction sites.

Transfections with the vaccinia-T7 RNA polymerase hybrid expression system

All experiments were performed using HeLa-T4 cells transfected with various receptor constructs using the vaccinia virus-T7 polymerase hybrid expression system (35). Subconfluent monolayers of HeLa-T4 cells maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 5% fetal bovine serum (Life Technologies, Inc.) were infected with vaccinia virus vTF7.3 expressing the bacteriophage T7 RNA polymerase (obtained under license from Dr. Bernard Moss, NIH), at a multiplicity of infection of 10, for 30 min in PBS/0.1% BSA. The various plasmid DNAs were incubated with transfectase (Sigma-Aldrich) at a ratio of 4–5 μg lipid per microgram of DNA in Opti-MEM 1 medium (Life Technologies, Inc.) for 20–30 min at room temperature. After aspirating the virus from the cells, the DNA/transfectase mixture was added, and the cells were incubated at 37 C in 5% CO_2 for 12–15 h. The amount of DNA used for transfection varied according to the size of the plates.

Metabolic labeling of transfected cells and immunoprecipitation of epitope-tagged receptors

HeLa-T4 cells grown in 35-mm plates were transfected with 5 μg DNA/plate for 12–15 h, then washed twice with PBS. Cells were starved in cysteine/methionine-deficient DMEM (ICN Biomedicals, Inc., Irvine, CA) for 30 min and then labeled with 50 μCi Express (NEN Life Science Products, Boston, MA)/plate in the same medium for 3 h at 37 C in 5% CO_2. The cells were harvested, washed twice with PBS, and resuspended in 400 μl RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulfate] containing 0.1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotonin. Cells were lysed through a series of four freeze-thaw cycles, and the lysates were centrifuged for 10 min to pellet residual cellular debris. The supernatant was separated into two fractions into which 1 μg/ml of the HA-specific 12CA5 ascites fluid (a gift from Dr. Robert A. Lamb, Northwestern University) or the anti-M2 monoclonal antibody against the flag epitope (Sigma-Aldrich) was added. Antibodies were incubated overnight at 4 C on a hematology mixer. The following day 30 μl of a 50% suspension of protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS were added to the tubes, and the incubation was continued for 45 min. The beads were washed seven times with 500 μl cold RIPA buffer and once with cold wash buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA]. The beads were then resuspended in 40 μl 2× SDS-PAGE sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.1% bromophenol blue]. The samples were boiled for 5 min before separation by SDS-PAGE using a Tris-glycine buffer with the Benchmark Prestained Protein Ladder (Invitrogen) as size markers. The gels were fixed in 20% methanol/7% acetic acid for 30 min, saturated with glacial acetic acid twice for 5 min each time, impregnated with 22% (wt/vol) 2,5-diphenyl-ox-azole in acetic acid for 45 min, dried, and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY). Receptor expression levels were quantified using the software package Scion Image 1.6 (NIH).

Immunofluorescence

HeLa-T4 cells cultured on 12-mm round coverslips (Fisher Scientific, Pittsburgh, PA) in 24-well plates were transfected with 500 ng DNA. Coverslips were washed with PBS, fixed in 1% paraformaldehyde for 30 min at 4 C, washed with PBS twice, and incubated with 1 μg/ml of the HA-specific 12CA5 ascites fluid or the anti-M2 monoclonal antibody

against the flag epitope in PBS containing 0.1% saponin for 1 h at room temperature. After extensive washing, the coverslips were incubated with 2 μg/ml fluorescein isothiocyanate-conjugated goat antimouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS containing 0.1% saponin for 1 h at room temperature. After extensive washing with PBS, the coverslips were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA). Photomicroscopy of cells was performed using a ×63 objective on a DM IRE2 inverted microscope (Leica Corp., Rockleigh, NJ) with equivalent exposure times.

Measurement of intracellular cAMP levels

HeLa-T4 cells were transfected in 12-well plates using 1 μg DNA/well overnight. The cells were washed twice in 1× PBS and then incubated in serum-free DMEM (Life Technologies, Inc.) with 0.1 mM isobutylmethylxanthine for 20 min at 37 C. Hormones in freshly warmed medium were added to the cells, and the incubation was continued for another 20 min at 37 C. Medium was removed, and cells were lysed with ice-cold 0.1 N HCl for 10 min on ice. The lysates were neutralized in 150 μl 50 mM Tris (pH 8.0) and 8 mM EDTA. Twenty-five microliters of neutralized lysate were used to assay cAMP in a competitive protein binding assay using [8-^{3}H]cAMP (Amersham Pharmacia Biotech) as a tracer. Lysate, tracer, and binding protein were incubated on ice for 2 h, followed by the addition of 50 μl activated charcoal solution [2.6% activated charcoal (Sigma-Aldrich), 50 mM Tris-HCl (pH 7.5), 4 mM EDTA, and 2% BSA]. Samples were centrifuged at 4 C for 2 min, and 100 μl supernatant were drawn off. The protein-bound [8-^{3}H]cAMP in the supernatant was measured by liquid scintillation counting using Cytoscint (ICN Biomedicals, Inc.), and a linear standard curve was performed in each assay. The assays were performed with triplicate samples.

Measurement of intracellular calcium release

Fluorometry was used to measure the release of calcium from intracellular stores after GHRH treatment. Transfected cells were lightly trypsinized and washed with DMEM (Life Technologies, Inc.) containing 5% serum (Life Technologies, Inc.). The cells were then washed with loading buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1 mM $CaCl_2$, and 1% BSA containing 147 μg/ml probenecid) and resuspended in 1.5 ml loading buffer containing 3.5 μg of the calcium indicator fluo-3/AM (Molecular Probes, Inc., Eugene, OR). After incubation on a hematology mixer for 30 min in the dark, the cells were centrifuged at 400 × *g* at 4 C for 1 min, washed in loading buffer to remove excess dye, repelleted, and resuspended in loading buffer supplemented with 1 mM CaCl and 1% BSA. Fluorometric emission at 530 nm from 1 ml transfected HeLa-T4 cells loaded with the calcium dye was monitored after treatment with 10 μM hormone, using a PC1 photon counting spectrofluorometer (ISS, Champaign, IL) with the excitation wavelength set at 515 nm. Before ligand treatment, the basal emission from transfected HeLa-T4 cells was measured and averaged. In plotting the ligand-induced calcium response, the averaged basal emission value was subtracted from all emission values.

Luciferase assay

After transfection and treatment, cells were washed twice with cold PBS, then lysed in sample buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, and 0.1% Triton X-100] on ice for 20–30 min with gentle shaking. One hundred microliters of the cell lysates were added to 400 μl assay buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 5 mM ATP, and 1 μg/ml BSA], 100 μl 1 mM luciferin (sodium salt) were added using an automatic injector, and emitted luminescence was measured using a 2010 luminometer (Analytical Bioluminescence, San Diego, CA) for 10 sec. Twenty microliters of the cell lysate were used for total protein determination using a protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, CA).

Results

Expression of a HA epitope-tagged rat GHRH receptor and a flag epitope-tagged rat GHS receptor

To allow for detection of the GHRH and GHS receptor proteins, epitope tags were added to the carboxyl termini of the receptors. The GHRH receptor had a HA epitope tag, and the GHS receptor had a flag epitope tag. Both receptors were expressed in HeLa-T4 cells using the vaccinia-T7 RNA polymerase hybrid expression system (35), and receptor protein expression was visualized through immunoprecipitation (Fig. 1A) and immunofluorescence (Fig. 1B). The GHRH and GHS receptors were found to be of the expected sizes of approximately 48 and 42 kDa, respectively (Fig 1A). For immunofluorescence, the HeLa-T4 cells were permeabilized with saponin after fixation with 1% paraformaldehyde to allow detection of the intracellular HA or flag epitope tags.

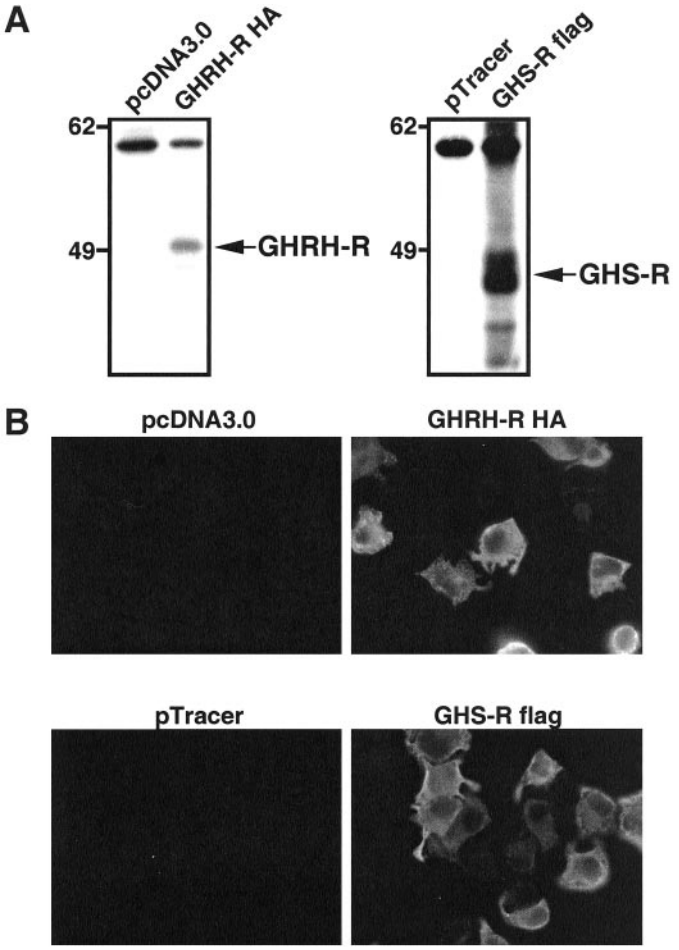

FIG. 1. Expression of the HA epitope-tagged GHRH receptor and the flag epitope-tagged GHS receptor. A, Immunoprecipitation of the epitope-tagged receptor constructs. Equivalent amounts of protein from metabolically labeled HeLa-T4 cells transfected with the pcDNA3.0 vector, the HA-tagged GHRH receptor, the pTracer vector, or the flag epitope-tagged GHS receptor were immunoprecipitated using the 12CA5 ascites fluid to the HA epitope or the anti-M2 monoclonal antibody to the flag epitope. Molecular mass markers (in kilodaltons) are indicated on the *left*. B, Immunofluorescent localization of the receptor constructs. Immunofluorescence of HeLa-T4 cells transfected with pcDNA3.0, the HA-tagged GHRH receptor, pTracer, or the flag-tagged GHS receptor was performed using 12CA5 ascites fluid to the HA epitope and anti-M2 monoclonal antibody against the flag epitope. All incubations occurred in the presence of 0.1% saponin to permeabilize the cells. Photographs were taken with the same exposure length and magnification using an inverted fluorescence microscope.

Both receptors were detected predominantly in the perinuclear and cell surface regions.

Functional characterization of the epitope-tagged rat GHRH and GHS receptors

To ensure that the GHS receptor is functional, the release of intracellular calcium (Fig. 2A) and activation of the MAPK pathway (Fig. 2B) were measured. HeLa-T4 cells transiently expressing the flag epitope-tagged GHS receptor were treated with the GHS agonist L692-429 or its inactive enantiomer L692-428. The release of intracellular calcium was measured using fluorometry with the calcium indicator dye fluo-3/AM. Untransfected HeLa-T4 cells were unresponsive to L692-429 treatment, whereas HeLa-T4 cells transiently expressing GHS receptors demonstrated an increase in fluorescent emission after L692-429 treatment. In contrast, treatment with the inactive enantiomer L692-428 had no effect on fluorescent emission from these transfected cells. As previously reported, treatment with GHRH had no effect on intracellular calcium release from HeLa-T4 cells transiently expressing the HA epitope-tagged GHRH receptor (34). An Elk-1 reporter gene-based assay was used to measure GHS activation of the MAPK pathway. This system includes a GAL4 UAS luciferase plasmid and a unique *trans*-activator plasmid that expresses a fusion protein consisting of the activation domain for Elk1 linked to the yeast GAL4 DNA-binding domain. HeLa-T4 cells were transfected with these two plasmids and the GHS receptor. A significant increase in luciferase activity after L692-429 treatment was seen, suggesting that activation of the GHS receptor stimulates the MAPK pathway. To determine whether GHS receptor activation has an effect on cAMP production, cAMP levels were measured in response to ligand treatment (Fig. 2C). There was no increase in cAMP levels after activation of the GHS

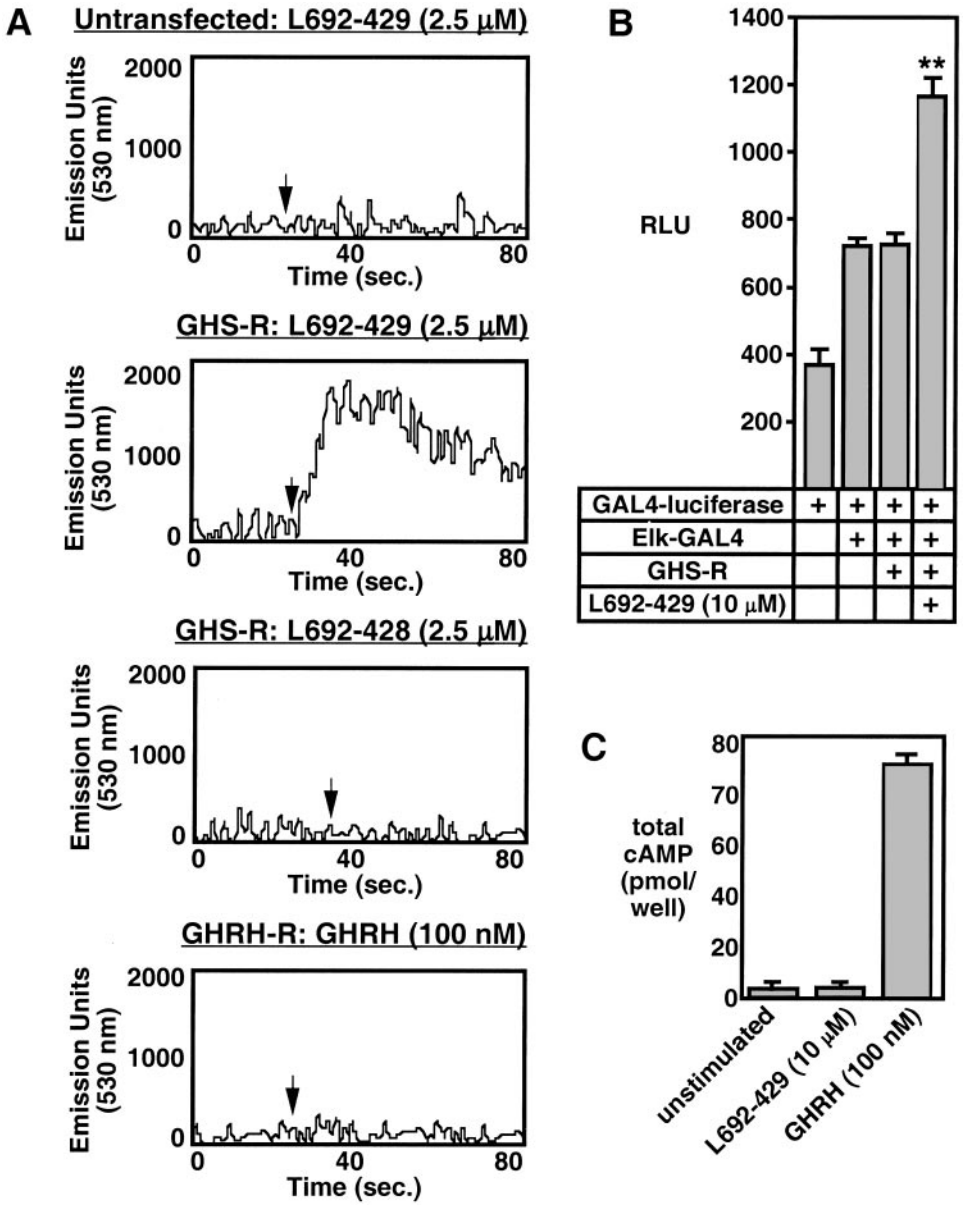

FIG. 2. Functional characterization of the HA epitope-tagged GHRH receptor and the flag epitope-tagged GHS receptor. A, Measurement of intracellular calcium release after activation of the GHS and GHRH receptors. HeLa-T4 cells untransfected or transfected with the receptor constructs were incubated with the calcium indicator dye fluo-3/AM and then stimulated with L692-429, its inactive enantiomer L692-428, or GHRH. The release of intracellular calcium was indicated by an increase in fluorescent emission at 530 nm. The *arrows* represent the onset of ligand treatment. B, Measurement of MAPK pathway activation using the PathDetect Trans-Reporting system (Stratagene, La Jolla, CA). The *pluses* indicate transfection with the GHS receptor, a GAL4-luciferase reporter gene, and an Elk-GAL4 *trans*-activator. Cells were treated with 10 μM L692-429 for 4 h (also indicated by a *plus*). The conditions were repeated in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (**, $P < 0.01$). RLU, Relative light units. C, Measurement of cAMP production in HeLa-T4 cells transfected with the flag-tagged GHS receptor or the HA-tagged GHRH receptor. The relative amount of intracellular cAMP produced in HeLa-T4 cells after treatment with 10 μM L692-429 or 100 nM GHRH is shown.

receptor, although as expected, cAMP levels were elevated in response to GHRH receptor activation.

Potentiation of GHRH-induced cAMP production by GHS receptor activation

To investigate the effect of GHS potentiation on GHRH-induced cAMP production in a homogeneous cell population, HeLa-T4 cells were transfected with both the GHRH and GHS receptors. Levels of cAMP were measured in the transfected cells after treatment with GHRH, L692-429, or both GHRH and L692-429 (Fig. 3A). There was an increase in cAMP levels in response to treatment with 1 nM and even more so with 100 nM GHRH. In contrast, the levels of cAMP were unaffected by treatment with 10 or 50 μM L692-429. Interestingly, cotreatment with GHRH and L692-429 elicited a potentiated cAMP response in the transfected cells. L692-429 treatment with 1 nM GHRH resulted in cAMP levels 3.3 times greater than those in response to GHRH treatment alone, whereas 100 nM GHRH plus L692-429 elicited a response 2.4 times greater than that with GHRH treatment alone. In the absence of the GHS receptor, there was no potentiation of the cAMP response (Fig. 3A). To determine receptor expression levels in the cotransfection experiments the GHRH and GHS receptors were immunoprecipitated from protein lysates of metabolically labeled HeLa-T4 cells using monoclonal antibodies to the HA- and flag epitope tags. Expression of each receptor was somewhat reduced in cells cotransfected with both receptors compared with that in cells transfected with individual receptors. GHRH receptor number was reduced to 63% of the control value, and GHS

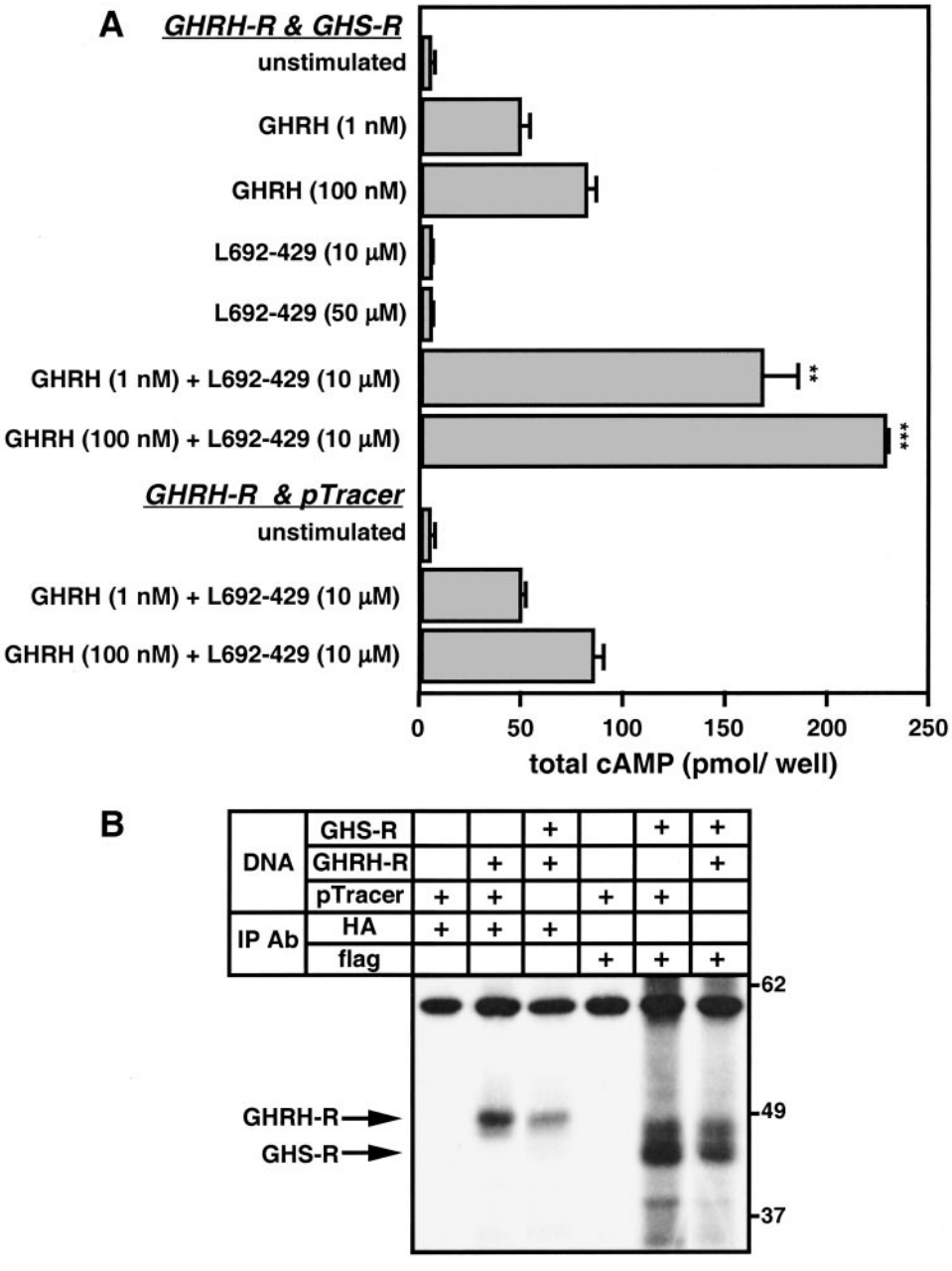

FIG. 3. GHS potentiation of GHRH-induced cAMP production. A, Measurement of cAMP production in HeLa-T4 cells transfected with the receptor constructs. HeLa-T4 cells were transfected with the GHRH and GHS receptors or with the GHRH receptor and the pTracer vector (indicated by *underlining* and *italics*). The cells were stimulated with increasing concentrations of GHRH, L692-429, or both GHRH and L692-429. Transfections were performed in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (**, $P < 0.01$; ***, $P < 0.005$). B, Immunoprecipitation of the epitope-tagged GHRH and GHS receptors. Equivalent amounts of protein from metabolically labeled HeLa-T4 cells transfected with the GHRH and GHS receptors (indicated by *plus*) were immunoprecipitated using antibodies to the HA or flag epitope tags (indicated by *plus*) and separated by SDS-PAGE on a 10% gel. Molecular mass markers (in kilodaltons) are indicated on the *right*.

receptor was reduced to 78% of control value in the cotransfection experiment (Fig. 3B).

A variety of GHSs potentate GHRH-induced cAMP production

As the GHSs are a family of structurally distinct compounds, and alternative isoforms of the GHS receptor have been recently characterized (36, 37), the effects of various synthetic and natural GHS ligands on GHRH-induced cAMP production were assessed. Cotreatment with GHRH and L692-585, L692-429, GHRP-6, or an analog of the endogenous GHS ligand, ghrelin-(1–5), produced a similar 2-fold increase in cAMP production beyond that attained with GHRH alone (Fig. 4). In contrast, cotreatment with the inactive GHS compound, L692-428, did not further increase cAMP production.

Dose-response curves of ghrelin-induced cAMP potentiation

To determine the concentration of ghrelin sufficient to potentiate GHRH-induced cAMP production, cAMP levels were measured in transfected cells treated with 100 nM GHRH and increasing concentrations of ghrelin-(1–5) (Fig. 5A). GHRH-induced cAMP production was potentiated by

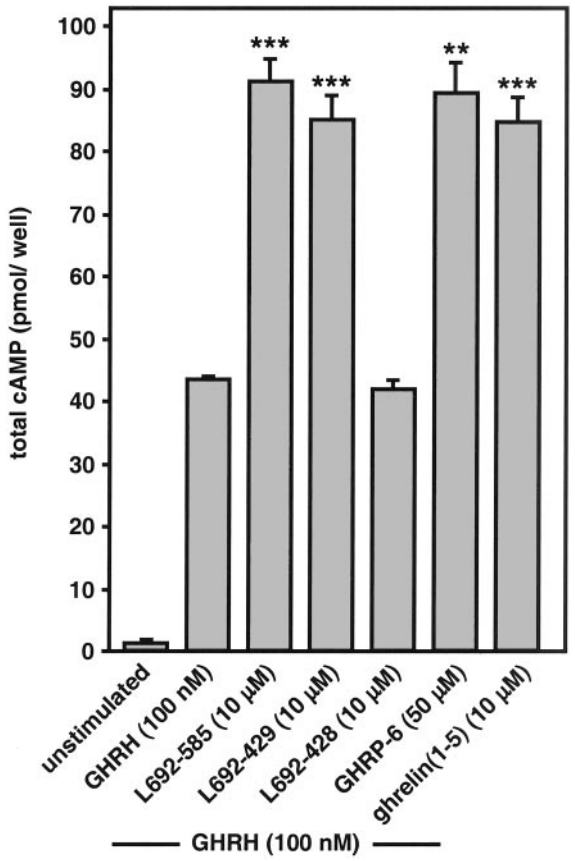

FIG. 4. Potentiation of GHRH-induced cAMP production by various GHSs. Production of cAMP was measured in HeLa-T4 cells transfected with the GHRH and GHS receptors and stimulated with GHRH or GHRH plus different GHSs. Conditions were repeated in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (**, $P < 0.01$; ***, $P < 0.005$).

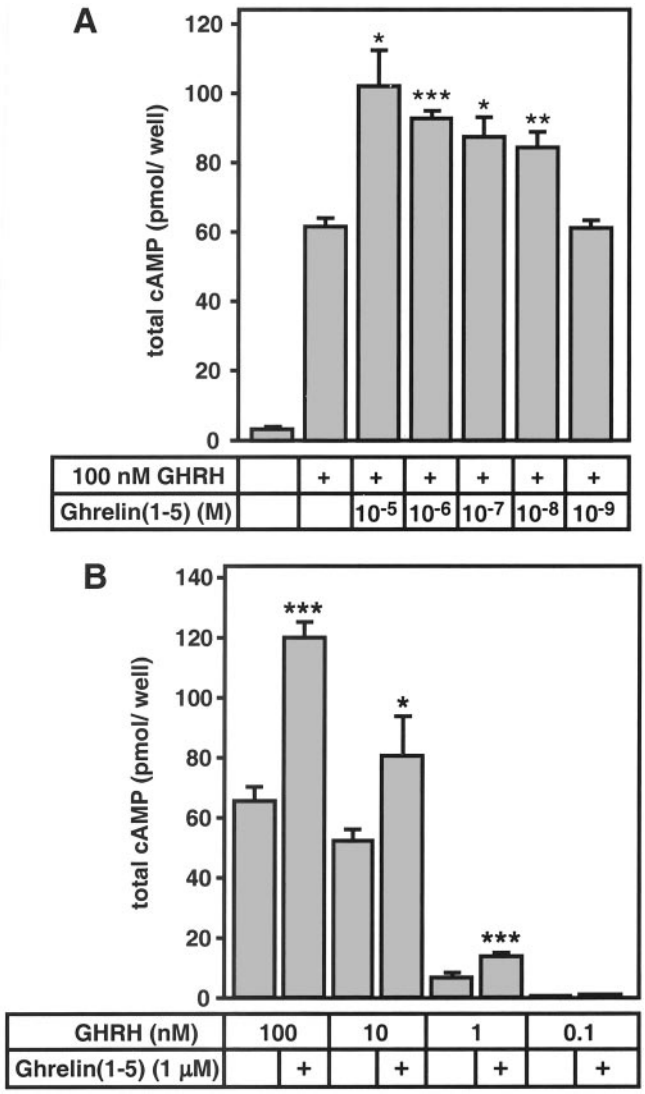

FIG. 5. Dose-response curves of ghrelin potentiation of GHRH-induced cAMP production. A, Measurement of cAMP production in HeLa-T4 cells transfected with both receptors in the presence of increasing concentrations of ghrelin-(1–5). Cells were transfected with the GHRH and GHS receptors and stimulated with 100 nM GHRH and increasing concentrations of ghrelin-(1–5). B, Measurement of cAMP production in HeLa-T4 cells transfected with both receptors in the presence of increasing concentrations of GHRH. Cells transfected with both receptors were stimulated with 1 μM ghrelin-(1–5) and increasing concentrations of GHRH. For A and B, conditions were repeated in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

ghrelin-(1–5) at concentrations ranging from 10 nM to 10 μM, but no effect was seen at 1 nM. To determine whether the ability of ghrelin to potentiate cAMP production is dependent on the concentration of GHRH, cAMP levels were measured in response to treatment with 1 μM ghrelin-(1–5) and increasing concentrations of GHRH (Fig. 5B). Although ghrelin-(1–5) potentiated cAMP production in response to treatment with GHRH at concentrations of 1, 10, and 100 nM, lower concentrations of GHRH failed to stimulate cAMP levels.

GHS potentiates the GHRH-induced cAMP response through a PKC-independent mechanism

Previous reports suggested that GHS activation of PKC resulted in potentiation of GHRH-induced cAMP production (30). To address the role of PKC in mediating this response, cAMP levels were measured in response to ligand stimulation after pretreatment with the PKC inhibitors GF109203X, RO31-8220, and staurosporine. GHRH-induced cAMP production is potentiated by GHS in the presence of each of these PKC inhibitors. cAMP production is potentiated 2.2-fold in the presence of GF109203X, 1.8-fold in the presence of RO31-8220, and 2.6-fold in the presence of staurosporine (Fig. 6, A and B). GHS continues to potentiate GHRH-induced cAMP production in the presence of a wide range of concentrations of these three PKC inhibitors (GF109203X, 1–100 μM; staurosporine, 0.01–1 μM; RO31-8220, 1–100 μM). The exception was 100 μM GF109203X, where there was no cAMP response to GHRH alone, suggesting that this high concentration of GF109203X had a toxic effect on the cells. Additionally, cAMP levels were measured after PKC was down-regulated following 12-*O*-tetraphorbol 12-myristate 13-acetate (TPA) pretreatment for 12 h. Although the overall cAMP response after TPA pretreatment was attenuated, the GHRH-induced cAMP response was potentiated 2.4-fold by GHS cotreatment (Fig. 6A). Similar results were observed in HeLa-T4 cells pretreated with TPA at concentrations of 60 nM, 3 nM, and 300 pM. To verify PKC inhibition by GF109203X in these cells, the activity of a TPA-responsive promoter-luciferase reporter construct (βA-Nae1) (38) was measured after TPA stimulation in the presence of GF109203X (Fig. 6C). GF109203X successfully inhibited TPA activation of the βA-Nae1 promoter construct, suggesting that PKC activity was blocked by

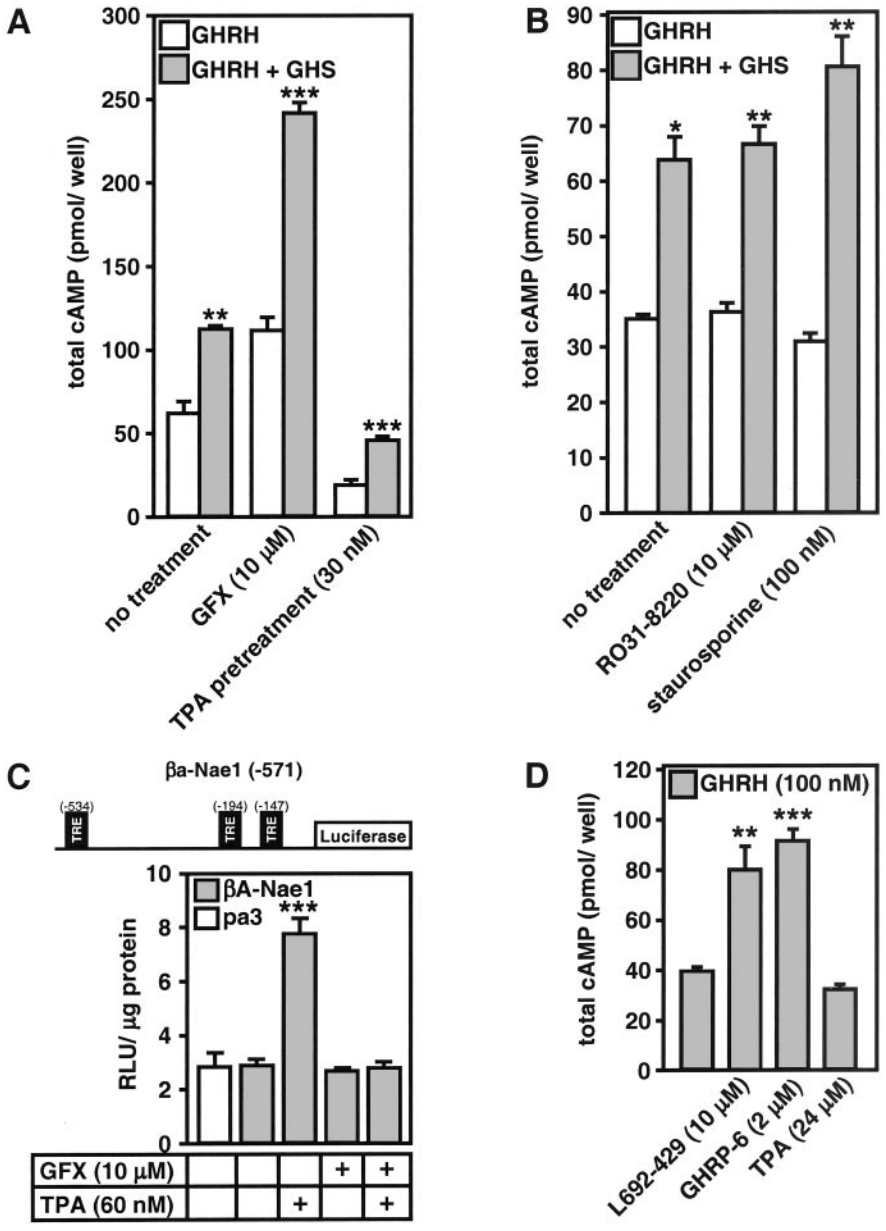

FIG. 6. GHS potentiation of GHRH-induced cAMP production in the presence of PKC inhibitors. A, Measurement of cAMP production in transfected HeLa-T4 cells in the presence of a PKC inhibitor or after PKC down-regulation. Cells were transfected with the GHRH and GHS receptors and stimulated with 100 nM GHRH or 100 nM GHRH plus 10 μM L692-429 after 30-min pretreatment with 10 μM GF109203X or 12-h pretreatment with 30 nM TPA. B, Measurement of cAMP production in transfected HeLa-T4 cells in the presence of PKC inhibitors. Cells were transfected with both receptors, pretreated with 10 μM RO31-8220 and 100 nM staurosporine for 30 min, and then stimulated with ligand. For the no treatment and RO31-8220 pretreatment conditions, the cells were stimulated with 100 nM GHRH or 100 nM GHRH plus 10 μM L692-585. For the staurosporine pretreatment condition, the cells were stimulated with 100 nM GHRH or 100 nM GHRH plus 10 μM L692-429. C, Measurement of luciferase production by the βA-Nae1 reporter construct in response to TPA in the presence of GF109203X. The βA-Nae1 construct has three TPA responsive elements (TRE) located at -147, -194, and -534. HeLa-T4 cells were transfected with the βA-Nae1 construct for 6 h, allowed to recover overnight, and then stimulated with 60 nM TPA in the presence or absence of 10 μM GF109203X. D, Measurement of cAMP production in HeLa-T4 cells after GHRH cotreatment with GHRP-6, L692-429, or TPA for 20 min. Cells were transfected with both receptors and then stimulated with 100 nM GHRH plus 10 μM L692-429, 2 μM GHRP-6, or 24 μM TPA. For A–D, conditions were repeated in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

the 30-min pretreatment with 10 μM GF109203X. Finally, to determine whether PKC activation was sufficient to potentiate the GHRH-induced cAMP response in these cells, cAMP production was measured in cells stimulated with GHRH and L692-429, GHRP-6, or TPA for 20 min (Fig. 6D). Although cAMP production was potentiated 2-fold in response to L692-429 treatment and 2.3-fold in response to GHRP-6 treatment, treatment with TPA had no significant effect on GHRH-induced cAMP production. These findings demonstrate that PKC activation after acute treatment with TPA is not sufficient to potentiate GHRH-induced cAMP production.

GHS potentiation of GHRH-induced cAMP production is unaffected by PLC inhibition

As previously mentioned, the GHS receptor couples to a G_q or G_{11} class G protein to activate the PLC signaling pathway. To determine the role of PLC in mediating the potentiated cAMP response, cAMP levels were measured in transfected HeLa-T4 cells after pretreatment with the PLC inhibitor, U73122. GHS treatment potentiated GHRH-induced cAMP production 1.9-fold in untreated cells and 1.7-fold in U73122-pretreated cells (Fig. 7A). GHS continued to potentiate GHRH-induced cAMP production by at least 2-fold after pretreatment with U73122 at concentrations ranging from 1–100 μM. To ensure that U73122 pretreatment successfully inhibited PLC, the release of intracellular calcium was measured in cells expressing the GHS receptor. Intracellular calcium release was reduced to 37.4% of the control value in cells pretreated with U73122 (Fig. 7B). The findings that PLC inhibition substantially reduced calcium release, but had no significant effect on cAMP potentiation, suggest that PLC and its second messenger pathways do not mediate this response.

GHS potentiation of GHRH-induced cAMP production is unaffected by sequestration of the G protein βγ-subunits

The G protein βγ-subunits can activate adenylyl cyclase types II, IV, and VII (39, 40). To determine whether the G protein βγ-subunits coupled to the GHS receptor mediate the potentiated cAMP response, cAMP levels were measured in cells cotransfected with a βγ sequestrant, the β-adrenergic receptor kinase 1 carboxyl terminus minigene (βARK1-ct) (41). HeLa-T4 cells were transfected with the GHRH and GHS receptors along with the βARK1-ct construct. Transfected cells were treated with 100 nM GHRH or 100 nM GHRH plus 10 μM L692-429. In the presence of the βγ sequestrant, GHS treatment still potentiated GHRH-induced cAMP production (Fig. 8A). GHS treatment potentiated cAMP production 3- or 1.9-fold in cells coexpressing the pTracer vector or the βARK1-ct construct, respectively. These findings suggest that GHS potentiates GHRH-induced cAMP production through a mechanism independent of the G protein βγ-subunits.

Similar experiments were performed using a different βγ sequestrant, the CD8-βARK. This βγ sequestrant is a Myc epitope-tagged βARK fused to the amino terminus of the CD8 lymphocyte-specific receptor, which includes four membrane-spanning domains that target the protein to the plasma membrane, where the G protein βγ-subunits are tethered (42). HeLa-T4 cells were transfected with the GHRH and GHS receptors in combination with the pcDNA3 vector or CD8-βARK. GHS continued to potentiate GHRH-induced cAMP production in the presence of CD8-βARK (Fig. 8B). The production of cAMP was potentiated 2-fold in cells coexpressing the vector and 2.3-fold in cells coexpressing CD8-βARK. To verify that the CD8-βARK protein is expressed, immunoprecipitation of protein lysates from metabolically

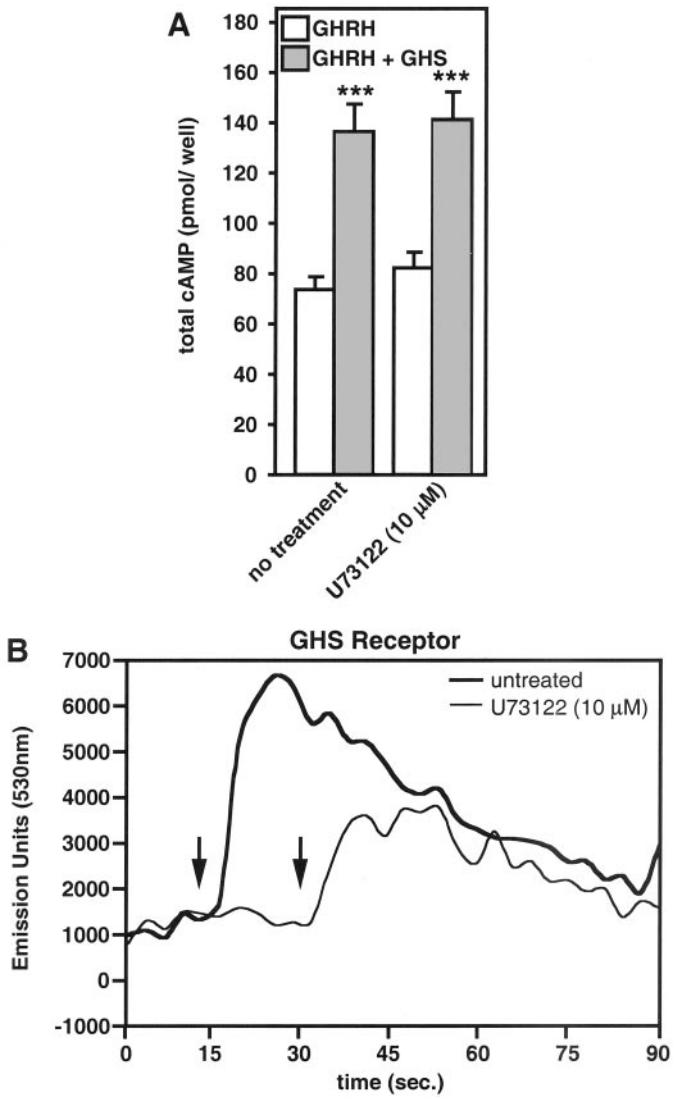

FIG. 7. GHS potentiation of GHRH-induced cAMP production in the presence of a PLC inhibitor. A, HeLa-T4 cells were transfected with both receptor constructs, pretreated with 10 μM U73122 for 20 min, and then stimulated with 100 nM GHRH or 100 nM GHRH plus 10 μM L692-585. Conditions were repeated in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (***, $P < 0.005$). B, Measurement of intracellular calcium release. HeLa-T4 cells were transfected with the GHS receptor, pretreated with 10 μM U73122 for 10 min, and then stimulated with 1 μM ghrelin-(1–5). The release of intracellular calcium was indicated by an increase in fluorescent emission at 530 nm. The *arrows* represent the onset of ligand treatment. Areas under the curve were calculated and used to assess U73122 inhibition of calcium release, which was reduced to 37.4% of control levels.

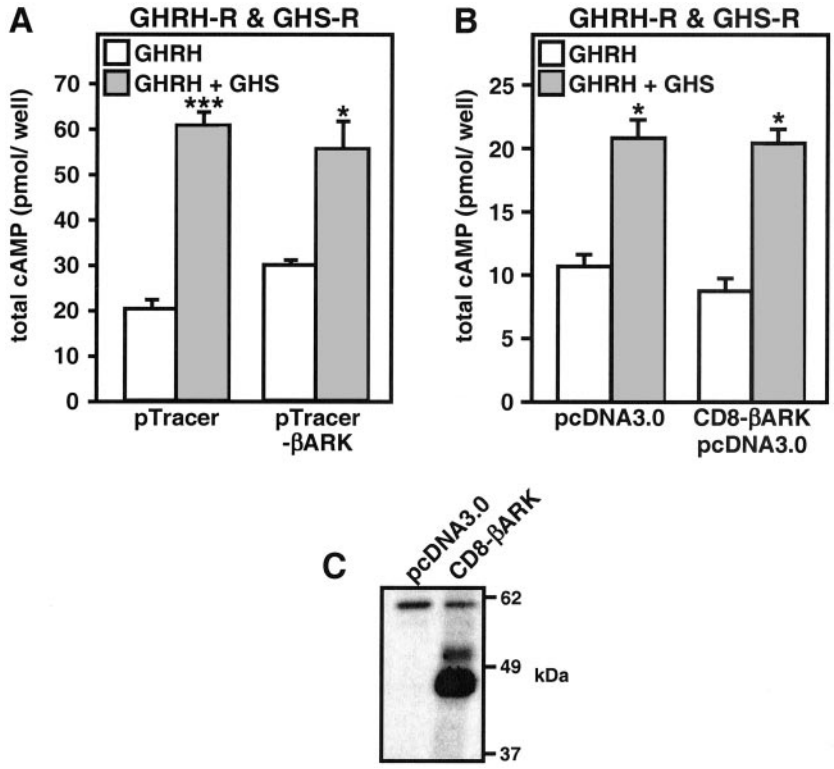

FIG. 8. GHS potentiation of GHRH-induced cAMP production in the presence of G protein $\beta\gamma$ sequestrants. A, Levels of cAMP were measured in HeLa-T4 cells expressing the GHRH and GHS receptors and a $\beta\gamma$ sequestrant, the carboxyl terminus of the β-adrenergic receptor kinase. Cells expressing both receptors were cotransfected with pTracer or βARK1-ct in pTracer and then stimulated with 100 nM GHRH or 100 nM GHRH plus 10 μM L692-429 for 20 min. B, Similar experiments were repeated using a different $\beta\gamma$ sequestrant, the CD8-βARK. HeLa-T4 cells expressing both receptors were cotransfected with the pcDNA3.0 vector or the CD8-βARK construct. Cells were stimulated with 100 nM GHRH in the presence or absence of 10 μM L692-429 for 20 min. For A and B, the conditions were repeated in triplicate, and the *error bars* represent the SEM. Statistical analysis was performed using paired *t* test (*, $P < 0.05$; ***, $P < 0.005$). C, Expression of CD8-βARK was determined by immunoprecipitation of protein lysates using the monoclonal antibody against the Myc epitope tag.

labeled HeLa-T4 cells was performed using an antibody against the Myc epitope tag (Fig. 8C). The Myc epitope-tagged CD8-βARK was found to be of the expected size of about 46 kDa.

Effect of GHS treatment on VIP-, secretin-, or forskolin-induced cAMP production

To address whether GHS could potentiate cAMP production induced by other related GPCRs, cAMP levels were measured in HeLa-T4 cells in which the receptors for VIP or secretin substituted for the GHRH receptor. Like the GHRH receptor, the VIP and secretin receptors stimulate AC activity and belong to the B-III subfamily of GPCRs (43). The amino acid sequences of the VIP and secretin receptors are 40% and 35% identical with that of the GHRH receptor (33). The level of cAMP in cells expressing the GHS and VIP receptors was slightly increased after cotreatment with both ligands compared with VIP alone, but this increase was not statistically significant (Fig. 9A). Likewise, no difference in cAMP levels was detected in cells expressing the GHS and secretin receptors after treatment with secretin or secretin plus GHS (Fig. 9B). These findings indicate that GHSs exhibit some degree of selectivity in potentiating cAMP production stimulated by the GHRH receptor.

Forskolin is a direct activator of adenylyl cyclase (44). To determine whether GHS treatment could potentiate forskolin-induced cAMP production, cells transfected with the GHRH and GHS receptors were stimulated with forskolin or forskolin plus GHS (Fig. 9C). Cotreatment with GHS had no effect on forskolin-induced cAMP production, suggesting that GHSs may interact with the GHRH signaling pathway upstream of AC activation.

Discussion

This paper reports that in a homogeneous cell population expressing the GHRH and GHS receptors GHRH-induced cAMP production is potentiated by GHS cotreatment. These findings demonstrate that GHRH and GHS act directly on the same cells to potentiate the second messenger pathway coupled to the GHRH receptor. Previous studies in dispersed rat anterior pituitary cells have shown that GHRP-6 and L692-429 treatments potentiate GHRH-induced cAMP production approximately 1.5-fold (13, 29, 30). In the transfected HeLa-T4 cells, GHS potentiation of GHRH-induced cAMP production was within the range of 1.5- to 3-fold. This response was seen with a variety of GHSs, including the benzolactam compounds L692-429 and L692-585, the peptide GHRP-6, and an analog of the endogenous GHS, ghrelin-(1–5). These GHSs vary in binding affinity for the GHS receptor and the 50% effective concentration values for GH secretion. Ghrelin has the greatest binding affinity for the receptor, followed by L692-585, GHRP-6, and L692-429 (45, 46), and the 50% effective concentration values of GH secretion for these GHSs are in the nanomolar range.

Given the pivotal importance of cAMP in mediating the ability of GHRH to stimulate GH synthesis and secretion, potentiation of cAMP production by GHS is likely to explain the synergism between GHRH and GHS in GH release observed in pituitary cells. Therefore, signaling studies were pursued in this model system to investigate GHS potentiation of GHRH-induced cAMP production. The GHS receptor signals through the PLC second messenger pathway. Early signaling studies suggested that PKC mediated GHS potentiation of GHRH-induced cAMP production and GH secretion (30, 47). One study found that treating rat pituitary cells

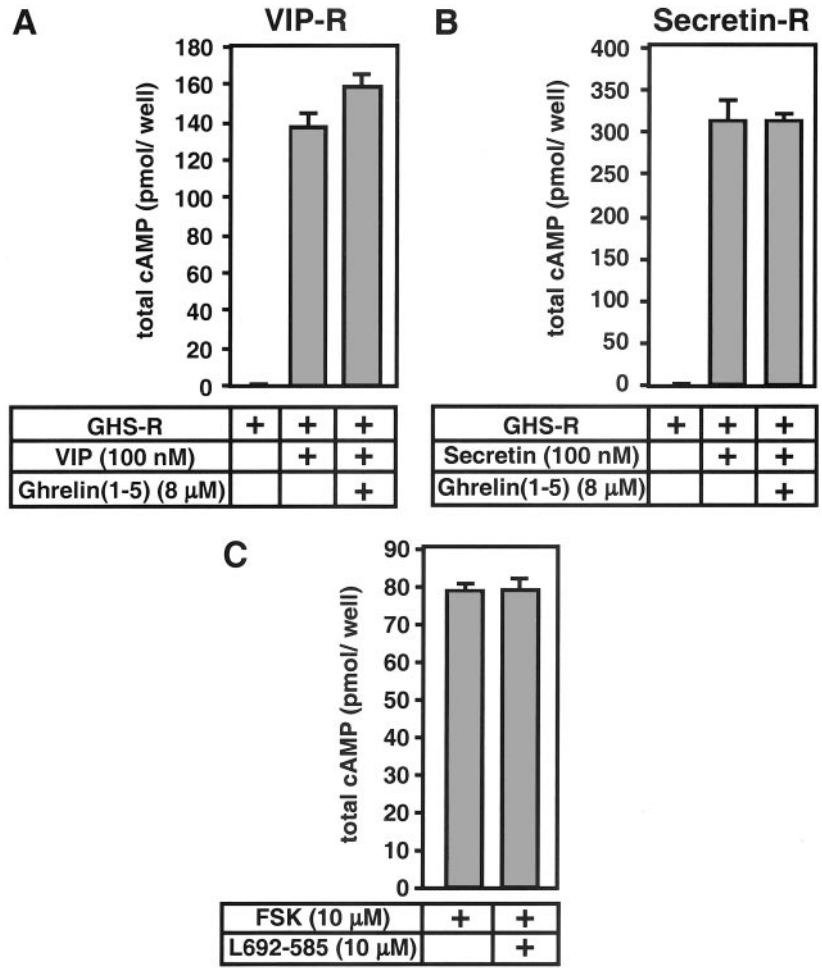

FIG. 9. Effect of GHS on VIP-, secretin-, and forskolin-induced cAMP production. A, Measurement of cAMP production in HeLa-T4 cells transfected with the VIP and GHS receptors. HeLa-T4 cells were transfected with VIP and GHS receptors and then treated with 100 nM VIP or 100 nM VIP plus 8 μM ghrelin-(1–5). B, Measurement of cAMP production in HeLa-T4 cells transfected with the secretin and GHS receptors. HeLa-T4 cells were transfected with the secretin and GHS receptors and then treated with 100 nM secretin or 100 nM secretin plus 8 μM ghrelin-(1–5). C, Measurement of cAMP production in HeLa-T4 cells transfected with the GHRH and GHS receptors. HeLa-T4 cells were transfected with both receptors and stimulated with 10 μM forskolin or 10 μM forskolin plus 10 μM L692-585. For A–C, samples were repeated in triplicate, and *error bars* represent the SEM. Statistical analysis was performed using paired *t* test.

with the phorbol ester PMA potentiated GHRH-induced cAMP production and GH secretion in a similar manner as GHRP-6 treatment (30). Furthermore, the potentiated cAMP and GH responses were eliminated by down-regulating PKC (30). In a separate study, the PKC inhibitor phloretin blocked GHS potentiation of GH secretion from somatotroph tumor cells expressing *gsp* oncogenes (47). These oncogenes are translated into $G\alpha_s$ proteins that lack endogenous GTPase activity, thereby resulting in constitutive activation of adenylyl cyclase and elevated levels of cAMP. The findings from these studies suggest that PKC may bridge the GHS and GHRH signaling pathways. In our model system, PKC inhibition or down-regulation had no effect on the ability of GHSs to potentiate GHRH-induced cAMP production. The PKC inhibitors GF109203X, RO31-8220, and staurosporine did not hinder the ability of GHS to potentiate the cAMP response. In addition, cAMP production was potentiated by GHS even after PKC down-regulation following a 12-h pretreatment with 30 nM TPA. In contrast, PKC down-regulation following a 24-h pretreatment with 1 μM PMA inhibited the potentiating effect of GHS on GHRH-induced cAMP production and GH secretion in dispersed rat anterior pituitary cells (30). The differences between these findings may reflect differences in the cell backgrounds or concentration/duration of the phorbol ester pretreatment. Finally, acute TPA treatment to activate PKC is unable to mimic the potentiating effect of GHS on GHRH-induced cAMP production. These results suggest that GHSs potentiate GHRH-induced cAMP production through a PKC-independent mechanism in this homogeneous population of cells expressing the cloned GHRH and GHS receptors.

GHS also potentiated GHRH-induced cAMP production in the presence of a PLC inhibitor. Inhibiting PLC should attenuate the entire downstream signal transduction pathway, including PKC activation and intracellular calcium mobilization. Like PKC, some Ca^{2+}/calmodulin isoforms can activate adenylyl cyclase, thereby providing an additional mechanism for cross-talk between the PLC/PKC and AC/cAMP signaling pathways. Adenylyl cyclase I, III, and VIII are activated by calmodulin (48–50). We confirmed inhibition of PLC by measuring intracellular calcium mobilization after activation of the GHS receptor. Although the calcium response to ghrelin stimulation was not completely eliminated, it was greatly attenuated, suggesting that PLC activity was largely inhibited. Yet, no effects on GHS potentiation of the cAMP response were observed under these conditions.

Some $\beta\gamma$-subunits of the heterotrimeric G protein can increase cAMP production. Specifically, these $\beta\gamma$-subunits interact with the carboxyl-half of adenylyl cyclase types II and IV to augment $G\alpha_s$-induced AC activity 10-fold (39). A recent

study reported the involvement of the G protein $\beta\gamma$-subunits in the synergistic activation of PKA signaling by the D2 dopamine and A2 adenosine receptors (51). However, in transfected HeLa-T4 cells coexpressing two different $\beta\gamma$ sequestrants, GHSs still potentiate GHRH-induced cAMP production, suggesting that the $\beta\gamma$-subunits are not involved in mediating the effect of GHS on cAMP production.

Cotreatment with GHS did not potentiate cAMP production in response to activation of other stimulatory G protein-coupled receptors belonging to the B-III subfamily. The VIP and secretin receptors have, respectively, 40% and 35% amino acid sequence identity with the GHRH receptor (33). The finding that VIP- or secretin-induced cAMP production is not affected by GHS cotreatment suggests that the actions of the GHS receptor are somewhat selective for the GHRH receptor. However, a recent report demonstrated that GHS can potentiate cAMP production stimulated by the D1 dopamine receptor in an *in vitro* model system of HEK293 cells expressing both receptors (52). Thus, it appears that the GHS receptor may more broadly impact cAMP signaling pathways activated by other G protein-coupled receptors. It remains to be determined whether similar or distinct mechanisms of interaction are used in these two systems.

Many studies have found that disruption of GHRH or its receptor reduces or completely eliminates the ability of GHSs to stimulate GH secretion. In rats, GHS-induced GH secretion was effectively blocked after GHRH antiserum treatment (10). In ovine pituitary cells, a GHRH receptor antagonist inhibited GH secretion induced by GHRP-2 (53). Similar findings were reported in human studies in which the GHRH antagonist (*N*-Ac-Tyr^1,D-Arg^2)GHRH-(1–29)NH_2 inhibited most of the GH response to GHRP-6 (54). Furthermore, the *little* mouse is resistant to GHS stimulation, suggesting that a functional GHRH receptor is necessary for GHS-induced GH secretion (55). A similar finding has been reported in a case study of patients with a homozygous mutation in the GHRH receptor gene. This study reported that an injection of hexarelin did not elevate plasma GH levels in these patients (56). Based on these findings, it has been suggested that GHS actions on the pituitary to release GH are dependent upon a functional GHRH receptor. The dependence of GHS signaling on the GHRH receptor may be mechanistically related to the synergistic effects of these two signaling pathways observed in our study.

Our signaling studies establish that blockade of the known signaling pathways downstream of GHS receptor activation fails to inhibit potentiation of GHRH receptor signaling. One plausible explanation for these results is that there is a direct interaction of the two receptors. Recent studies have shown that GPCRs can homo- or heterodimerize such that the characteristics of the receptor complexes, including binding, internalization, and signaling, are different from the characteristics of each receptor individually. Often the signaling of GPCR heterodimers results in potentiation of the second messenger pathways associated with each receptor in the complex. Both the dopamine and somatostatin receptors couple to inhibitory G proteins. In a heterodimer complex, inhibition of cAMP production by the dopamine and somatostatin receptors is potentiated (57). Likewise, heterodimers of the δ and κ opioid receptors potentiate inhibition of cAMP production (58). Heterodimerization of the bradykinin receptor, a G_q protein-coupled receptor, and the angiotensin receptor, a GPCR that binds G_i and G_q proteins, potentiates angiotensin-induced G_i turnover and inositol phosphate production (59).

The findings from signaling studies identifying dominant negative GHRH receptors also provide support for the proposal that GHRH receptors form dimers. In two separate studies, coexpression of the GHRH receptor with a truncated receptor diminished GHRH-induced cAMP production (60, 61). Furthermore, the binding of GHRH was reduced by coexpression of this truncated receptor (61). One explanation for this effect is that the truncated receptor is forming a homodimer or homooligomer complex with the GHRH receptor, such that GHRH binding and signaling are attenuated. In addition to forming homodimers, we speculate that the GHRH receptor may be forming heterodimers with the GHS receptor. However, our preliminary studies using chemical cross-linking and immunoprecipitation have not detected heterodimer formation between the GHRH and GHS receptors. There may be technical limitations to detecting such heterodimers, including very low stoichiometry of dimer formation, instability of the dimers during the immunoprecipitation step, or inaccessibility of the epitope tags in a dimeric complex. Alternatively, the observed synergy may be independent of direct receptor interaction and may involve alterations in unidentified components of the signaling pathway leading to cAMP production and GH secretion. The model system here should facilitate further investigation to distinguish between these possible mechanisms of receptor synergy.

Acknowledgments

We thank Dr. Scott Feighner (Merck Research Laboratories, Rahway, NJ) for providing the benzolactam compounds, Dr. Bernard Moss (NIH, Bethesda, MD) for the use of the Vaccinia-T7 polymerase system, Dr. Robert Lamb (Northwestern University) for the 12CA5 monoclonal antibody against the HA epitope, Dr. J. Silvio Gutkind (NIH) for the CD8-βARK construct, and Drs. Dan Linzer and Jon Levine for comments on the manuscript. We thankfully acknowledge the use of instruments in the Keck Biophysics facility at Northwestern University (http://www.biochem.northwestern.edu/Keck/keckmain.html).

Received July 1, 2002. Accepted August 22, 2002.

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This work was supported by NIH Training Grant HD-07068 (to S.R.C.) and NIH Grant DK-48071 to (to K.E.M.).

References

1. **Devesa J, Lima L, Tresguerres AF** 1992 Neuroendocrine control of growth hormone secretion in humans. Trends Endocrinol Metab 3:175–182
2. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
3. **Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR** 2000 The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325–4328
4. **Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS** 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719
5. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S** 2001 A role for ghrelin in the central regulation of feeding. Nature 409:194–198

6. **Bowers CY** 1996 Xenobiotic growth hormone secretagogues: growth hormone releasing peptides. In: Bercu BB, Walker RF, eds. Growth hormone secretagogues. New York: Springer-Verlag; 9–28
7. **Bowers C, Momany F, Reynolds G, Chang D, Hong A, Chang K** 1980 Structure-activity relationships of a synthetic pentapeptide that specifically releases growth hormone *in vitro*. Endocrinology 106:663–667
8. **Bowers CY, Reynolds GA, Durham D, Barrera CM, Pezzoli SS, Thorner MO** 1990 Growth hormone (GH)-releasing peptide stimulates GH release in normal men and acts synergistically with GH-releasing hormone. Journal of Clinical Endocrinology, Metabolism 70:975–982
9. **Bowers CY, Momany FA, Reynolds GA, Hong A** 1984 On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology 114:1537–1545
10. **Bowers C, Sartor A, Reynolds G, Badger T** 1991 On the actions of the growth hormone-releasing hexapeptide, GHRP. Endocrinology 128:2027–2035
11. **Bowers CY** 1993 GH releasing peptides: structure and kinetics. J Pediatr Endocrinol 6:21–31
12. **Deghenghi R, Cananzi MM, Torsello A, Battisti C, Muller EE, Locatelli V** 1994 GH-releasing activity of hexarelin, a new growth hormone releasing peptide, in infant and adult rats. Life Sci 54:1321–1328
13. **Cheng K, Chan WW, Butler B, Wei L, Schoen WR, Wyvratt Jr MJ, Fisher MH, Smith RG** 1993 Stimulation of growth hormone release from rat primary pituitary cells by L-692, 429, a novel non-peptidyl GH secretagogue. Endocrinology 132:2729–2731
14. **Smith RG, Cheng K, Schoen WR, Pong SS, Hickey G, Jacks T, Butler B, Chan WW, Chaung LY, Judith F** 1993 A nonpeptidyl growth hormone secretagogue. Science 260:1640–1643
15. **Jacks T, Hickey G, Judith F, Taylor J, Chen H, Krupa D, Feeney W, Schoen W, Ok D, Fisher M** 1994 Effects of acute and repeated intravenous administration of L-692,585, a novel non-peptidyl growth hormone secretagogue, on plasma growth hormone, IGF-1, ACTH, cortisol, prolactin, insulin, and thyroxine levels in beagles. J Endocrinol 143:399–406
16. **Patchett AA, Nargund RP, Tata JR, Chen MH, Barakat KJ, Johnston DB, Cheng K, Chan WW, Butler B, Hickey G** 1995 Design and biological activities of L-163, 191 (MK-0677): a potent, orally active growth hormone secretagogue. Proc Natl Acad Sci USA 92:7001–7005
17. **Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML** 2001 Circulating ghrelin levels are decreased in human obesity. Diabetes 50: 707–709
18. **Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K** 2000 Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun 276:905–908
19. **Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S** 2001 Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 280:904–907
20. **Lee HM, Wang G, Englander EW, Kojima M, Greeley Jr GH** 2002 Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. Endocrinology 143:185–190
21. **Bednarek MA** 2000 Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem 43:4370–4376
22. **Howard A, Feighner S, Cully D, Arena J, Liberator P, Rosenblum C, Hamelin M, Hreniuk D, Palyha O, Anderson J, Paress P, Diaz C, Chou M, Liu K, McKee K, Pong S, Chaung L, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji D, Dean D, Melillo D, Van der Ploeg L** 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974–977
23. **Day R, Dong W, Panetta R, Kraicer J, Greenwood MT, Patel YC** 1995 Expression of mRNA for somatostatin receptor (sstr) types 2 and 5 in individual rat pituitary cells. A double labeling *in situ* hybridization analysis. Endocrinology 136:5232–5235
24. **Kumar U, Laird D, Srikant CB, Escher E, Patel YC** 1997 Expression of the five somatostatin receptor (SSTR1–5) subtypes in rat pituitary somatotrophes: quantitative analysis by double-layer immunofluorescence confocal microscopy. Endocrinology 138:4473–4476
25. **Mezey E, Hunyady B, Mitra S, Hayes E, Liu Q, Schaeffer J, Schonbrunn A** 1998 Cell specific expression of the sst2A and sst5 somatostatin receptors in the rat anterior pituitary. Endocrinology 139:414–419
26. **Hataya Y, Akamizu T, Takaya K, Kanamoto N, Ariyasu H, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K, Nakao K** 2001 A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. J Clin Endocrinol Metab 86:45–52
27. **Penalva A, Carballo A, Pombo M, Casanueva F, Dieguez C** 1993 Effect of growth hormone (GH)-releasing hormone (GHRH), atropine, pyridostigmine, or hypoglycemia on GHRP-6-induced GH secretion in man. J Clin Endocrinol Metab 76:168–171
28. **Guillaume V, Magnan E, Cataldi M, Dutour A, Sauze N, Renard M, Razafindraibe H, Conte-Devolx B, Deghenghi R, Lenaerts V** 1994 Growth hormone (GH)-releasing hormone secretion is stimulated by a new GH-releasing hexapeptide in sheep. Endocrinology 135:1073–1076
29. **Cheng K, Chan W, Barreto AJ, Convey E, Smith R** 1989 The synergistic effects of His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 on growth hormone (GH)-releasing factor-stimulated GH release and intracellular adenosine 3′, 5′-monophosphate accumulation in rat primary pituitary cell culture. Endocrinology 124:2791–2798
30. **Cheng K, Chan W, Butler B, Barreto AJ, Smith R** 1991 Evidence for a role of protein kinase-C in His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2-induced growth hormone release from rat primary pituitary cells. Endocrinology 129:3337–3342
31. **Ishihara T, Shigemoto R, Mori K, Takahashi K, Nagata S** 1992 Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron 8:811–819
32. **Ishihara T, Nakamura S, Kaziro Y, Takahashi T, Takahashi K, Nagata S** 1991 Molecular cloning and expression of a cDNA encoding the secretin receptor. EMBO J 10:1635–1641
33. **DeAlmeida VI, Mayo KE** 1998 Identification of binding domains of the growth hormone-releasing hormone receptor by analysis of mutant and chimeric receptor proteins. Mol Endocrinol 12:750–765
34. **Miller TL, Godfrey PA, Dealmeida VI, Mayo KE** 1999 The rat growth hormone-releasing hormone receptor gene: structure, regulation, and generation of receptor isoforms with different signaling properties. Endocrinology 140: 4152–4165
35. **Fuerst TR, Earl PL, Moss B** 1987 Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol Cell Biol 7:2538–2544
36. **Bodart V, Bouchard JF, McNicoll N, Escher E, Carriere P, Ghigo E, Sejlitz T, Sirois MG, Lamontagne D, Ong H** 1999 Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. Circ Res 85: 796–802
37. **Ong H, McNicoll N, Escher E, Collu R, Deghenghi R, Locatelli V, Ghigo E, Muccioli G, Boghen M, Nilsson M** 1998 Identification of a pituitary growth hormone-releasing peptide (GHRP) receptor subtype by photoaffinity labeling. Endocrinology 139:432–435
38. **Ardekani AM, Romanelli JC, Mayo KE** 1998 Structure of the rat inhibin and activin β_A-subunit gene and regulation in an ovarian granulosa cell line. Endocrinology 139:3271–3279
39. **Tang WJ, Gilman AG** 1991 Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Science 254:1500–1503
40. **Yoshimura M, Ikeda H, Tabakoff B** 1996 mu-Opioid receptors inhibit dopamine-stimulated activity of type V adenylyl cyclase but enhance dopamine-stimulated activity of type VII adenylyl cyclase. Mol Pharmacol 50:43–51
41. **Koch WJ, Hawes BE, Inglese J, Luttrell LM, Lefkowitz RJ** 1994 Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G$\beta\gamma$-mediated signaling. J Biol Chem 269:6193–6197
42. **Crespo P, Cachero TG, Xu N, Gutkind JS** 1995 Dual effect of β-adrenergic receptors on mitogen-activated protein kinase. Evidence for a $\beta\gamma$-dependent activation and a Gα_s-cAMP-mediated inhibition. J Biol Chem 270:25259–25265
43. **Segre G, Goldring S** 1993 Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone and glucagon belong to a newly discovered G-protein-linked receptor family. Trends Endocrinol Metab 4:309–314
44. **Seamon KB, Daly JW** 1986 Forskolin: its biological and chemical properties. Adv Cyclic Nucleotide Protein Phosphorylation Res 20:1–150
45. **Smith R, Van der Ploeg L, Howard A, Feighner S, Cheng K, Hickey G, Wyvratt MJ, Fisher M, Nargund R, Patchett A** 1997 Peptidomimetic regulation of growth hormone secretion. Endocr Rev 18:621–645
46. **Hosoda H, Kojima M, Matsuo H, Kangawa K** 2000 Purification and characterization of rat des-Gln^{14}-ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. J Biol Chem 275:21995–22000
47. **Adams EF, Lei T, Buchfelder M, Bowers CY, Fahlbusch R** 1996 Protein kinase C-dependent growth hormone releasing peptides stimulate cyclic adenosine 3′,5′-monophosphate production by human pituitary somatotropinomas expressing gsp oncogenes: evidence for crosstalk between transduction pathways. Mol Endocrinol 10:432–438
48. **Choi EJ, Wong ST, Dittman AH, Storm DR** 1993 Phorbol ester stimulation of the type I and type III adenylyl cyclases in whole cells. Biochemistry 32:1891–1894
49. **Cali JJ, Zwaagstra JC, Mons N, Cooper DM, Krupinski J** 1994 Type VIII adenylyl cyclase. A Ca^{2+}/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J Biol Chem 269:12190–12195
50. **Wayman GA, Wei J, Wong S, Storm DR** 1996 Regulation of type I adenylyl cyclase by calmodulin kinase IV in vivo. Mol Cell Biol 16:6075–6082
51. **Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S, Janak PH, Gordon AS, Diamond I** 2002 $\beta\gamma$ dimers mediate synergy of dopamine D2 and adenosine A2 receptor-stimulated PKA signaling and regulate ethanol consumption. Cell 109:733–743
52. **Jiang H, Smith R,** The synergistic effect of the growth hormone secretagogue receptor on the dopamine D1 receptor signal transduction pathway. Program of the 84th Annual Meeting of The Endocrine Society, San Francisco, CA, 2002, p 541 (Abstract P3-20)
53. **Wu D, Chen C, Katoh K, Zhang J, Clarke IJ** 1994 The effect of GH-releasing peptide-2 (GHRP-2 or KP 102) on GH secretion from primary cultured ovine pituitary cells can be abolished by a specific GH-releasing factor (GRF) receptor antagonist. J Endocrinol 140:R9–13

54. **Pandya N, DeMott-Friberg R, Bowers CY, Barkan AL, Jaffe CA** 1998 Growth hormone (GH)-releasing peptide-6 requires endogenous hypothalamic GH-releasing hormone for maximal GH stimulation. J Clin Endocrinol Metab 83:1186–1189
55. **Jansson J-O, Downs T, Beamer W, Frohman L,** The dwarf 'little' (*lit/lit*) mouse is resistant to growth hormone (GH)-releasing peptide (GH-RP-6) as well as to GH-releasing hormone (GRH). Program of the 68th Annual Meeting of The Endocrine Society, Anaheim, CA, 1986, p 130 (Abstract 397)
56. **Maheshwari HG, Silverman BL, Dupuis J, Baumann G** 1998 Phenotype and genetic analysis of a syndrome caused by an inactivating mutation in the growth hormone-releasing hormone receptor: dwarfism of Sindh. J Clin Endocrinol Metab 83:4065–4074
57. **Rocheville M, Lange DC, Kumar U, Patel SC, Patel RC, Patel YC** 2000 Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science 288:154–157
58. **Jordan BA, Devi LA** 1999 G-Protein-coupled receptor heterodimerization modulates receptor function. Nature 399:697–700
59. **AbdAlla S, Lother H, Quitterer U** 2000 AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. Nature 407: 94–98
60. **Motomura T, Hashimoto K, Koga M, Arita N, Hayakawa T, Kishimoto T, Kasayama S** 1998 Inhibition of signal transduction by a splice variant of the growth hormone-releasing hormone receptor expressed in human pituitary adenomas. Metab Clin Exp 47:804–808
61. **Horikawa R, Fujita K, Tanaka T, Mastromarino A, Gaylinn BD,** A growth hormone-releasing hormone (GHRH) receptor mutation that acts as a dominant negative. Program of the 83rd Annual Meeting of The Endocrine Society, Denver, CO, 2001, p 170 (Abstract P1-97)