Growth hormone-releasing hormone as an agonist of the ghrelin receptor GHS-R1a

Felipe F. Casanueva[a,b,c,1], Jesus P. Camiña[a,c], Marcos C. Carreira[c], Yolanda Pazos[a,c], Jozsef L. Varga[d], and Andrew V. Schally[d]

[a]Laboratory of Molecular and Cellular Endocrinology, Research Area, Complejo Hospitalario Universitario de Santiago (CHUS), Santiago de Compostela, Spain; [b]Department of Medicine, School of Medicine, University of Santiago de Compostela, Santiago de Compostela, Spain; [c]CIBER de Fisiopatología de la Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, Spain; and [d]Veterans Affairs Medical Center, 1201 NW 16th Street, Research Service (151), and University of Miami Miller School of Medicine, Departments of Pathology and Medicine, Division of Hematology and Oncology, Miami, FL 33125

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Ghrelin synergizes with growth hormone-releasing hormone (GHRH) to potentiate growth hormone (GH) response through a mechanism not yet fully characterized. This study was conducted to analyze the role of GHRH as a potential ligand of the ghrelin receptor, GHS-R1a. The results show that hGHRH(1–29)NH_2 (GHRH) induces a dose-dependent calcium mobilization in HEK 293 cells stably transfected with GHS-R1a an effect not observed in wild-type HEK 293 cells. This calcium rise is also observed using the GHRH receptor agonists JI-34 and JI-36. Radioligand binding and cross-linking studies revealed that calcium response to GHRH is mediated by the ghrelin receptor GHS-R1a. GHRH activates the signaling route of inositol phosphate and potentiates the maximal response to ghrelin measured in inositol phosphate turnover. The presence of GHRH increases the binding capacity of ^{125}I-ghrelin in a dose dependent-fashion showing a positive binding cooperativity. In addition, confocal microscopy in CHO cells transfected with GHS-R1a tagged with enhanced green fluorescent protein shows that GHRH activates the GHS-R1a endocytosis. Furthermore, the selective GHRH-R antagonists, JV-1–42 and JMR-132, act also as antagonists of the ghrelin receptor GHS-R1a. Our findings suggest that GHRH interacts with ghrelin receptor GHS-R1a, and, in consequence, modifies the ghrelin-associated intracellular signaling pathway. This interaction may represent a form of regulation, which could play a putative role in the physiology of GH regulation and appetite control.

Ghrelin | Ghrelin receptor | GHRH

The recognition of ghrelin as the natural ligand for the growth hormone secretagogue receptor type 1a (GHS-R1a) added a new element to the complex physiological regulation for growth hormone (GH) secretion and clarified some of its aspects that were previously not fully understood (1, 2). However, this system is not as simple as it was initially thought. Ghrelin and its receptor became recognized not only for stimulating GH release but also for the findings that they exert an important role on several aspects of energy homeostasis and perhaps contribute to the metabolic syndrome (3, 4). One of the peculiar features of ghrelin and GH secretagogues (GHS) is the synergistic relationship with growth hormone-releasing hormone (GHRH) in the release of GH (5–7). The mechanism of this synergistic interaction remains a subject of debate in view of the fact that ghrelin and GHS act on both the hypothalamus and the pituitary. The evidence that this interaction requires a functional hypothalamus was obtained from patients with tumoral mass producing a hypothalamo–pituitary disconnection in whom GH secretion is preserved after GHRH stimulation while GHS/ghrelin-induced GH secretion is blocked and the synergistic action between GHRH and GHS is absent (8, 9). Similar findings were observed in children with neonatal stalk transection (10). These observations led to the hypothesis that the action of ghrelin/GHS is mediated through an unknown hypothalamic factor with GH-releasing capabilities. GHRH was proposed as a candidate based on the observation that GH-releasing action of GHS is blunted in the presence of GHRH receptor antagonists (11). An increased release of GHRH into hypophyseal portal blood after GHS administration has also been shown in sheep (12). Furthermore, the requirement of a functional GHRH system for GHS was observed in patients with GHRH receptor deficiency who showed a selective absence of GH response to GHS (13). In contrast to an action through GHRH, the other proposed mechanism points to a direct effect of ghrelin on pituitary somatotropes that synergizes with GHRH. Indeed, biochemical studies performed in cultured pituitary cells showed a synergistic effect on cAMP production and GH release after co-treatment with GHRH and GHS (14–18). However, it remained unclear whether the potentiated cAMP and GH response arises from the direct actions of GHS on the somatotrope cells. Based on the synergistic effect of ghrelin and GHRH on GH release, we formulated the working hypothesis that GHRH may directly activate the ghrelin receptor GHS-R1a. Such direct activation could be induced either by canonical binding, through the formation of a heterodimer GHS-R1a/GHRH-receptor or by unidentified components of the signaling pathway associated with each hormone. Therefore, the targets of this work were to determine a) whether GHRH can bind, activate, and internalize the ghrelin receptor GHS-R1a, and b) the cell biology mechanisms involved in such events.

Results

Effects of GHRH on Intracellular Calcium Mobilization in HEK 293 Cells Expressing the Ghrelin Receptor. Exposure of HEK 293 cells that stably express the ghrelin receptor GHS-R1a (HEK-GHSR1a) to hGHRH(1–29)NH_2 (GHRH, 200 nmol/l) evoked a biphasic increase of $[Ca^{2+}]_i$, an effect that was not observed in HEK 293 wild-type cells (Fig. 1*A*). The calcium peak was due to an initial redistribution from intracellular stores, as shown by the absence of the peak in cells pretreated for 30 minutes with 50 nmol/l thapsigargin ([Supporting Information (SI) Fig. S1*A*)]. The GHRH-induced calcium mobilization was not affected after a pretreatment with PMA (1 μmol/l, 5 minutes before stimulus), a characteristic shared with ghrelin-activated calcium mobilization through GHS-R1a (Fig. S1*B*). The GHRH-induced $[Ca^{2+}]_i$ rise was dose dependent (Fig. 1*B*), with a half maximal response at 5 nmol/l and saturation at 200 nmol/l. The calcium response

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[1]To whom correspondence may be addressed. E-mail: felipe.casanueva@usc.es or andrew.schally@va.gov.

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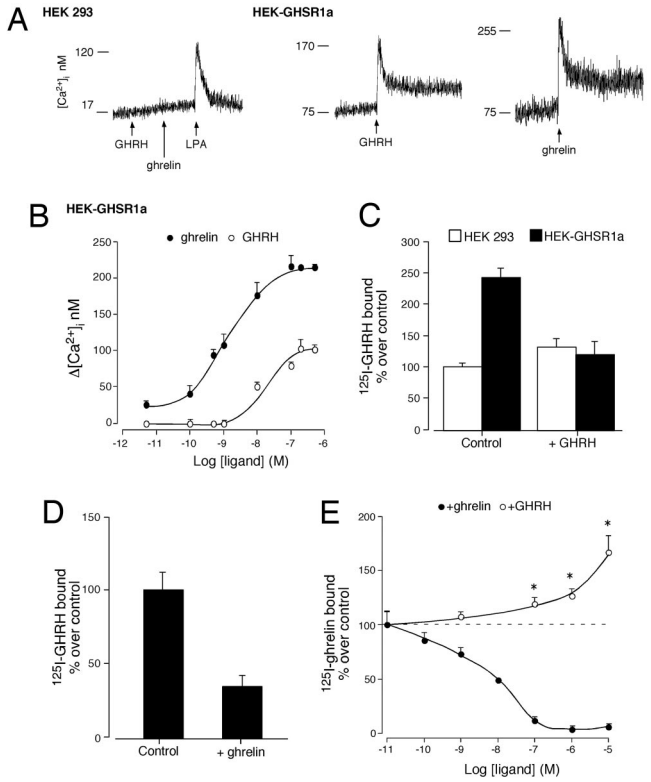

Fig. 1. (*A*) Effects of GHRH (200 nmol/l), ghrelin (100 nmol/l), and LPA (1 μmol/l) on intracellular calcium mobilization in HEK 293 wild-type (*left panel*) or HEK-GHSR1a (*right panel*) cell lines. Calcium mobilization traces are representative of a series of 20 calcium assays for each cell line. (*B*) Dose-response of GHRH- and ghrelin-induced Ca^{2+} transients. The results are expressed as mean ± SE of three independent experiments. (*C*) Displacement of ^{125}I-GHRH by GHRH (1 μmol/l) in HEK 293 (open bars) and HEK-GHSR1a (filled bars) cell lines. (*D*) Displacement of ^{125}I-GHRH by ghrelin (1 μmol/l) in HEK-GHSR1a cell line. (E) Binding property of ^{125}I-ghrelin in the presence of increasing amounts of GHRH (10^{-11}-10^{-5} mol/l) or ghrelin (10^{-11}-10^{-5} mol/l). For *panels C-E,* data are represented as a percentage of control, binding in the absence of unlabeled competitor in HEK 293 cell line (mean ± SE of three independent experiments performed in duplicate; $*P < 0.05$).

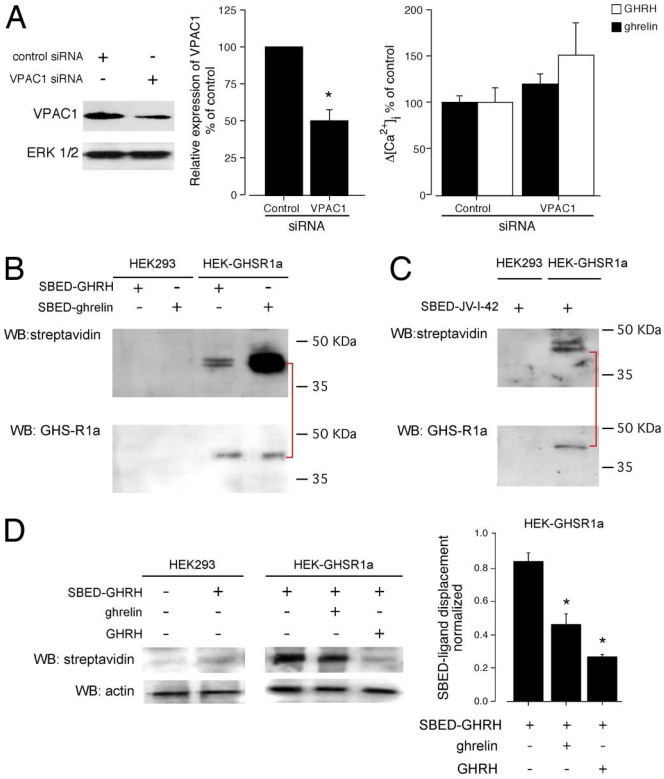

Fig. 2. (*A*) Effect of siRNA-mediated suppression of VPAC1 expression on the calcium response induced by GHRH in HEK-GHSR1a cell line. The efficiency of the silencing treatments was analyzed by Western blot for VPAC1 receptor (*left panel*), quantified by densitometry and expressed as percentage of control (mean ± SE of four independent experiments; $*P < 0.05$) (*middle panel*). The intracellular calcium responses to GHRH (200 nmol/l) and ghrelin (100 nmol/l) were evaluated and expressed as percentage of control (mean ± SE of four independent experiments). (*B*) The cross-linker Sulfo-SBED was coupled to GHRH, ghrelin, or JV-1–42 (*C*) and then incubated with HEK 293 and HEK-GHSR1a cell lines. Samples were separated on a 10% SDS/PAGE, transferred to nitrocellulose membranes, and incubated with streptavidin-HRP or anti-GHS-R1a antibodies. Red lines indicate the Sulfo-SBED-labeled protein bands identified by the anti-GHS-R1a antibody. (*D*) The cross-linker Sulfo-SBED was coupled to GHRH in the presence of nonlabeled ghrelin or GHRH and then incubated with HEK-GHSR1a cells. Samples were separated on a 10% SDS/PAGE, transferred to nitrocellulose membranes, incubated with streptavidin-HRP or anti-actin antibodies, and quantified by densitometry (mean ± SE of four independent experiments; $*P < 0.05$)

to GHRH was one order of magnitude smaller than that of ghrelin that showed a half-maximal value at 0.5 nmol/l with saturation at 100 nmol/l (Fig. 1*B*).

GHRH Binding Studies. Membranes from HEK 293 wild-type cells exhibited a nonspecific binding for ^{125}I-GHRH that was not displaced by cold GHRH (1 μmol/l). However, HEK-GHSR1a membranes showed a higher binding capacity of ^{125}I-GHRH (2.5-fold) that was displaced by unlabeled GHRH (1 μmol/l) (Fig. 1*C*). Furthermore, bound ^{125}I-GHRH was displaced by ghrelin (1 μmol/l; Fig. 1*D*). Unexpectedly, the presence of increasing amounts of GHRH (10^{-11}–10^{-5} mol/l) exerted a significant and dose dependent increase in the ^{125}I-ghrelin binding capacity in HEK-GHSR1a cells (Fig. 1*E*).

Reverse Transcription-Polymerase Chain Reaction Analysis of GHRH, Vasoactive Intestinal Peptide, and Pituitary Adenylate Cyclase-Activating Polypeptide Receptors in HEK-GHSR1a Cell. The expression of mRNA for the full-length GHRH receptor was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) as previously described (19). No PCR product corresponding to hGHRH receptor was visualized in HEK-GHSR1a cells, whereas a 820-bp band was observed in human normal brain tissue used as positive control (Fig. S1*C*). To ascertain whether the observed actions could be exerted on endogenous vasoactive intestinal peptide (VIP) or pituitary adenylate cyclase-activating polypeptide (PACAP) receptors (20, 21), the mRNA expression for VIP receptor type type 1 (VPAC1), VIP receptor type 2 (VPAC2), and PACAP receptor type 1 (PAC1) was also evaluated by RT-PCR. A 447-bp PCR product was detected in HEK-GHSR1a cells with primers corresponding to VPAC1. No PCR products corresponding to VPAC2 or PAC1 were visualized in HEK-GHSR1a cells (Fig. S1*D*). Distinct PCR products, 447-bp and 332-bp bands corresponding to VPAC1 and VPAC2, respectively, were detected in human gastric carcinoma cell line (KATO-III used as positive control (Fig. S1*D*). A 161-bp band for PAC1 was observed in normal human stomach tissue (Fig. S1*D*). On the basis of these results, siRNA was used to reduce the expression of endogenous VPAC1 in HEK-GHSR1a cells. siRNA targeting VPAC1 reduced the expression of VPAC1 by 50%, but the GHRH- or ghrelin-induced $[Ca^{2+}]_i$ rise was similar to that observed in the presence of a nontargeting control siRNA (Fig. 2*A*).

Identification of GHRH-GHS-R1a Interaction by Chemical Cross-Linking. Sulfo-SBED is a trifunctional cross-linking reagent that allows affinity-based enrichment of cross-linked species. Sulfonated *N*-hydroxysuccinimide ester on this reagent reacts with primary amines on the ligands GHRH, ghrelin, or the selective GHRH antagonist JV-1–42; thereby, biotinylated ligand is obtained in a first step. During the second step, photosensitive aryl azide moiety, linked by a disulfide bond on this cross-linker, is captured by the receptor from the plasma membrane because of the receptor affinity and the proximity of the moiety to the receptor (within a distance of 10 Å; Fig. S2). Subsequent analysis of biotin-labeled ligand-receptor complexes by Western blot using streptavidin-horseradish peroxidase (HRP) revealed two proteins with close molecular mass (between 40–50 KDa) for both biotin labeled ghrelin and GHRH in HEK-GHSR1a cells, which were absent in HEK 293 wild-type cells. By reprobing the same blots with an anti-GHS-R1a antibody, the presence of the ghrelin receptor was confirmed indicating a specific interaction (Fig. 2*B*). The presence of the same protein bands in the assays for biotin labeled JV-1–42 in HEK-GHSR1a cells supports the view that GHRH interacts in a specific manner with the ghrelin receptor (Fig. 2*C*). The cross-linking procedure used also identified a protein band not recognized with the anti-GHS-R1a antibody. This fact seems to be due to the nonspecific crosslinking between prelabel ligand and the receptor through the UV light-activatable aryl azide group that prevents, in some manner, the interaction with the antibody. Control experiments in the absence or presence of excess of unlabeled ligand showed that SBED-GHRH was displaced by ghrelin and GHRH (Fig. 2*D*).

Evaluation of GHRH Agonists (JI-34 and JI-36) and Antagonists (JV-1–42 and JMR-132) on Intracellular Calcium Mobilization in HEK 293 cells Expressing the Ghrelin Receptor. Addition of the potent GHRH agonists JI-34 or JI-36 (400 nmol/l) to HEK-GHSR1a cells evoked a biphasic increase of $[Ca^{2+}]_i$, an effect that was not observed in HEK 293 wild-type cells (Fig. 3*A*). This increase of $[Ca^{2+}]_i$ was not detected with the two selective GHRH antagonists JV-1–42 and JMR-132 in HEK-GHSR1a cells (data not shown). However, preincubation (20 seconds) of cells with the JV-1–42 resulted in a strong dose-dependent inhibition of the ghrelin- and GHRH-induced calcium mobilization (Fig. 3*B*), with an inhibition at 500 nmol/l of 60% and 90% for ghrelin and GHRH, respectively. A similar dose-dependent inhibition was observed after pretreatment with the JMR-132 for both ghrelin- and GHRH-induced $[Ca^{2+}]_i$ rise (60% and 95% inhibition at 500 nmol/l, respectively; Fig. 3*C*). Under these conditions, ^{125}I-ghrelin binding capacity was partially displaced by both JV-1–42 (60% displacement at 1 μmol/l) and JMR-132 (45% displacement at 1 μmol/l) in whole HEK-GHSR1a cells (Fig. 3*D*).

GHRH-Induced Calcium Signaling. As shown in Fig. 4*A*, GHRH (1 μmol/l) induced a low but significant formation of inositol phosphates (IP) from labeled PtdIns(4,5)P_2 in HEK-GHSR1a cells. As expected, this effect was clearly higher after ghrelin treatment (500 nmol/l). Co-administration with both ghrelin and GHRH showed a significant additive effect on IP production compared with GHRH or ghrelin alone (Fig. 4*A*). In contrast, neither ghrelin (500 nmol/l) nor GHRH (1 μmol/l) treatment caused a significant effect on cAMP production (Fig. 4*B*). The effect of GHRH on ghrelin-induced $[Ca^{2+}]_i$ rise was further monitored in a Schild-type analysis. As Fig. 4*C* shows, GHRH acted as an enhancer of ghrelin signaling, as dose of 100 nmol/l of GHRH, which stimulated the $[Ca^{2+}]_i$ rise up to 40% of the maximal ghrelin response, shifted the dose-response curve for ghrelin approximately twofold to the left [EC_{50} for ghrelin 8.9×10^{-10} nmol/l; EC_{50} for ghrelin+GHRH (100 nmol/l) 4.4×10^{-10} nmol/l].

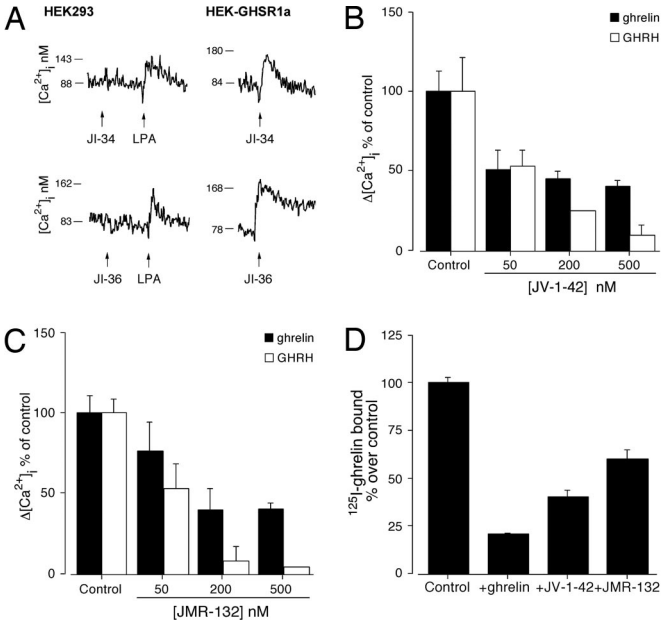

Fig. 3. (*A*) Effects of GHRH analogs JI-34 and JI-36 (400 nmol/l) on intracellular calcium mobilization in HEK 293 wild-type (left panel) and HEK-GHSR1a (right panel) cell lines. (*B*) Effect of the GHRH antagonist JV-1–42 on the calcium responses induced by ghrelin. (*C*) Effect of the GHRH antagonist JMR-132 on the calcium responses induced by ghrelin. (*D*) Displacement of ^{125}I-ghrelin by ghrelin (1 μmol/l), JV-1–42 (1 μmol/l), and JMR-132 (1 μmol/l) in HEK-GHSR1a cell line. For (*B*) and (*C*), data are expressed as percentage of control (mean ± SE of four independent experiments). For (*D*), data are represented as a percentage of control, binding in the absence of unlabeled competitor (mean ± SE of three independent experiments performed in duplicate).

Assessment of the Effects of GHRH on GHS-R1a Endocytosis. Internalization of the ghrelin receptor was evaluated in CHO cells stably expressing the GHSR1a- enhanced green fluorescent protein (EGFP) (CHO-GHSR1a-EGFP) by confocal microscopy. In the resting cells, fluorescence associated with the receptor was predominantly confined to plasma membrane (Fig. 4*D*). After exposure to GHRH (200 nmol/l) for 20 minutes, the fluorescence almost completely disappeared from the plasma membrane to be redistributed to a population of intracellular vesicles throughout the cytoplasm. No redistribution was observed with des-*n*-octanoyl ghrelin (100 nmol/l), PACAP (200 nmol/l), or VIP (200 nmol/l) (Fig. 4*D*). After 60 minutes of GHRH incubation, fluorescent vesicles were observed in a perinuclear localization and were again concentrated near the plasma membrane after 120 minutes. Ligand specificity of the GHSR1a endocytosis was suggested by the fact that ghrelin (100 nmol/l) induced a similar decrease in cell-surface labeling associated with a redistribution of fluorescence to scattered cytoplasmic vesicles (Fig. 4*D*).

Discussion

In the present study, we provide the first evidence that GHRH specifically binds to the ghrelin receptor GHS-R1a, increasing the binding capacity of its natural ligand. This binding activates the signaling route of inositol phosphate, leading to an intracellular calcium rise, and finally leads to a GHRH-mediated ghrelin receptor endocytosis. Furthermore, GHRH augments the maximal response to ghrelin in respect to inositol phosphates turnover through G_q-associated signal transduction that increases the potency of ghrelin, at least on intracellular calcium rise.

To consider a true ligand-receptor interaction, two requirements must be fulfilled: namely, specific binding and biological

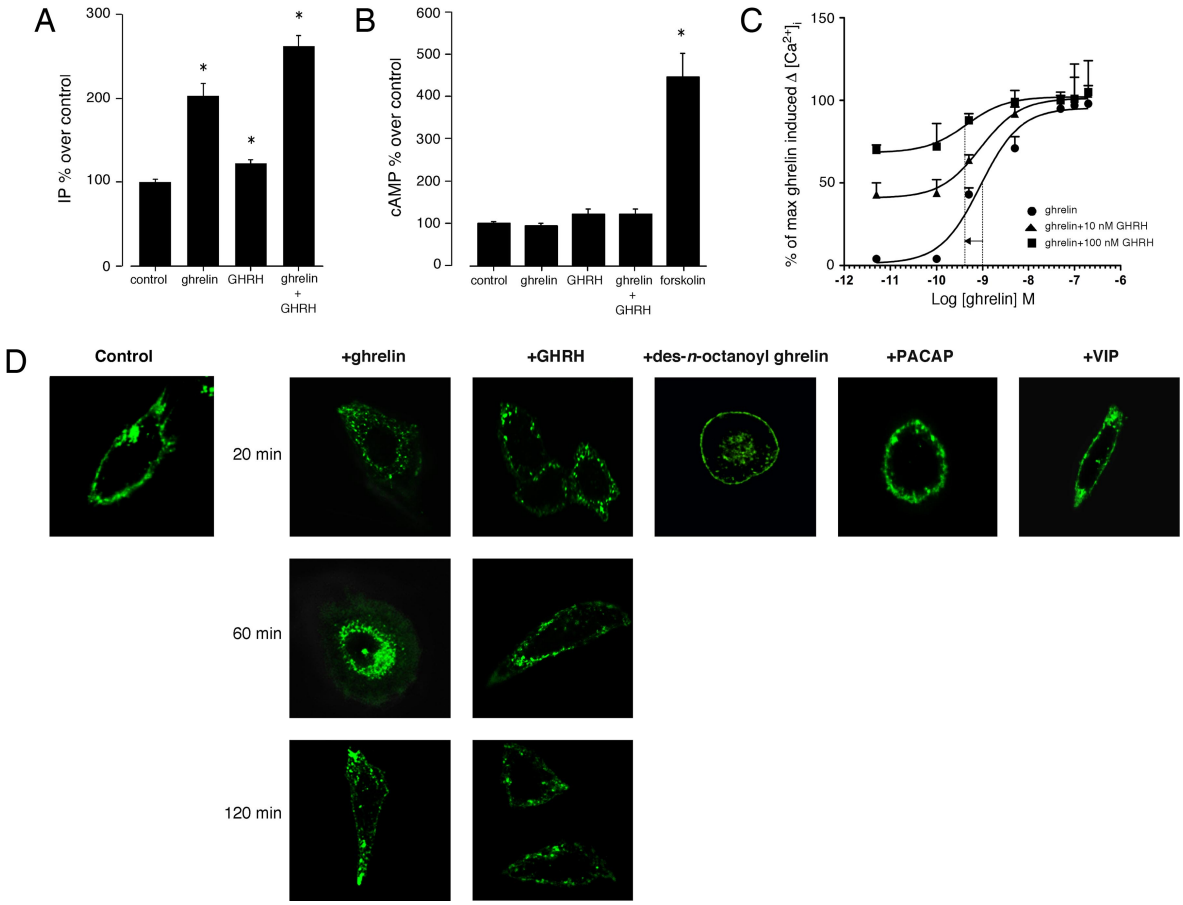

Fig. 4. (*A*) Effects of ghrelin (500 nmol/l), GHRH (1 μmol/l) and ghrelin plus GHRH on PtdIns (4,5)P_2 hydrolysis from *myo*-[2-^{3}H]inositol-labeled HEK-GHSR1a cell line. (*B*) Effects of ghrelin (500 nmol/l), GHRH (1 μmol/l) and ghrelin+GHRH on cAMP in HEK-GHSR1a cell line. Results (mean ± SE, $n = 6$) are expressed as percentage of control (unstimulated) (*$P < 0.05$). (*C*) Dose-response curves for ghrelin in the absence (circles) and presence of GHRH in concentrations of 10 nmol/l (triangles) and 100 nmol/l (squares). Results (mean ± SE, $n = 6$) are expressed as percentage of the maximum of ghrelin-induced $[Ca^{2+}]_i$ rise. (*D*) Kinetics of endocytosis of GHSR-1a-EGFP in CHO cells after stimulation with ghrelin (100 nmol/l), GHRH (200 nmol/l), des-*n*-octanoyl ghrelin (100 nmol/l), PACAP (200 nmol/l) or VIP (100 nmol/l). In absence of ligand, the fluorescent labeling appeared at the cell surface (Control). After a 20-minute incubation with ghrelin or GHRH, the fluorescent labeling was visualized inside cells. After a 60-minute incubation, perinuclear localization was observed for both ligands. After 120-minute incubation, the fluorescent vesicles were concentrated near the membrane, and a moderate labeling appeared at the cell surface.

effect. In this work, GHRH showed specific binding sites in cells transfected with the ghrelin receptor GHS-R1a displaceable with unlabeled GHRH and no binding in wild-type cells. However, GHRH appears to be not only a simple ligand of the GHS-R1a but also an allosteric enhancer of ghrelin action. ^{125}I-GHRH was displaced by unlabeled ghrelin; but, interestingly, the binding capacity of ^{125}I-ghrelin was augmented by increased amounts of unlabeled GHRH. Considering the complexity of binding studies with GHRH, because of its lipophilicity, selective cross-linking studies were performed. Cross-linking reagent, sulfo-SBED, linked GHRH to a 40–50 KDa protein that reacted with anti-GHS-R1a antibody proving the identity of the cross-linked complex as GHRH-GHSR1a in HEK-GHSR1a cells. This complex was further displaced by unlabeled GHRH and ghrelin. Western blot analysis, after cross-linking of the selective GHRH-R antagonist JV-1–42 to HEK-GHSR1a cells, confirmed the cross-linked protein as GHS-R1a. In accordance with the binding activity, GHRH activates the rise in intracellular calcium through the activation of signaling route of inositol phosphates in HEK-GHSR1a cells. This result might be explained by the endogenous expression of the VPAC1 detected by RT-PCR and Western blot analysis in HEK-GHSR1a cells. However, the functionality of GHRH was not affected after depleting cellular levels of this receptor by transfecting siRNA specially directed against this isoform, ruling out this hypothesis. Furthermore, the GHRH actions here reported were not under the regulation of PKC, since phorbol ester PMA failed to suppress the GHRH-induced calcium response such as occurs for ghrelin-induced signal (22). As a proof of concept hGHRH analogs, JI-34 and JI-36, were able to induce a rise in intracellular calcium. In the same line, the selective hGHRH-R antagonists, JV-1–42 and JMR-132 (23–26), behave as antagonists of the ghrelin receptor GHS-R1a. Finally, and quite remarkably, GHRH activated the endocytosis of the ghrelin receptor. The GHRH/GHSR1a complex progressively disappears from the plasma membrane after 20 minutes exposure to GHRH and accumulates in the perinuclear region after 60 minutes. This observation fits in well with the kinetics of receptor endocytosis described for ghrelin in the present and previous works (27). It is important to emphasize that this effect is different from that described for adenosine in the same cellular model (22). The binding analyses showed the same number of adenosine binding sites in both HEK 293 wild-type and HEK-GHSR1a cells corresponding to a similar endogenous expression of adenosine receptors, types 2b and 3, in both cell lines. In addition, adenosine failed to induce the GHS-R1a endocytosis, demonstrating that adenosine modulates but does not bind to ghrelin receptor.

Thus, GHRH is able to bind to the ghrelin receptor and does not compete for binding against ghrelin. Instead, GHRH increases the affinity of ghrelin, displaying positive binding cooperativity. Furthermore, GHRH increased the maximal response to ghrelin with respect to inositol phosphates turnover through G_q-associated signal transduction. This is in accordance with the concept of GHRH being an allosteric modulator (28, 29). This enhancer function is further supported by the fact that GHRH decreased the EC_{50} for ghrelin, at least as measured in the calcium assay. Similar positive allosteric modulation was described for CGP7930 on the $GABA_B$ receptor, which functions as partial agonist increasing maximal signaling response for the natural ligand (30). On the melanocortin receptors, Zn^{2+} potentiates the response for the natural ligand acting also as partial agonist (31). Furthermore, on the ghrelin receptor GHS-R1a, the non-peptidyl growth hormone secretagogue L-692,429 appears to belong to this group of ligands, being both an agonist and a positive modulator (32). L-692,429 increases the maximal response to ghrelin acting as a full agonist in stimulation of G_q-mediated IP turnover. The effects observed in the present study for GHRH with respect to ghrelin binding are markedly different from those described for L-692,429 which competes with the natural ligand of this receptor. With regard to molecular mechanisms, it is not possible from the present data to establish a model of GHRH action on the ghrelin receptor. A homodimeric model was recently proposed for the ghrelin receptor, in which, because of negative cooperativity, the binding of ghrelin occurs only in one subunit, preventing another ghrelin molecule from binding to the other subunit (32). On the basis of this model, we suggest that GHRH might be able to bind in a multivalent form. When GHRH is present alone, it might bind to one subunit of the dimeric receptor interacting with the orthosteric site (main ghrelin binding site) that determines the agonist properties. However, when ghrelin is also present, GHRH occupies an "allosteric site," acting as co-agonist that stabilizes the ghrelin binding. This implies that GHRH allows two ghrelin molecules to bind at the same time in the two subunits, which might explain the increase in ghrelin binding capacity observed in the present study.

In conclusion, the studies described here suggest that GHRH is an allosteric modulator of the ghrelin receptor GHS-R1a. The complete elucidation of this phenomenon may provide the basis for understanding the regulatory mechanisms on GH secretion and appetite control. In addition, this might indicate that GHRH may contribute to the variety of physiological events in which ghrelin is implicated as the regulator of energy homeostasis.

Materials and Methods

Peptides. Human ghrelin and des-*n*-octanoyl ghrelin were obtained from Global Peptide. hGHRH (1–29)NH_2 was purchased from Serono Laboratory (Madrid, Spain). Lysophosphatidic acid (LPA, oleoyl), human VIP, and PACAP were obtained from Sigma (St. Louis, MO). The synthesis of hGHRH antagonists JV-1–42 and JMR-132, was previously described (23, 26, 33–35). The hGHRH agonists JI-34 and JI-36 were synthesized by the same methods (36). ^{125}I-ghrelin and ^{125}I-GHRH were obtained from Amersham GE Healthcare.

Cell Cultures. The HEK 293 cell line stably transfected with the GHS-R1a (HEK-GHSR1a), the CHO cell line stably transfected with the GHS-R1a conjugated with enhanced green fluorescent protein fluorophore (CHO-GHSR1a-EGFP) and human gastric cancer cell line, KATO-III, were cultured as previously described (27, 37).

Calcium Measurements. Intracellular calcium measurements were performed using the fluorescent calcium probe fura-2 pentaacetoxymethylester (Molecular Probes) as previously described (27).

Membrane Binding Assays. HEK-293 and HEK-GHSR1a cells were homogenized in homogenization buffer (containing, in mmol/l: Tris-HCl, 50, pH 7.4; ethylenediaminetetraacetic acid (EDTA), 5; $MgCl_2$, 5; supplemented with 30 μg/ml bacitracin) on ice. The homogenate was centrifuged at 500 $\times$ *g* for 10 minutes at 4 °C. The supernatant containing the crude membrane fraction was again centrifuged at 70.000 $\times$ *g* for 50 minutes at 4 °C. The pellet was washed twice with cold homogenization buffer. The protein concentration was determined using QuantiPro BCA assay kit (Sigma-Aldrich, St. Louis, MO). For the competition binding assay, 50 μg of membrane homogenates were incubated (in triplicate) in the absence or presence of excess unlabeled competitor GHRH (1 μmol/l) and ghrelin (1 μmol/l) with 100.000 cpm/tube of ^{125}I-GHRH or ^{125}I-ghrelin for 1 hour at 4 °C in a final volume of 500 μl of binding buffer (containing, in mmol/l: Tris-HCl, 50, pH 7.4; EDTA, 5; $MgCl_2$, 5, supplemented with 1% bovine serum albumin (BSA) and 30 μg/ml bacitracin). After incubation, the membrane homogenates were pelleted at 12,000 $\times$ *g* for 2 minutes at 4 °C and washed twice with binding buffer. Finally, the bottoms of the tubes, containing the pellets, were cut off and counted in a γ-counter. Before experiments, tubes were treated with silicone to avoid the nonspecific binding.

Whole-Cell Binding Assays. Confluent monolayer cells were resuspended (5–6 $\times$ 10^5 cells per aliquot) in binding buffer (containing: Dulbecco's modified Eagle's medium [DMEM], pH 7.4, plus 1% [wt/vol] BSA) supplemented with 100.000 cpm/aliquot of ^{125}I-ghrelin in the absence or presence of unlabeled GHRH (1 μmol/l), ghrelin (1 μmol/l), or GHRH antagonists (JV-I-42 and JMR-132; 1 μmol/l). After incubation, cell surface radioligand was determined by incubating the cells in 0.5 ml ice-cold acid buffer. Finally, the cells were centrifuged and supernatants were counted in a γ-counter.

RNA Isolation and RT-PCR. A 1-μg quantity of total RNA, isolated using Quick Prep Micro mRNA Purification Kit (Amersham GE Healthcare), was used in reverse transcription reactions with Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen). The resulting total cDNA was then used in PCR to measure the mRNA levels of full-length GHRH-receptor as follows: sense: 5′-TTCTGC GTGTTG AGC CCG TTA C-3′; antisense: 5′-CAC GTG CCA GTG AAG AGC ACG G-3′, VPAC1: sense: 5′-TGTTCTACGGTTCTGTGAAGA-3′; antisense: 5′-AGCACCCAT AATCCTCAAAAT-3′; VPAC2: sense: 5′-AAGCAG-GAAAC ATAAAGC-3′; antisense: 5′- TAGAGAACGTCGTCCTTGACC-3′; and PAC1 [sense: 5′- CTCTGCTGTGAGACCTTC-3′; antisense: 5′- CCACAGAGCTGT-GCTGTCAT-3′. The mRNA levels of hprt or actin were used as internal controls. The PCR was performed in 40 cycles, each consisting of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 45 seconds, with a final extension of 72 °C for 7 minutes. The amplified fragments were separated on 2% agarose gels and visualized by ethidium bromide staining.

siRNA and Cell Transfection. For VPAC1, three different strands for each siRNA were used: 5′-CCAUCUUGGUAAACUUCAUtt-3′, 5′-GGAGUACACUACAUCAU-GUtt-3′, 5′- GGAGAGCACACCUAUCUUAtt-3′. A nonsilencing RNA duplex was used as a control (Santa Cruz Biotechnologies). For siRNA experiments, HEK-GHSR1a cells (40–50% confluent on 100-mm dishes) were transfected with 0.72 nmol siRNA, using siRNA transfection reagent (Santa Cruz Biotechnologies). Forty-eight hours later, cells were used for calcium assays. Only experiments with validated silencing were analyzed. VAPC1 expression was determined by Western blotting using anti-VAPC1 rabbit policlonal (1:1000) antibody (Santa Cruz Biotechnologies).

Measurement of Inositol Phosphate. The total [^{3}H]-inositol phosphates (IP) were analyzed essentially as previously described (38).

Measurement of Intracellular cAMP levels. HEK-GHSR1a cells were treated with 3-isobutyl-1-methylxanthine (IBMX) (1 mmol/l) for 30 minutes, followed by 30 minutes with test agents. Cells were lysed and the cAMP assay was performed using cAMP-[^{3}H] Biotrak Assay from Amersham GE Healthcare.

Chemical Cross-Linking. The heterotrifunctional cross-linker Sulfo-SBED Biotin label transfer reagent (Pierce Biotechnology, IL) was coupled to GHRH, ghrelin, and GHRH antagonist JV-I-42 (100 μg each) at low stoichiometry (1:1). The reactions were stopped by addition of Tris 2 mol/l, and cross-linked peptides were incubated with HEK 293 and HEK-GHSR1a cells for 5 minutes at 4 °C. After incubation, the cells were exposed at 365 nm at 4 °C for 10 minutes and then lysed in ice-cold lysis buffer. Protein concentration of lysates was evaluated with the QuantiPro BCA assay kit. The same amount of protein of each sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were incubated with streptavidin (Pierce Biotechnology, IL), horseradish peroxidase-conjugated (1:1000), or anti-GHSR1a rabbit polyclonal (Santa Cruz Biotechnologies, CA) (1:1000). Sig-

nals were visualized using an enhanced chemiluminescence detection system (Amersham GE Healthcare, UK).

Confocal Microscopy. The cells were examined with a Leica TCS SP 2 (Leica Microsystems, Heidelberg, Germany) confocal laser scanning microscope mounted on a Leica DM IRBE inverted microscope equipped with an argon/krypton laser. EGFP fluorescence was detected with excitation at 488 nm, using a RSP 500 (dichroic) mirror and the spectrophotometer set to acquire emission between 530 and 560 nm. Optical sections (1024 × 1024) of individual cells were taken at the equatorial level (level of the nucleus), using a 63 × 1.32 NA oil-immersion objective. The receptor internalization studies were performed essentially as described (27).

Data Analysis. The results were expressed as the mean ± SE. Differences between means were evaluated by analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant.

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