Ghrelin Stimulates Porcine Somatotropes

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Summary and Implications

Ghrelin is an endogenous ligand for growth hormone secretagogue receptor (GHS-R) and is predominantly produced by the stomach and lower amounts in the hypothalamus and various peripheral tissues. Ghrelin is a potent stimulator of growth hormone (GH) secretion from the pituitary *in vivo* and *in vitro*. GH secretion from the pituitary also is controlled by two hypothalamic peptides: stimulatory GH-releasing hormone (GHRH) and inhibitory somatostatin-14 (SRIH). GH participates in its own rhythmic secretion through feedback action on GHRH and SRIH neurons. The mechanism of action of GHS is not established. The present study examined the signal transduction pathways of ghrelin in isolated porcine somatotropes. The ability of ghrelin to induce an increase in the intracellular Ca^{2+} concentration – $[Ca^{2+}]_i$ – somatotropes was examined in dispersed porcine pituitary cells using a calcium imaging system. Somatotropes were functionally identified by application of human growth hormone releasing hormone (hGHRH). Ghrelin increased the $[Ca^{2+}]_i$ in a dose-dependent manner in 98% of the cells that responded to. In the presence of (*D*-Lys^3)-GHRP-6, a specific receptor antagonist of GHS-R, the increase in $[Ca^{2+}]_i$ evoked by ghrelin was decreased. Pretreatment of cultures with somatostatin or neuropeptide Y reduced the ghrelin-induced increase of $[Ca^{2+}]_i$. The stimulatory effect of ghrelin on somatotropes was greatly attenuated in low-calcium saline and blocked by nifedipine, an L-type calcium channel blocker, suggesting involvement of calcium channels. In a zero Na^+ solution, the stimulatory effect of ghrelin on somatotropes was decreased, suggesting that besides calcium channels, sodium channels are also involved in ghrelin-induced calcium transients. Either SQ-22536, an adenylyl cyclase inhibitor, or U73122, a phospholipase C inhibitor, decreased the stimulatory effects of ghrelin on $[Ca^{2+}]_i$ transiently, indicating the involvement of adenylyl cyclase-cyclic adenosine monophosphate and phospholipase C inositol 1,4,5-trisphosphate pathways. The non-peptidyl GHS, L-692,585 (L-585), induced changes in $[Ca^{2+}]_i$ similar to those observed with ghrelin. Application of L-585 after ghrelin did not have additive effects on $[Ca^{2+}]_i$. Preapplication of L-585 blocked the stimulatory effect of ghrelin on somatotropes. Our results suggest that the actions of ghrelin and synthetic GHS closely parallel each other, in a manner that is consistent with an increase of hormone secretion. An understanding of the molecular mechanisms by which ghrelin and GHS modulate GH secretion is of particular interest in the regulation of GH for muscle accretion and somatic growth.

Introduction

Growth hormone (GH) secretion from the pituitary gland is controlled by two hypothalamic peptides: stimulatory GH-releasing hormone (GHRH) and inhibitory somatostatin-14 (SRIH). GH participates in its own rhythmic secretion through feedback action on GHRH and SRIH neurons. Peptidyl and nonpeptidyl compounds, known as GH secretagogues (GHS), can also stimulate the GH secretion both *in vivo* and *in vitro*. The mechanism of action of GHS is not fully established. There is both a direct effect on the anterior pituitary gland and an indirect effect on the hypothalamus. The direct effect of GHS on GH secretion is through an interaction with a specific receptor named GHS-R which is distinct from the GHRH receptor. The GHS-R, a typical G-protein-coupled seven-transmembrane receptor, has been cloned in pigs, humans, and rats.

Ghrelin, an endogenous ligand for GHS-R, is an 28-amino-acid peptide with an n-octanoyl modification at Ser^3 residue that is essential for its biological activity. Ghrelin is predominantly produced by the stomach, whereas lower amounts are derived from hypothalamus and various peripheral tissues. Ghrelin receptors (GHS-R) are widely distributed, e.g., in pituitary, hypothalamus, stomach, heart, blood vessels, lung, pancreas, intestine, kidney, adipose tissue, and immune system. This suggests that ghrelin can have peripheral and central effects in addition to having a powerful effect on the secretion of GH. Moreover, ghrelin signals directly to the hypothalamic regulatory nuclei to control energy homeostasis and, therefore, might regulate the growth process in an integrated manner.

Ghrelin has been demonstrated to induce GH secretion from the pituitary *in vivo* and *in vitro*. The effects of ghrelin exhibit a similar time course for GH release to that of other GHS. Several studies confirm that the actions of ghrelin and peptidyl GHS closely parallel each other.

The present study examined intracellular Ca^{2+} mobilization and the signal transduction pathways of ghrelin in isolated porcine somatotropes and the interaction between ghrelin and nonpeptidyl GHS, L-692,585 (L-585) (1).

Materials and Methods

Experimental animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for these experiments. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Preparation of Cell Cultures

Newborn pigs, 1-8 days of age (n = 26), were sacrificed and the pituitary glands were immediately removed and collected in cold sterile EBSS solution (4°C). Primary cell cultures from neonatal anterior pituitary gland were established. Tissue from 2 animals were incubated for 50 min at 37°C in 2 ml of EBSS-papain solution (1.54 mg/ml) and followed by trypsin inhibitor solution (1 mg/ml). After being rinsed, once with EBSS solution and once with DMEM-0.1% BSA medium, the tissue was mechanically dispersed and the supernatant containing cells was removed and filtered through a sterile filter. The cells were plated onto poly-*L*-lysine (0.1 mg/ml; 100,000 kD) coated glass coverslips (at a density of 2×10^5 cells). Cultures were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere. Studies examining the effects of ghrelin on changes in the intracellular calcium concentrations – $[Ca^{2+}]_i$ – in somatotropes were carried out after 2 days in culture. The presence of somatotropes was confirmed by immunocytochemical methods.

Immunocytochemistry Staining

After fixation with 4% paraformaldehyde for 30 min at room temperature, the cells were incubated for 30 min in a 50% goat serum solution containing 1% BSA and 100 m*M L*-lysine to block nonspecific binding and 0.4% Triton X-100 to permeabilize the membrane. To stain the somatotropes, cultures of anterior pituitary gland were incubated with polyclonal antiporcine GH antibody (dilution 1:50,000). Antibody visualization was accomplished by using a Vectastain ABC kit (Vector Laboratories) and the nickel-enhanced 3,3'-diaminobenzidine method.

Intracellular Calcium Imaging

The effect of secretagogues on $[Ca^{2+}]_i$ was evaluated by ratiometric imaging techniques. The cells were loaded with fura-2/AM for 40–60 min at 37° C. Coverslips containing pituitary cells were washed with Hepes-buffered solution and further incubated for 10 min at 37° C to allow deesterification of fura-2/AM. All image processings and analyses were performed using an Attoflour system (Atto Bioscience; Rockville, Md., USA) with a Zeiss microscope. Using wavelengths of 340 and 380 nm, fura-2/AM was excited, and the emitted light was analyzed at 520 nm.

Statistics

For every treatment, sister cultures were used as controls in order to minimize the effect of variability on individual responses. Multiple comparisons were carried out by one-way Anova followed by the Newman-Keuls multiple-comparison test. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Stimulatory Effect of Ghrelin on $[Ca^{2+}]_i$ in Cultured Porcine Somatotropes

The presence of somatotropes in pituitary cell cultures was confirmed by immunocytochemical staining with an antibody raised against GH. GH-immunoreactive cells comprised 40% of the total pituitary cells in cultures. An increase in $[Ca^{2+}]_i$ above baseline following hGHRH application functionally identified GH cells. Of the cells that responded to hGHRH, 98% also responded to 1 μ*M* ghrelin applied 10 min after the application of hGHRH. Perfusion application of 1 μ*M* ghrelin for 2 min produced a prompt transient increase in $[Ca^{2+}]_i$ of 57 $\pm$ 3.0 n*M* (n = 227; $p < 0.01$; mean $\pm$ standard error; fig. 1a). A similar calcium response was observed in previous work after treatment of isolated porcine somatotropes with nonpeptidyl GHS, L-692,585 (L-585).

The second application of 1 μ*M* ghrelin 30 min after the first application increased the $[Ca^{2+}]_i$ in 75% of the cells that responded to the first application (fig. 1a). Application of the nonselective stimulus potassium (50 m*M*) after hGHRH and ghrelin resulted in an elevated $[Ca^{2+}]_i$ by 93 $\pm$ 6.2 n*M* ($p < 0.01$) in all cells that responded to hGHRH and ghrelin (fig. 1a).

The effect of ghrelin on calcium transient in cultured porcine somatotropes was dose dependent (fig. 1b).

Receptor Mediation of the Stimulatory Effect of Ghrelin on $[Ca^{2+}]_i$

To determine whether the effect of ghrelin was receptor mediated, experiments were performed with (*D*-Lys^3)-GHRP-6, a specific antagonist of GHS-R. In the presence of (*D*-Lys^3)-GHRP-6, the increase in $[Ca^{2+}]_i$ evoked by ghrelin (1 μ*M*) was delayed for about 100 seconds and decreased as compared with the response in controls. These results support the GHS-R, mediating the effect of ghrelin increasing the $[Ca^{2+}]_i$ in cultured porcine somatotropes.

SRIH Decreases the Effect of Ghrelin

Application of SRIH at concentrations of 10 μ*M* decreased $[Ca^{2+}]_i$ from 92 $\pm$ 3.5 to 69 $\pm$ 2.5 n*M* (n = 115; $p < 0.01$). In the presence of 10 μ*M* SRIH, the increase in $[Ca^{2+}]_i$ evoked by perfusion application of 1 μ*M* ghrelin for 2 min was decreased (78 $\pm$ 4.1 n*M*, n = 121, vs. 48 $\pm$ 2.7 n*M*, n = 103; $p < 0.01$).

NPY Decreases the Effect of Ghrelin

NPY has a role in the regulation of GH secretion, and thus the ability of ghrelin to evoke changes in $[Ca^{2+}]_i$ in the presence of NPY was examined. In the presence of NPY, the increase in $[Ca^{2+}]_i$ evoked by 1 μ*M* ghrelin for 2 min was decreased as compared with the response in the control cultures (63 $\pm$ 3.5 n*M*, n = 140, vs. 39 $\pm$ 2.1 n*M*, n = 128, $p < 0.01$). Thus, NPY influenced the stimulatory effects of ghrelin.

Role of Membrane Depolarization and Calcium Channels in Ghrelin Effects

The stimulatory effect of ghrelin was significantly decreased in calcium-depleted saline. In low-Ca^{2+} HEPES, only 42% of the cells that responded to hGHRH also responded to ghrelin. These results suggest that extracellular calcium has an important role in the effect of ghrelin on the GH-secretory cells.

To investigate the contribution of voltage-dependent Ca^{2+} channels in calcium influx evoked by ghrelin, experiments were performed in the presence of nifedipine, an antagonist of the L-type calcium channel. In the presence of 100 n*M* nifedipine, 1 μ*M* ghrelin evoked a response in 90% of the cells (64 of 71) that responded to hGHRH. The average increase in $[Ca^{2+}]_i$ was significantly smaller (60 $\pm$ 2.4 vs. 39 $\pm$ 2.2 n*M*; $p < 0.01$), and the duration of the sustained phase of calcium increase was brief.

Signal Transduction Pathways, Phospholipase C (PLC), and Adenylyl Cyclase-Cyclic Adenosine 3',5'-Monophosphate (AC-cAMP) Are Activated by Ghrelin

The involvement of intracellular Ca^{2+} stores in the action of ghrelin was further investigated in experiments with U73122, a selective inhibitor of PLC. Pretreatment of cultures for 10 min with 5 μ*M* U73122 significantly decreased the effect of 1 μ*M* ghrelin on calcium transient implying an involvement of the PLC-IP_3 pathway in the ghrelin action.

To determine whether ghrelin activates the cAMP pathway, cell cultures were pretreated with SQ-22536, an AC inhibitor. After 10 min of the application of SQ-22536 at a concentration 100 μ*M*, the amplitude of the $[Ca^{2+}]_i$ increase evoked by 1 μ*M* ghrelin was significantly decreased suggesting activation of AC by ghrelin.

Results and Discussion

In the present study, we have shown that ghrelin has a direct, dose-dependent stimulatory effect on the $[Ca^{2+}]_i$ in isolated porcine somatotropes. It is presumed that this increase in $[Ca^{2+}]_i$ evoked by ghrelin is followed by GH release. Similarly, in previous experiments on isolated porcine somatotropes with simultaneous measurements of GH release and calcium transients, we have shown that L-585, a nonpeptidyl GHS, evoked an increase in $[Ca^{2+}]_i$ that coincided with the GH release.

The amplitude of the increase in $[Ca^{2+}]_i$ evoked by ghrelin was similar to that with hGHRH, with the latter at the ten-fold higher concentration. This may suggest that ghrelin is more potent than GHRH in stimulating a rapid release of GH. There was no evidence for any heterologous desensitization between hGHRH and ghrelin.

Somatostatin decreased, but did not abolish the effect of ghrelin on calcium transient in porcine somatotropes. The effects of SRIH on somatotropes are mediated either by inhibiting the cAMP formation or via decreased Ca^{2+} influx as a result of an increase in K^+ conductance and hyperpolarization of somatotropes. GHSs, and probably ghrelin, can behave as functional SRIH antagonists by inhibiting K^+ channels and depolarizing the plasma membrane of the somatotropes.

It has been suggested that NPY suppresses the release of GH by reciprocal regulation of GHRH and SRIH neurons. Indeed, centrally administered NPY inhibited the GH secretion in pigs. In the present study, pretreatment of cultured porcine pituitary cells with NPY decreased the effect of ghrelin on $[Ca^{2+}]_i$.

Depletion of extracellular calcium greatly diminished but did not completely abolish the stimulatory effect of ghrelin on porcine somatotropes. The latter suggests that while ghrelin does mobilize Ca^{2+} from intracellular stores, Ca^{2+} influx has the major contribution to calcium transient. In somatotropes, the major Ca^{2+} channels are the voltage-gated T and L types. Perfusion of porcine cells with nifedipine decreased the effect of ghrelin. This indicates an involvement of L-type Ca^{2+} channels in calcium influx induced by ghrelin and that influx of calcium is a crucial step in the action of ghrelin, as in the action of L-585.

The stimulatory effect of ghrelin was decreased in a zero Na^+ environment, suggesting that ghrelin can depolarize somatotropes, at least partially, through sodium channels. cAMP, protein kinases A and C, and PLC are possible signaling systems involved in the action of GHS and are likely involved in the ghrelin action. In our experiments, U73122, a PLC inhibitor, decreased the effect of ghrelin on calcium transient, indicating the involvement of the PLC/IP_3 pathway in ghrelin action on porcine somatotropes. Pretreatment of porcine somatotropes with SQ-22536, an AC inhibitor, also decreased the stimulatory effect of ghrelin on calcium transient, implying that the binding of ghrelin to GHS-R activates AC.

In the present study, we have for the first time investigated interactions between ghrelin and the nonpeptidyl GHS, L-585. To examine interactions between ghrelin and L-585, we applied these agonists either in succession or simultaneously. L-585 had no additive effect on $[Ca^{2+}]_i$ when applied 10 min after ghrelin. Moreover, the response evoked by L-585 was almost completely abolished when applied in the presence of 100 n*M* ghrelin, a concentration that alone did not have any effect on $[Ca^{2+}]_i$. The stimulatory effect of

ghrelin was blocked when it was applied during the plateau phase of the L-585 action.

Simultaneous application of ghrelin and L-585 did not have additive effects on calcium transient. Indeed, the change in $[Ca^{2+}]_i$ was reduced as compared with either GHS alone. Together, our experiments with ghrelin and L-585 indicate antagonistic effects between these two agonists. Competition between ghrelin and L-585 probably occurs at the GHS-R on somatotropes which may involve binding to the different but overlapping domains on the same receptor.

In summary, ghrelin has a direct stimulatory effect on porcine somatotropes. During the first phase of the ghrelin-induced calcium transient, activation of different signal transduction pathways results from calcium mobilization from internal stores, whereas a second prolonged phase results from calcium influx as a consequence of somatotrope depolarization by ghrelin acting on Na^+ and K^+ channels and the activation of calcium channels by acting on different second messengers.

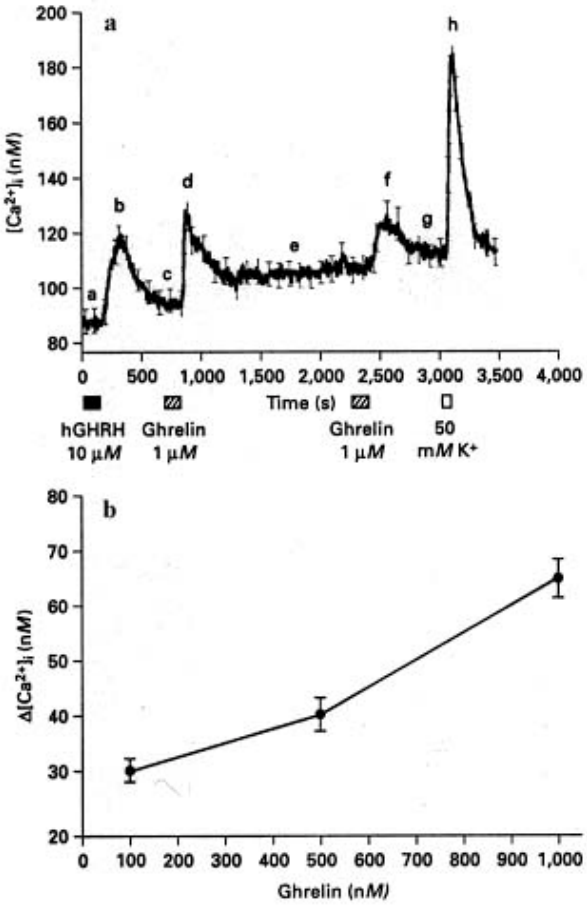

Fig. 1. Stimulatory effect of ghrelin on the $[Ca^{2+}]_i$ in porcine somatotropes. a Somatotropes were functionally identified by a 2-min application of 10 μ*M* hGHRH. Subsequent administration of 1 μ*M* ghrelin evoked an increase in $[Ca^{2+}]_i$ in 98% of the somatotropes. The repeated administration of ghrelin after 30 min did not have an additive effect on the $[Ca^{2+}]_i$, increase, suggesting the existence of homologous desensitization of GHS-R. Application of 50 m*M* K^+ increased the $[Ca^{2+}]_i$ in all cells that responded to hGHRH and ghrelin. Panels of images of calcium transient were taken at the time indicated on the time course histogram. **b** Application of ghrelin at concentrations of 100 n*M*, 500 n*M*, and 1 μ*M* dose dependently increased the $[Ca^{2+}]_i$ in isolated porcine somatotropes. The application of 500 n*M* ghrelin for 2 min evoked a smaller response than application of 1 μ*M* ghrelin in cultures run in parallel ($p < 0.01$), while the application of 100 n*M* ghrelin for 2 min evoked a smaller response than application of 500 n*M* ghrelin in cultures run in parallel ($p < 0.05$).

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Reference

1. Aleksandra Glavaski-Joksimovic, Ksenja Jeftinija, Colin G. Scanes, Lloyd L. Anderson, Srdija Jeftinija. Stimulatory effect of ghrelin on isolated porcine somatotropes. Neuroendocrinology 2003;77:366-378