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Intracellular Signaling Mechanisms Mediating Ghrelin-Stimulated Growth Hormone Release in Somatotropes

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Ghrelin is a newly discovered peptide that binds the receptor for GH secretagogues (GHS-R). The presence of both ghrelin and GHS-Rs in the hypothalamic-pituitary system, together with the ability of ghrelin to increase GH release, suggests a hypophysiotropic role for this peptide. To ascertain the intracellular mechanisms mediating the action of ghrelin in somatotropes, we evaluated ghrelin-induced GH release from pig pituitary cells both under basal conditions and after specific blockade of key steps of cAMP-, inositol phosphate-, and Ca^{2+}-dependent signaling routes. Ghrelin stimulated GH release at concentrations ranging from 10^{-10} to 10^{-6} M. Its effects were comparable with those exerted by GHRH or the GHS L-163,255. Combined treatment with ghrelin and GHRH or L-163,255 did not cause further increases in GH release, whereas somatostatin abolished the effect of ghrelin. Blockade of phospholipase C or protein kinase C inhibited ghrelin-induced GH secretion, suggesting a requisite role for this route in ghrelin action. Unexpectedly, inhibition of either adenylate cyclase or protein kinase A also suppressed ghrelin-induced GH release. In addition, ghrelin stimulated cAMP production and also had an additive effect with GHRH on cAMP accumulation. Ghrelin also increased free intracellular Ca^{2+} levels in somatotropes. Moreover, ghrelin-induced GH release was entirely dependent on extracellular Ca^{2+} influx through L-type voltage-sensitive channels. These results indicate that ghrelin exerts a direct stimulatory action on porcine GH release that is not additive with that of GHRH and requires the contribution of a multiple, complex set of interdependent intracellular signaling pathways. (*Endocrinology* 144: 5372–5380, 2003)

GHRELIN IS A RECENTLY discovered peptide that was initially isolated from rat stomach (1). Since then, ghrelin has been shown to be expressed in a variety of tissues including the pituitary (2), hypothalamus (1–4), kidney (5), heart (6), pancreas (7), immune system (8), testis (9), ovary (10), and placenta (11). Accordingly, a number of distinct functions has been reported for ghrelin so far, from stimulation of gastric acid secretion and motility (12) to inhibition of testicular secretion of testosterone (9). Nevertheless, early studies about ghrelin were focused on its effects on GH secretion by pituitary somatotropes, owing to the fact that this peptide was originally discovered by its ability to bind and activate the receptor for the family of synthetic GH secretagogues (GHS). Indeed, ghrelin stimulates GH release, both *in vitro* and *in vivo*, in a broad range of species including human (13–15), rodents (1, 16–20), swine (21), birds (22, 23), amphibians (24), and fish (25, 26). Moreover, available evidence suggests that ghrelin acts on somatotropes in a manner similar, if not identical, to that reported for GHSs such as GH-releasing peptide (GHRP)-6, hexarelin, or other peptidergic members of the family as well as for nonpeptidyl synthetic compounds (27). These results, together with the abundant expression of GHS-receptors (GHS-Rs) in the pituitary (28–31) has led to the idea that ghrelin, from either the hypothalamus or stomach or from both sources, could play a relevant role in the physiological regulation of GH secretion from somatotropes (reviewed in Ref. 32). In this scenario, ghrelin would have to interact in a coordinated manner with the primary hypothalamic regulators of somatotrope function, the stimulator GHRH and the inhibitor somatostatin (SRIF) (33).

Both GHRH and SRIF act on pituitary somatotropes through specific G protein-coupled receptors (34). Activation of the GHRH receptor in somatotropes is primarily linked to the adenylate cyclase (AC) signaling pathway leading to cAMP accumulation and activation of protein kinase A (PKA) and extracellular Ca^{2+} entry through voltage-sensitive Ca^{2+} channels (VSCC) (35), whereas the inhibitory effect of SRIF on GH release is mainly exerted through blockade of these intracellular routes (36–38). In contrast, the stimulatory action of GHSs on GH secretion appears to be mostly dependent on the activation, through a G protein-coupled GHS-R (28, 29), of the phospholipase C (PLC)/inositol phosphate/protein kinase C (PKC) system (34, 39). In particular, we previously observed that the PLC/PKC signaling pathway is required for both peptidyl (GHRP-6) and nonpeptidyl (L-163,255) GHSs to induce GH release from pig somatotropes (40–42). At present, there is a lack of information on the intracellular mechanisms that mediate the stimulatory action of the endogenous GHS-R ligand, ghrelin, on GH release in porcine or other vertebrate species. In the present study, we aimed at ascertaining this issue by determining,

Abbreviations: AC, Adenylate cyclase; FBS, fetal bovine serum; GHRP, GH-releasing peptide; GHS, GH secretagogue; GHS-R, receptor for GH secretagogue; IBMX, 3-isobutyl-1-methylxanthine; MEM, minimal essential medium; pGH, porcine GH; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; SRIF, somatostatin; VSCC, voltage-sensitive Ca^{2+} channel.

first, the effects of ghrelin alone or in combination with GHRH and SRIF on *in vitro* GH release from pig somatotropes, and second, by analyzing the signaling routes employed by ghrelin to act on pig somatotropes.

Materials and Methods

Reagents

Porcine GH (pGH; USDA-B-1, AFP-11716C) was kindly supplied by Dr. A. F. Parlow, from the Pituitary Hormones and Antisera Center, Harbor-University of California-Los Angeles Medical Center. D-valine modified minimal essential medium (MEM), collagenase type V, trypsin type I, soybean trypsin inhibitor I, DNase I, antibiotic-antimycotic solution, BSA, 3-isobutyl-1-methylxanthine (IBMX), thapsigargin, nifedipine, verapamil, and all other reagents were purchased from Sigma Chemical Co. (London, UK), unless otherwise specified. Fetal bovine serum (FBS) was obtained from Sera-Lab Ltd. (Crawley Down, UK). [^{3}H]-cAMP assay kit was from Amersham International (Aylesbury, UK). Indo-1/AM and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Synthetic human ghrelin was purchased from Bachem Ltd. (Merseyside, UK). SRIF (1–14), GHRH (1–29), and rabbit antisera to ovine (for immunocytochemical studies) or porcine (for enzyme immunoassays) GH were obtained from UCB Bioproducts (Brain L'Alleud, Belgium). L-163,255 was kindly provided by Merck Research Laboratories (Rahway, NJ). The inhibitors MDL-12,330A and U-73122 were obtained from Research Biochemicals International (Natick, MA), and H89 and phloretin were from Calbiochem Corp. (San Diego, CA). Tissue culture plasticware was from Costar (Cambridge, MA), and alphanumeric microgrid coverslips were from Eppendorf (Netheler, Germany).

Stock solutions of ghrelin, MDL-12,330A, H89, and thapsigargin were prepared with distilled deionized water, whereas phloretin and nifedipine were dissolved in ethanol. U-73122 was dissolved in dimethyl sulfoxide. Aliquots of concentrated stock solutions were stored at 20 C until use, when they were diluted to final concentrations in MEM. The highest concentration of ethanol or dimethyl sulfoxide used was always less than 0.1%, which had no effect on basal porcine GH release.

Animals and tissue

Prepubertal female Large White-Landrace pigs (5–6 months old) were obtained from a local slaughterhouse. Animals were killed by exsanguination after brief electrical stunning, and pituitary glands were immediately removed and transferred to sterile cold (4 C) MEM supplemented with 0.1% BSA and antibiotic-antimycotic solution. In the laboratory, pituitaries were washed twice with fresh medium, and the posterior lobes were discarded.

Pituitary cell dispersion and culture

Isolated cells from the porcine anterior pituitary were obtained using a dispersion protocol as previously described (43, 44). Briefly, for each experiment, three to four anterior pituitaries were pooled, minced, and enzymatically dissociated by sequential incubation in MEM supplemented with 0.3% trypsin (type I), 0.1% collagenase (type V), 0.1% soybean trypsin inhibitor I, 2 μg/ml DNase I, and Ca^{2+}/Mg^{2+}-free salt solution with EDTA (2 and 1 mM). Finally, the tissues were mechanically dispersed using a siliconized Pasteur pipette until a homogeneous cellular suspension was obtained. After each step, the cellular suspension was centrifuged at 60 $\times$ *g* for 5 min. Cellular viability, as estimated by the trypan blue test, was always above 90%.

For secretion experiments, dispersed pituitary cells were plated at a density of 300,000 cells/100 μl MEM onto 24-well culture plates and incubated at 37 C in a 5% CO_2, in 1 ml culture medium supplemented with 10% FBS and 0.1% gentamicin sulfate. After a 3-d culture period, the medium was removed and cells were preincubated in 1 ml serum-free MEM for 2 h to stabilize basal GH secretion. Then cells were incubated for 30 min with the corresponding test substances as follows: 1) ghrelin alone at doses ranging from 10^{-14} to 10^{-6} M; 2) GHRH (10^{-8} M) alone or in combination with ghrelin (10^{-8} M); 3) the synthetic GHS L-163,255 (10^{-8} M) alone or in combination with ghrelin (10^{-8} M); and 4) SRIF (10^{-7} M) alone or in combination with ghrelin (10^{-8} M). In another set of experiments, the contribution of different intracellular signaling routes to ghrelin-induced GH release from porcine somatotropes was evaluated by incubating cultured pituitary cells for 30 min with 10^{-8} M ghrelin in the presence of specific blockers of key intracellular signaling enzymes or Ca^{2+} channels. Specifically, U-73122 (5×10^{-6} M), phloretin (25 μM), MDL-12,330A (10^{-5} M), and H89 (15 μM) were used to inactivate PLC, PKC, AC, and PKA, respectively. In addition, the effect of the selective blocker of L-type VSCC nifedipine (1 μM) as well as that of the endoplasmic reticulum Ca^{2+}-ATPase pump inhibitor thapsigargin (100 nM) on GH release was also tested. The concentrations of inhibitors employed in each case were selected on the basis of their ability to effectively block the corresponding enzyme or Ca^{2+} channel in porcine somatotropes without modifying basal porcine GH release (44–46). In all the experiments, to ensure that the corresponding routes were blocked at the beginning of the treatments, inhibitors were added to the incubation medium at the concentrations indicated 90 min before ghrelin challenge. Medium samples were collected at the end of the experiments, centrifuged at 6000 $\times$ *g* for 5 min, and the supernatants were stored at -20 C until hormone determination.

Enzyme immunoassay

The pGH levels in the culture media were measured with a homologous enzyme immunoassay procedure described previously (43, 44).

cAMP measurement

Dispersed pituitary cells were plated in 6-well tissue culture plates at a density of 2×10^6 cells/well in 2 ml of MEM-FBS as described elsewhere for measurement of intracellular cAMP accumulation (44, 46). After 3 d of culture in MEM-FBS, cells were incubated for 30 min in MEM containing 1 mM IBMX to prevent enzymatic degradation of cAMP. Thereafter, cells were incubated for an additional 30-min period in MEM-IBMX in the presence or absence of 10^{-8} M ghrelin and/or 10^{-8} M GHRH. Medium was then aspirated and wells were scraped in 0.01 M PBS with 4 mM EDTA. Aliquots for determination of protein were removed, and samples were sonicated (3 min), boiled (5 min), and centrifuged (10,000 rpm, 5 min). The supernatant was removed and stored at -20 C until cAMP determination by a competitive radioreceptor assay according to the instructions of the commercial supplier.

$[Ca^{2+}]_i$ measurement

For $[Ca^{2+}]_i$ studies, dispersed anterior pituitary cells were plated on microgrid coverslips coated with poly-L-lysine (0.25 mg/ml) at a density of 50,000 cells/coverslip in 2 ml MEM-FBS. After 3–4 d of culture, analysis of $[Ca^{2+}]_i$ dynamics in single cells was evaluated by microfluorimetry as described previously for porcine pituitary cells (47, 48). Briefly, on the day of Ca^{2+} measurements, cells were loaded with the fluorescent dye indo-1 (acetoxymethylester form; 5 μM) and Pluronic F127 (0.02%) in culture medium for 30 min at 37 C in the dark and then maintained for a second 30-min period in MEM alone to allow hydrolysis of indo-1. $[Ca^{2+}]_i$ was monitored by a dual-wavelength microfluorimetry system (Nikon Corp., Tokyo, Japan). Fluorescence emission of indo-1 induced by excitation at 355 nm was recorded at two wavelengths (405 and 485 nm) by separate photometers, and both 405- and 485-nm signals and the 405/485 ratio were continuously monitored by a software FASTINCA 1.03 (Nikon Corp.). $[Ca^{2+}]_i$ was calculated from the formula established by Grynkiewicz *et al.* (49), using the calibration constants previously established for porcine pituitary cells (47, 48). Ghrelin (10^{-5} M) was ejected at 4-sec pulses in the vicinity of randomly selected cells with the aid of a low-pressure ejection system. To counteract the rapid dissolution of the peptide in the medium during the pulse (44), the concentration of ghrelin tested was higher than that employed in GH release experiments. After $[Ca^{2+}]_i$ measurements, somatotropes in the coverslips were identified by immunocytochemistry using an antibody against ovine GH as described elsewhere (47, 48), and recorded cells were localized on the alphanumeric grid of the coverslips.

Statistical analysis

Data are expressed as the mean $\pm$ SEM of the number of experiments indicated in each figure. A minimum of three or four replicate wells per

treatment were tested in each experiment for cAMP measurements and secretion experiments, respectively. To avoid variability between experiments, samples from each experiment were analyzed in the same assay and expressed as a percentage of the corresponding control value. A one-way ANOVA followed by a statistical test for multiple comparisons (Duncan's multiple range test and critical ranges) were applied to compare experimental treatments. For Ca^{2+} experiments, a paired Student's *t* test was used. Statistical analysis was assessed by the program Statistica for Windows (Statsoft Inc., Tulsa, OK). Differences were considered significant at $P < 0.05$.

Results

Effect of ghrelin on GH release

Incubation of cultured porcine pituitary cells with increasing doses of ghrelin for 30 min revealed that the peptide stimulated basal GH release from somatotropes at a concentration equal to or above 10^{-10} M (Fig. 1A; $P < 0.05$, n = 5). In particular, the three highest doses of ghrelin tested (10^{-10} M, 10^{-8} M, and 10^{-6} M) caused significant, maximal increases of GH secretion. Accordingly, a stimulatory concentration of 10^{-8} M ghrelin was chosen to further analyze the action of the peptide on porcine somatotropes.

Interaction of ghrelin with regulators of somatotrope function

Comparison of the effect of ghrelin 10^{-8} M with that evoked by the same dose of GHRH demonstrated that both peptides induced similar increases of GH release (Fig. 1B; $P < 0.05$, n = 8). Concurrent exposure of porcine adenohypophyseal cell cultures to equimolar concentrations of ghrelin and GHRH induced neither an additive nor a synergistic increase in GH release when compared with the effects evoked by each peptide alone (Fig. 1B; $P < 0.05$, n = 8). Likewise, ghrelin and L-163,255 similarly increased GH release above control values (Fig. 1C; $P < 0.05$, n = 5), and when administered together the secretory response of porcine somatotropes did not significantly differ from that evoked by either compound alone (Fig. 1C). In addition, SRIF 10^{-7} M, which did not

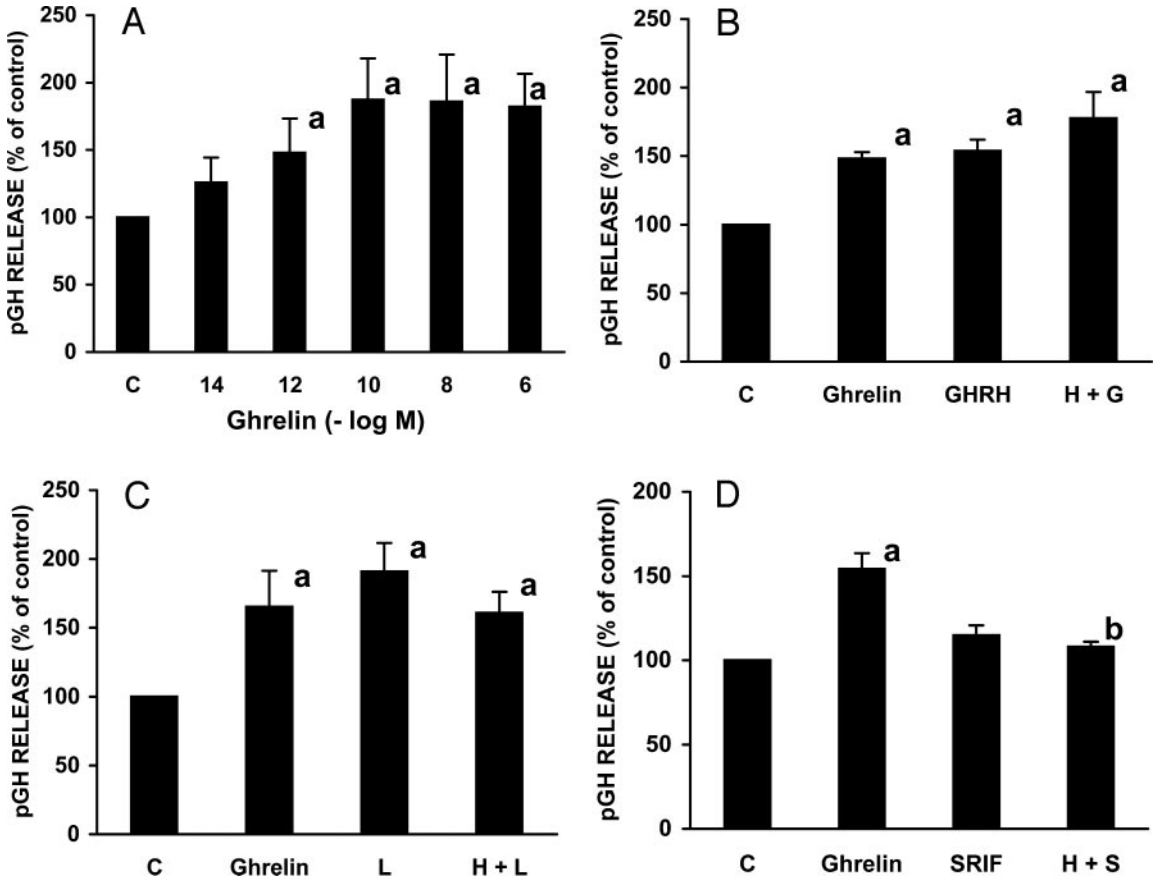

FIG. 1. Effect of ghrelin, alone (A) or in combination with GHRH (B), the synthetic GHS L-163,255 (L; C), or SRIF (D) on GH release from cultured porcine pituitary cells. After 3 d of culture in MEM-FBS, cells were equilibrated for 2 h in serum-free MEM and then incubated in the absence (C, control) or presence of the corresponding test substances for 30 min. At the end of the incubations, culture media were recovered and GH released was evaluated by enzyme immunoassay. Data are expressed as a percentage of basal values in control cultures (100%) and are the mean (±SEM) of the number of experiments indicated for each figure. At least four replicate wells were evaluated per treatment in each experiment. A, Secretory response of porcine somatotropes to increasing doses of ghrelin (10^{-14} to 10^{-6} M) (absolute value for control cultures 177.8 ± 38.8 ng GH/ml; n = 5). B, Response of porcine pituitary cells to treatment with ghrelin (10^{-8} M) or GHRH (10^{-8} M) alone or with a combination of both peptides (10^{-8} M each) (control value 46.6 ± 9.7 ng GH/ml; n = 8). C, Effect of ghrelin (10^{-8} M) or L-163,255 (L, 10^{-8} M) alone or in combination (10^{-8} M each) on GH release (control value 58.1 ± 13.8 ng GH/ml; n = 5). D, GH release from cultured pig pituitary cells after treatment with ghrelin (10^{-8} M) or SRIF (10^{-7} M) alone or with a combination of both peptides (10^{-8} and 10^{-7} M, respectively) (control value 50.0 ± 7.7 ng GH/ml; n = 4). a, $P < 0.05$ *vs.* corresponding control; b, $P < 0.05$ *vs.* ghrelin alone.

modify basal GH release by itself, completely blocked the stimulatory effect of ghrelin on GH release from porcine somatotropes (Fig. 1D; $P < 0.05$, n = 4).

Involvement of the PLC/PKC pathway in the response of somatotropes to ghrelin

Neither the specific inhibitor of PLC, U-73122, nor that of PKC, phloretin, had any effect on basal GH secretion from pig pituitary cell cultures (Fig. 2, A and B). In contrast, both compounds similarly abolished the secretory response of porcine somatotropes to 10^{-8} M ghrelin (Fig. 2, A and B; $P < 0.05$, n = 4).

Involvement of the AC/PKA pathway in the response of somatotropes to ghrelin

The presence of the specific inhibitor of AC, MDL-12,330A, suppressed the stimulatory effect caused by 10^{-8} M ghrelin on pig GH release, whereas the inhibitor alone did not modify basal GH secretion (Fig. 3A; $P < 0.05$, n = 4). Similarly, blockade of PKA activity by H89, which had no significant effect on the spontaneous secretory activity of porcine somatotropes, completely blunted ghrelin-induced GH release (Fig. 3B; $P < 0.05$, n = 4).

Effect of ghrelin on cAMP production

Treatment of porcine pituitary cultures with 10^{-8} M ghrelin significantly increased cAMP concentration with respect to control value (Fig. 4; $P < 0.05$, n = 5). Likewise, 10^{-8} M GHRH induced a significant increase in basal cAMP levels (Fig. 4; $P < 0.05$, n = 5). Moreover, when both peptides were administered together, intracellular cAMP levels were significantly higher than those evoked by either ghrelin or GHRH alone (Fig. 4; $P < 0.05$, n = 5). Specifically, simultaneous administration of ghrelin and GHRH to porcine pituitary cells in culture resulted in an additive increase in intracellular cAMP levels when compared

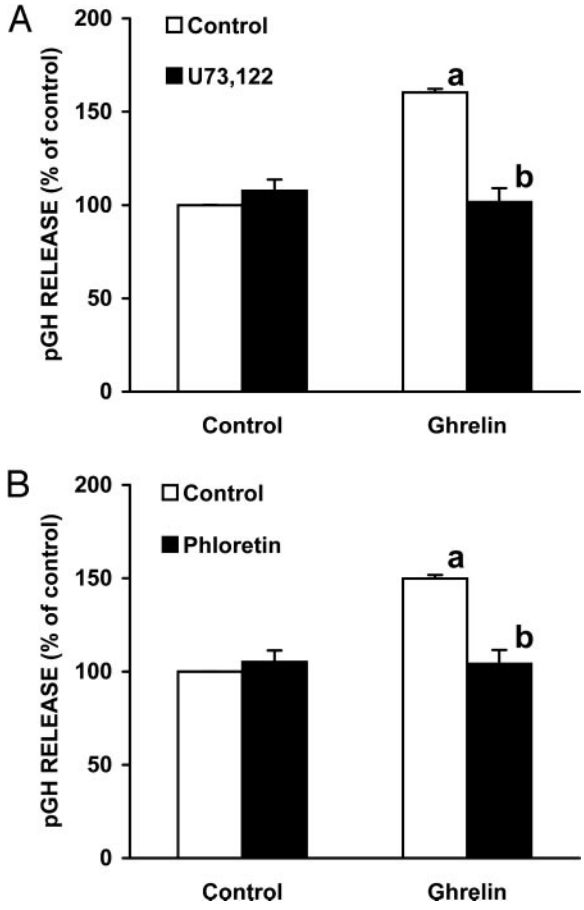

FIG. 2. Effects of inhibition of PLC or PKC on ghrelin-stimulated GH release. Dispersed porcine pituitary cells were cultured for a 3 d-period in MEM-FBS. On the day of the experiment, cells were equilibrated for 2 h in serum-free MEM and then incubated with or without (A) the PLC inhibitor U-73122 (5 mM) or (B) the PKC blocker phloretin (25 μM). Thereafter, cells were exposed to a 30-min challenge with 10^{-8} M ghrelin alone or in the presence of the corresponding inhibitor and GH released was evaluated. Inhibitors were added to the incubation medium 90 min before ghrelin treatment. Data are mean (±SEM) of four independent experiments, each performed in quadruplicate. a, $P < 0.05$ *vs.* corresponding control (100%; 18.5 ± 1.6 and 48.7 ± 2.5 ng GH/ml for A and B, respectively); b, $P < 0.05$ *vs.* ghrelin alone.

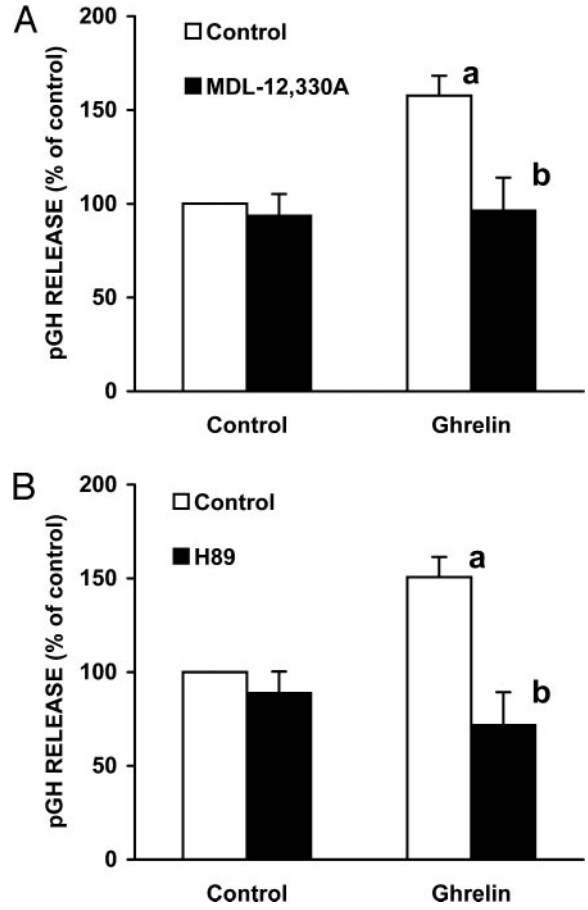

FIG. 3. Secretory response of porcine pituitary cells to ghrelin after blockade of AC or PKA. Cultures were treated for 30 min with ghrelin (10^{-8} M) alone or in the presence of (A) the AC inhibitor MDL 12,330 A (10 mM) or (B) the PKA blocker H89 (15 μM), and GH released into the culture medium was evaluated. Inhibitors were added to the incubation medium 90 min before ghrelin treatment. Data are mean (±SEM) of four independent experiments, each performed in quadruplicate. See Fig. 5 for further details. a, $P < 0.05$ *vs.* corresponding control (100%; 21.9 ± 4.5 and 99.9 ± 1.1 ng GH/ml for A and B, respectively); b, $P < 0.05$ *vs.* ghrelin alone.

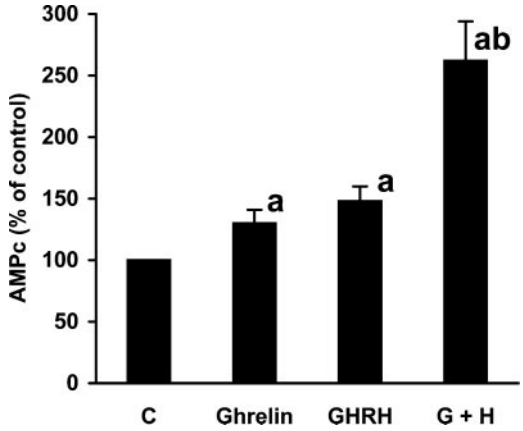

FIG. 4. Effects of ghrelin and/or GHRH on intracellular cAMP accumulation in cultured porcine pituitary cells. After 3 d of culture in MEM-FBS, cells were equilibrated for 2 h in serum-free medium and then incubated with 1 mM IBMX during 30 min. Then cells were challenged with either ghrelin (10^{-8} M) or GHRH (10^{-8} M) or a combination of both peptides (10^{-8} M each) in the presence of IBMX and incubated for 30 min. Thereafter, cAMP production was measured. Each *bar* represents the mean (±SEM) of five independent experiments, each performed in triplicate. Data are expressed as a percentage of the control value (C, 100%; 25.4 ± 3.1 pmol/mg protein). a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* ghrelin or GHRH alone.

with the effect caused by each peptide when administered alone.

Contribution of extra- and intracellular Ca^{2+} to the response of somatotropes to ghrelin

As shown in Fig. 5A, ghrelin-induced GH release was inhibited after blocking extracellular Ca^{2+} entry though L-type VSCC with nifedipine ($P < 0.05$, n = 4). Conversely, depletion of intracellular Ca^{2+} stores by thapsigargin had no effect on GH secretion, either alone or in combination with ghrelin (Fig. 5B; $P < 0.05$, n = 3).

Effect of ghrelin on $[Ca^{2+}]_i$

Microfluorimetric analysis of the response of single porcine pituitary cells to ghrelin showed that the peptide increased $[Ca^{2+}]_i$ in 87.5% of somatotropes, as identified by immunocytochemistry (14 of 16 cells; n = 3). In all responsive somatotropes, ghrelin induced a spike-type response (Fig. 6) characterized by a sharp increase in $[Ca^{2+}]_i$, which then rapidly returned to baseline. Specifically, $[Ca^{2+}]_i$ values increased from 192.7 ± 20.9 nM before ghrelin administration (basal $[Ca^{2+}]_i$) to 402.2 ± 51.4 nM (maximal $[Ca^{2+}]_i$; $P < 0.05$, n = 3) after a 4-sec pulse of ghrelin 10^{-5} M, thus resulting in a 124.5 ± 24.9% increase in $[Ca^{2+}]_i$ (n = 14).

Discussion

This study demonstrates for the first time that ghrelin stimulates GH release from porcine somatotropes through the activation of multiple signaling cascades, which include, at least, inositol phosphate, cAMP, and extracellular Ca^{2+}-dependent mechanisms. This indicates that ghrelin activates and requires a unique, complex set of intracellular signals to act on somatotropes, which is partially different from those employed by synthetic GHSs.

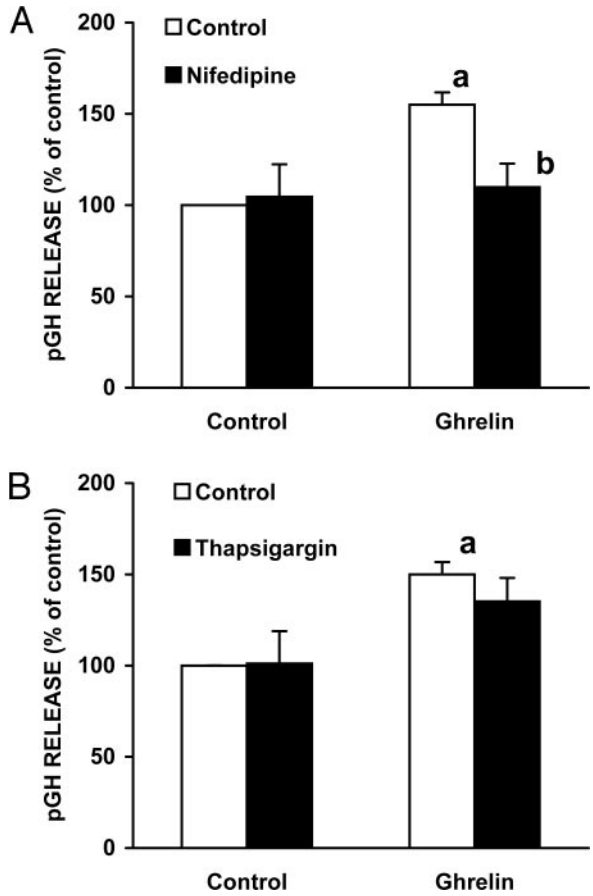

FIG. 5. Contribution of extra- and intracellular Ca^{+2} to the stimulatory effect of ghrelin on GH release from porcine somatotropes. Role of extracellular Ca^{+2} was investigated by incubating porcine pituitary cells in the presence of nifedipine (1 mM) alone or in combination with 10^{-8} M ghrelin (A), whereas the participation of Ca^{2+} stores from the endoplasmic reticulum was assessed in cultures of cells treated with thapsigargin (100 nM) alone or together with 10^{-8} M ghrelin (B). Cells were treated with the corresponding substances during 30 min and GH release was evaluated thereafter. Data are mean (±SEM) from four (for nifedipine) or three (for thapsigargin) experiments, each performed in quadruplicate (100%; 38.5 ± 1.6 and 20.9 ± 4.3 ng GH/ml for A and B, respectively). a, $P < 0.05$ *vs.* control; b, $P < 0.05$ *vs.* ghrelin alone.

The stimulatory effect of ghrelin on GH secretion from cultured somatotropes obtained from prepubertal female pigs was significant in a 10^{-10} to 10^{-6} M concentration range, which is comparable with the rank order of sensitivity reported for this peptide in somatotropes of other species (1, 18, 50) and is 2 orders of magnitude higher than that reported very recently for pig GH release in cells derived from barrows (*i.e.* castrated male pigs) (21). In addition, the GH-releasing potency of ghrelin on porcine somatotropes was similar to that observed for GHRH at an equimolar concentration (10^{-8} M), which is in agreement with that previously found for rat pituitary cells in static culture (1). In contrast, pig GH release from barrow-derived pituitary cell cultures was higher in response to GHRH than to rat or human ghrelin (21), thereby suggesting gender differences in the response of porcine somatotropes to GHRH and/or ghrelin, as has been previously described for other species (51). How-

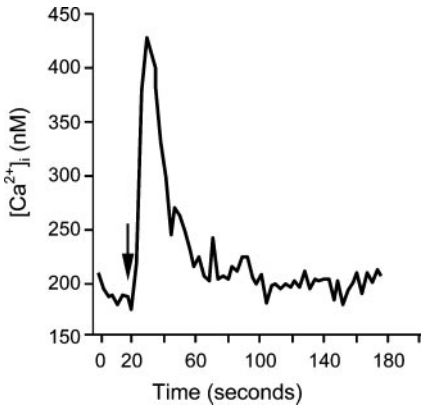

FIG. 6. Effect of ghrelin on $[Ca^{2+}]_i$ in cultured porcine somatotropes. The figure depicts a representative profile of the $[Ca^{2+}]_i$ rise induced by ghrelin in a single somatotrope identified *post facto* by immunocytochemistry. The *arrow* indicates onset of ghrelin (10^{-5} M; 4 sec) application.

ever, methodological differences may also contribute to these differences because 30-min incubations were used in our study, whereas 2-h incubations were used for barrow-derived cells. Our present results also demonstrate that ghrelin is as potent as the synthetic nonpeptidyl GHS L-163,255 in stimulating porcine GH release, which also agrees with previous data obtained after similar *in vitro* treatment of porcine pituitary cells with the prototype of peptidyl GHS, GHRP- 6 (52).

Previous results from our group (40, 52, 53, reviewed in Ref. 42) indicate that combined treatment of porcine pituitary cultures with GHRH and a GHS (either GHRP-6 or L-163,255) results in an additive stimulation of GH release. In contrast with this finding, when ghrelin was administered to porcine pituitary cells in culture together with GHRH, the amount of GH released by somatotropes was similar to that obtained in cultures treated with either peptide alone. This lack of additive or synergistic interaction between ghrelin and GHRH on GH release compares well with that reported for rat somatotropes in culture (50), in which the lack of interaction contrasted with the well-established additive or synergistic action exerted *in vitro* by several GHSs on GHRH-induced GH release in this species (54). On the other hand, our results clearly differ from the additive interaction described recently for ghrelin and GHRH on pig GH release in somatotropes from barrows (21).

As mentioned earlier, gender differences may account for the modest response of barrow somatotropes to ghrelin as well as for its distinct interaction with GHRH. These considerations notwithstanding, it seems clear that the lack of interaction between ghrelin and GHRH observed here or the moderate additive effects found in barrows (21) or between GHRP-6 and GHRH in somatotropes from prepubertal female pigs (52) is in striking contrast with the potent synergistic GH stimulatory response caused *in vivo* by coadministration of GHRH with synthetic GHS [*e.g.* pigs (55), rats (56), and human (54, 57)] or ghrelin [human (14), rat (58)]. Thus, when viewed as a whole, these results indicate that the pituitary somatotrope does not seem to be the primary direct site wherein GHRH and ghrelin (and, perhaps, synthetic GHSs) implement their strong, cooperative stimulation of GH release. In line with this notion, it has been shown that both ghrelin and GHSs exert at least part of their effects on GH release through a hypothalamic GHRH pathway. Indeed, central or peripheral administration of GHSs (59, 60) or ghrelin (61) in rats increases neuronal activity in the hypothalamic arcuate nucleus, in which GHRH-containing neurons expressing GHS-Rs are located (20, 62, 63). On the other hand, in pigs, central administration of the nonpeptidyl GHS L-163,255 seems to reduce SRIF release into the portal circulation (64). In fact, the stimulatory action of ghrelin on GH release *in vitro* is clearly blunted in the presence of SRIF in pigs (our current data and Ref. 21), as in rats (58), thus supporting the view that SRIF may antagonize the effects of ghrelin acting at the level of the pituitary gland (20).

Similar to that observed for GHRH, combined administration of ghrelin and the nonpeptidyl GHS L-163,255 to pig somatotropes did not stimulate GH release above the secretion levels induced by either compound alone. These results were not unexpected because similar data had been previously obtained from *in vivo* studies with the peptide and the GHS hexarelin in human (14). Moreover, this would be consistent with ghrelin and GHSs acting on somatotropes through the same GHS-R and/or intracellular signal transduction pathways. Consistent with this idea, ghrelin-induced GH release from porcine somatotropes was completely blocked in the presence of specific inhibitors of either PLC or PKC, thus suggesting that, as previously proposed for GHSs in swine (41, 42) and other species (32, 54), the PLC/PKC pathway is the primary signaling system mediating the stimulatory action of ghrelin on somatotropes. Despite this, we found that, besides this signaling cascade, the AC/PKA pathway is also required for ghrelin to increase porcine GH release because blockade of any of these enzymes fully suppressed the secretory response of somatotropes to the peptide.

These results differ from those observed previously on this same cell model for GHRP-6 or L-163,255 (reviewed in Ref. 42), which may help to explain the divergent interaction between these GHSs and ghrelin with GHRH (additive *vs.* nonadditive, respectively) on pig GH release. Interestingly, the contribution of AC/PKA to ghrelin-induced GH secretion in swine is reminiscent to that displayed by ovine somatotropes in response to the peptidyl GHS GHRP-2 (65, 66). Indeed, both MDL-12,330 and Rp-cAMP, a cAMP antagonist for PKA regulatory subunits, inhibited GHRP-2-induced GH release in enriched populations of ovine somatotropes (65). Moreover, GHRP-2 increased cAMP levels in ovine pituitary cell cultures (65). Likewise, our present results also revealed that ghrelin treatment increased cAMP production in cultures of porcine pituitary cells to levels comparable with those evoked by GHRH, in a similar manner as that found previously for GHRP-6 in this same cell model (41, 42). Furthermore, ghrelin acted additively with GHRH in increasing cAMP levels in porcine somatotropes, which is also in accordance with that reported for GHRP-6 in this species (41, 42) and GHRP-2 in sheep (65, 66).

Although the mechanisms underlying this additive interaction between ghrelin (or synthetic GHSs) and GHRH on the cAMP pathway in somatotropes is still unclear, a recent, elegant study by Cunha and Mayo (67) in HeLa-T4 cells

transfected with the GHS-R provides a plausible scenario wherein molecular interactions between this receptor and that of GHRH would play a major role (67). Taken together, these and our present results strongly suggest that the AC/PKA pathway could play a pivotal role in the response of somatotropes to both ghrelin and GHSs, either when acting alone or in combination with GHRH. In line with this notion are our findings demonstrating a lack of additive interaction between ghrelin and GHRH on GH release, which is likely related to the ability of both peptides to activate common signaling pathways in porcine somatotropes.

In somatotropes, as in most endocrine cells, activation of different signaling cascades eventually converges in causing a rise in $[Ca^{2+}]_i$ to induce hormone release. Consistent with this concept, we observed that ghrelin caused a potent increase in $[Ca^{2+}]_i$ in a high proportion of immunoidentified somatotropes. Furthermore, our data show that extracellular Ca^{2+} entry through L-type VSCC is essential for the secretory response of porcine somatotropes to ghrelin, whereas the Ca^{2+} released from thapsigargin-sensitive estrogen receptor stores does not contribute significantly to the stimulatory action of ghrelin on GH secretion in swine. When these results and those found here by microfluorimetric measurement of $[Ca^{2+}]_i$ in single somatotropes are analyzed together, it seems reasonable to suggest that the $Ca^{2+}{}_i$ rise observed after ghrelin administration results primarily from ghrelin-induced Ca^{2+} entry through L-type VSCC. This observation differs partially from that reported for synthetic GHSs on somatotropes from swine or other species (42, 68–72). Thus, both extracellular Ca^{2+} influx and mobilization of intracellular Ca^{2+} stores contribute to the stimulatory effect of L-163,255 (42) and GHRP-6 (69, 70) on GH release by porcine and rat somatotropes, respectively, yet the hexapeptide requires only external Ca^{2+} to exert its stimulatory effect on porcine GH release (42). Besides, in contrast to that found here for ghrelin, all the GHSs tested to date mostly induce a biphasic $Ca^{2+}{}_i$ rise consisting of a rapid increase in $[Ca^{2+}]_i$ followed by a sustained plateau phase above basal levels, although a lower proportion of somatotropes display single spike-type or plateau-type profiles in response to these compounds (42, 68, 71, 72). A recent report by Glavaski-Joksimovic *et al.* (73) has shown that ghrelin increases $[Ca^{2+}]_i$ already in somatotropes from neonatal pigs. In line with our findings on GH release, the stimulatory effect of ghrelin on $[Ca^{2+}]_i$ dynamics in somatotropes from neonates was shown to be dependent on extracellular Ca^{2+} as well as on both PLC and AC activation, thus supporting the view that multiple signaling pathways are required for ghrelin to stimulate somatotrope cell function.

In summary, our results show that the response of porcine somatotropes to ghrelin relies on the activation of three distinct systems of second messengers, including the AC/PKA, PLC/PKC, and extracellular Ca^{2+} systems. In this regard, one of the most intriguing results derived from our studies was the observation that blockade of any of these pathways completely abolished ghrelin-induced GH release, demonstrating the required participation of all of them for ghrelin to promote hormone secretion. This suggests that the three intracellular routes are functionally interdependent and that they might operate in somatotropes in a sequential manner. As an example, Ca^{2+} channel activation and subsequent extracellular Ca^{2+} entry could be the end point of the intracellular signaling system used by ghrelin because both PKA or PKC have been found to modulate VSCC function (39, 74, 75). The existence of cross-talk among signaling systems in somatotropes has also been described between Ca^{2+} and PKA, via calmodulin (76) as well as between PKA- and PKC-related pathways (65, 66). Thus, the cascade of intracellular signaling events driving GH release from pig somatotropes in response to ghrelin is more complex than previously envisioned. Experiments are underway in our laboratory aimed at elucidating the precise contribution of additional signaling components in this system as well as at clarifying the hierarchy of the mechanisms activated by ghrelin in this cell type.

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