Mechanism of action of the growth hormone secretagogue, L-692,585, on isolated porcine somatotropes

A Glavaski-Joksimovic¹, K Jeftinija¹, A Jeremic¹, L L Anderson^{1,2}
and S Jeftinija¹

Neuroscience Program, Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, 1098 Veterinary Medicine Building, Ames, Iowa 50011, USA

¹Department of Animal Science, Iowa State University, Ames, Iowa 50011, USA

(Requests for offprints should be addressed to S Jeftinija; Email: sjeftini@iastate.edu)

Abstract

The effects of a GH secretagogue, L-692,585 (L-585), and human GH-releasing hormone (hGHRH) on calcium transient and GH release were investigated in isolated porcine pituitary cells using calcium imaging and the reverse hemolytic plaque assay (RHPA). Somatotropes were functionally identified by the application of hGHRH. All cells that responded to hGHRH responded to L-585 application. Perfusion application of 10 μ M hGHRH and L-585 for 2 min resulted in an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) of 53 ± 1 nM (mean $\pm$ S.E.M.) ($P < 0.01$) and 68 ± 2 nM ($P < 0.01$) respectively. The L-585 response was characterized by an initial increase in $[Ca^{2+}]_i$ followed by a decline to a plateau level above the baseline. Concurrent calcium imaging with RHPA indicated that the L-585-evoked increase in $[Ca^{2+}]_i$ coincided with GH release. L-585 significantly increased the percentage of plaque-forming cells (24 ± 3 vs $40 \pm 6\%$; $P < 0.05$) and mean area of plaques (1892 ± 177 vs $3641 \pm 189 \mu m^2$; $P < 0.01$) indicating increased GH release. Substance P (SP) analogue ([D-Arg¹, D-Phe⁵, D-Trp^{7,11}]-SP) blocked, and the hGHRH receptor antagonist ((Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷,

D-Arg²⁸, Homoarg²⁹)-GRF (1–29) amide) decreased the stimulatory effect of hGHRH. These failed to block the stimulatory effect of L-585, suggesting a different receptor for L-585 from the GHRH receptor. The hGHRH-induced calcium transients and initial peak increase induced by L-585 were significantly decreased by removal of calcium from the bathing medium or the addition of nifedipine, an L-calcium channel blocker. The plateau component of L-585-induced calcium change was abolished by removal of calcium and nifedipine. These results suggest an involvement of calcium channels in GH release. Either SQ-22536, an adenylate cyclase inhibitor, or U73122, a phospholipase C (PLC) inhibitor, blocked the stimulatory effects of hGHRH and L-585 on $[Ca^{2+}]_i$ transient, indicating the involvement of adenylate cyclase-cAMP and PLC-inositol triphosphate pathways. These results further suggested that calcium mobilization from internal stores during the first phase of the L-585 response induced an increase in $[Ca^{2+}]_i$ whereas calcium influx during the second phase is a consequence of somatotrope depolarization.

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Introduction

Growth hormone (GH) secretion is controlled by hypothalamic stimulatory- and inhibitory-releasing hormones, GH-releasing hormone (GHRH) and GH release-inhibiting hormone or somatostatin (SS). GH also participates in its own rhythmic secretion through feedback action on GHRH and SS neurons (Chan *et al.* 1996). Pulsatile GH secretion can be stimulated and amplified by novel compounds known as GH secretagogues (GHS) (Casanueva & Dieguez 1999). The mechanism of the action of GHS is not fully established. GHS-elicited GH secretion involves both a direct effect and an indirect effect

on the hypothalamus, suggesting that exogenous GHS may induce the release of another hypothalamic factor with GH-releasing capabilities (U-factor) (Bowers *et al.* 1991, Casanueva & Dieguez 1999). Cloning of the human GHS receptor (Howard *et al.* 1996) and the isolation and characterization of an endogenous GHS, designated as ghrelin (Kojima *et al.* 1999), implicates GHS as a new physiological system in growth regulation by stimulating feeding and release of GH (Nakazato *et al.* 2001).

The GH-releasing peptides (GHRPs) were the first identified compounds in the class of GHS. Bowers *et al.* (1991) reported the discovery of a series of peptides derived from Leu- and Met-enkephalins that specifically

released GH from the pituitary gland of humans and other animals. The biological action of GHRPs was considered different from GHRH. Benzolactam and spiroindolamine GHS have been developed with improved oral bioavailability and pharmacokinetic properties (Smith *et al.* 1993, 1997); L-692,429 was the first described non-peptidyl GHS (Smith *et al.* 1993). L-692,585 (L-585) is a non-peptidyl GHS 10- to 20-fold more potent than L-692,429, as based on *in vitro* and *in vivo* studies and with no detectable change in receptor affinity (Jacks *et al.* 1994, Schoen *et al.* 1994). The GHS activity of L-585 has been reported in several species including beagles (Jacks *et al.* 1994), sheep (Guillaume *et al.* 1994) and swine (Hickey *et al.* 1996). *In vivo* experiments have shown that GHS in combination with GHRH augments GH release, and it is suggested that L-585 acts directly on somatotropes causing GH release (Smith *et al.* 1993). Besides direct effects on somatotropes, L-585 exerts central effects and requires an intact hypothalamic-pituitary axis for optimal GH release (Fairhall *et al.* 1996, Hickey *et al.* 1996). Experiments in the pig *in vivo* have shown that L-585 also stimulates GH secretion by acting in combination with GHRH to interrupt the inhibitory tone of SS (Hickey *et al.* 1996).

The present studies have examined the signal transduction mechanism of the GHS, L-585, on calcium transient and GH release from isolated porcine somatotropes. An understanding of the molecular mechanisms by which GHS modulate GH secretion is of particular interest because of their biochemical simplicity and efficacy of oral administration.

Materials and Methods

Chemicals

Fetal bovine serum, horse serum (HS), minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Earle's balanced salt solutions (EBSS) and guinea pig complement were purchased from Gibco-Invitrogen Co. (Carlsbad, CA, USA). HEPES, ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), papain solution, trypsin inhibitor, penicillin-streptomycin solution, poly-L-lysine, L-cysteine, L-glutamine, sodium pyruvate, bovine serum albumin (BSA), nifedipine, and staphylococcal protein-A were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Ovine red blood cells were purchased from Colorado Serum (Denver, CO, USA). Glucose, sodium bicarbonate (NaHCO_3), paraformaldehyde, and chromium chloride were obtained from Fisher Chemical (Fair Lawn, NJ, USA). Fura 2/AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). The ABC kit was obtained from Vector Laboratories (Burlingame, CA, USA). U73211 and SQ-22536 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). SS, SP analogue (D-Arg¹, D-Phe⁵, D-Trp^{7,11})-SP,

and GHRH antagonist (Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹)-GRF (1-29) amide (human) were obtained from Bachem California, Inc. (Torrance, CA, USA). Human (h) GHRH and anti-porcine GH antibody were gifts from Dr A F Parlow, NIDDK National Hormone and Pituitary Program (Torrance, CA, USA). L-585 was a gift from Dr G J Hickey, Merck Research Laboratories, Rahway, NJ, USA.

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, were killed with electrical shock and decapitated. Pituitary glands were immediately removed. The total number of animals used was 32. The pituitary glands were collected in cold sterile EBSS solution (4 °C). Anterior lobes were transferred to a sterile cold (4 °C) MEM-0.1% BSA medium. Primary cell cultures from neonatal anterior pituitary gland were established using a modified method of Huettner & Baughman (1986). Tissues from two animals were incubated for 50 min at 37 °C in 2 ml EBSS-papain solution (1.54 mg/ml). After incubation, the tissue was rinsed with EBSS solution and incubated for 5 min in 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 in DMEM-0.1% BSA medium by triturating through a fire-polished glass pipette. The undigested tissue was allowed to sediment. The supernatant containing cells was removed and filtered through a sterile filter. Cells were plated onto poly-L-lysine (0.1 mg/ml; 100 000 kDa)-coated glass coverslips (at a density of 2×10^5 cells). Cells were allowed to attach to coverslips and, after 3-4 h, DMEM-0.1% BSA medium was exchanged with DMEM medium supplemented with 10% HS and 1 ml penicillin-streptomycin solution per 100 ml medium. Cultures were maintained at 37 °C in a humidified 5% CO₂/95% air atmosphere. Experiments 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, cells were incubated for 30 min in a 50% goat serum solution containing 1% BSA and 100 mM L-lysine to block non-specific binding and 0.4% Triton

X-100 to permeabilize the membrane. To stain the somatotropes, cultures of anterior pituitary gland were incubated in polyclonal anti-porcine GH antibody (dilution 1:50 000). Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) and the nickel-enhanced 3,3' diaminobenzidine method (Jeftinija *et al.* 1992).

Intracellular calcium imaging

The effect of secretagogues on intracellular calcium concentration $[Ca^{2+}]_i$ was evaluated by ratiometric imaging techniques (Parpura *et al.* 1994). Cells were loaded with Fura 2-AM for 40–60 min at 37 °C. One microliter of 25% (w/w) of Pluronic F-127 was mixed with 4 nM of AM ester to aid solubilization of the ester into aqueous medium. Coverslips containing pituitary cells were washed with HEPES-buffered solution and further incubated for 10 min at 37 °C to allow de-esterification of Fura 2-AM. All image processing and analyses were performed using an Attofluor system (Atto Bioscience, Rockville, MD, USA) with a Zeiss microscope. Background subtraction and ratio images were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz *et al.* (1985). Using wavelengths of 340 and 380 nm, Fura 2-AM was excited and the emitted light was collected at 520 nm.

Reverse hemolytic plaque assay (RHPA) for measurement of GH release in culture medium

The RHPA was established according to the method of Taylor & Clark (1994). Staphylococcal protein-A was coupled to ovine red blood cells using 0.1% $CrCl_3$. After dissociation, pituitary cells were incubated for 48 h to allow them to recover biological responsiveness. After incubation, the cells were briefly exposed to trypsin (2.5 mg trypsin in 10 ml MEM–0.1% BSA) to detach them from the culture dish. Following gentle trituration by a fire-polished glass pipette, the cells were separated by centrifugation (1500 *g* for 10 min), washed twice in MEM–0.1% BSA, and resuspended in DMEM–0.1% BSA. The working cell dilution was 2.5×10^5 cells/ml. Equal amounts of the single secretory cell suspension and a 50% protein-A-labeled erythrocyte solution were mixed. The mixture was infused into Cunningham slide chambers and incubated at 37 °C in 95% air/5% CO_2 for 50 min. Pretreatment of the glass surface with poly-L-lysine (0.1 mg/ml; 100 000 kDa) ensured anchorage of the cells. After incubation, the chambers were rinsed with DMEM–0.1% BSA. The monkey anti-porcine GH serum, diluted 1:60 in DMEM–0.1% BSA, was then infused into the chambers alone or with L-585 (100 nM). The optimal incubation period was established by a preliminary study in which the maximum percentage of plaque-forming GH cells was achieved at 3 h. Plaque formation was initiated by infusion of guinea pig

complement in a final dilution of 1:40 in DMEM–0.1% BSA, and the reaction was terminated after 50 min by the infusion of 1% solution of glutaraldehyde. Omission of any of the assay reagents, i.e. antiserum, complement, protein-A, resulted in failure to form a hemolytic plaque.

In every experiment, each treatment (with secretagogue or vehicle) was run in triplicate. Three separate experiments were performed. On each slide, the percentage of plaque-forming cells (% PFC) was determined by counting the number of plaques among 200 pituitary cells. The mean plaque area (MPA) of 100 plaques/slide was measured by using a calibrated ocular reticule. The total secretion index, representing integrated hormone secretion per 100 pituitary cells, was calculated by multiplying the % PFC and MPA (Goth *et al.* 1992, Mitani *et al.* 1996).

Combination of RHPA and measurement of $[Ca^{2+}]_i$

A 1:1 mixture of protein-A-conjugated ovine red blood cells and pituitary cells was incubated for 1 h in Cunningham slide chambers, and then loaded with Fura 2-AM for 40 min at 37 °C. Fura 2-AM was washed from the chamber with DMEM–0.1% BSA. Twenty minutes later the presence of pituitary cells was confirmed under the fluorescent microscope. Calcium measurements were performed using consecutive excitation at 340 and 380 nm. After recording a baseline of $[Ca^{2+}]_i$ for 60 s, anti-porcine GH antibody and secretagogue (10 μ M L-585) were both introduced into the chamber. Changes in $[Ca^{2+}]_i$ were monitored for 15 min. After recording, cells in the chambers remained on the microscope for 3 h. Complement was introduced into the chamber, leading to lysis of red blood cells adjacent to GH-secreting pituitary cells. These plaques retrospectively identified the somatotropes.

Statistical analysis

Independent data are expressed as means $\pm$ S.E.M. Simple comparisons between two groups were made by Student's *t*-test. Multiple comparisons were carried out by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered as statistically significant at $P < 0.05$.

Results

Morphological identification of somatotropes in culture

Somatotropes in pituitary cell culture were confirmed by immunocytochemical staining. GH immunoreactive cells comprised 40% of the total pituitary cells in cultures. Somatotropes were approximately 10 μ m in diameter (Fig. 1A).

Functional identification of somatotropes in culture

Changes in the intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) mediate a variety of biological responses in both

A

GH positive cells

B

C

D

excitable and non-excitable cells. GH cells were functionally identified by a $[Ca^{2+}]_i$ increase above the baseline following hGHRH application. Perfusion with hGHRH (10 μ M) for 2 min significantly increased $[Ca^{2+}]_i$ by 53 ± 1 nM (means $\pm$ S.E.M.; $n=578$; $P<0.01$; Fig. 1B). Of the cells that responded to hGHRH, all (100%) also responded to L-585, which was applied 10 min after the application of hGHRH. Perfusion with 10 μ M L-585 for 2 min produced a prompt transient increase in $[Ca^{2+}]_i$, followed by the sustained decline to a plateau above the basal level. The initial increase in intracellular calcium reached a peak of 68 ± 2 nM ($n=578$; $P<0.01$) approximately 80 s after the onset of L-585 application (Fig. 1B). Potassium at a concentration of 50 mM, a non-selective stimulus, elevated $[Ca^{2+}]_i$ by 53 ± 1 nM ($P<0.01$) in all cells that responded to hGHRH and L-585 (Fig. 1B). Some cells only responded to the (non-specific) stimulation with 50 mM K^+ whereas others failed to respond to any stimuli (Fig. 1B).

L-585 induced release of GH in a dose-dependent manner

To demonstrate that calcium transient in cultured pituitary cells after application of L-585 coincided with GH secretion, we performed experiments where RHPA and $[Ca^{2+}]_i$ measurements were done concurrently. Hemolytic plaques were formed only around cells that on calcium imaging responded to L-585, which identified them as GH-secreting somatotropes (Fig. 1C).

Before quantitatively studying the effects of L-585 on GH release, preliminary experiments were performed in which the maximum effective dose (ED_{max}) of L-585 for GH secretion was determined. When incubated with 10 nM, 100 nM, and 10 μ M L-585, cultured pig somatotropes dose-dependently released GH with the ED_{max} of 100 nM. Using RHPA, the % PFC, and their MPA after 3 h incubation, were quantified. A total secretion index (TSI) was calculated by multiplying % PFC and MPA. Under basal conditions, GH plaques were formed in $24 \pm 3\%$ of the pituitary cells in culture obtained from newborn pigs. The similar % PFC (about 30%) under basal conditions was observed in experiments with rat somatotropes (Niimi *et al.* 1994, Mitani *et al.* 1996). The average size of the GH plaques in control cultures treated with vehicle was $1892 \pm 177 \mu m^2$. Incubation with 100 nM L-585 significantly increased the number and size of

plaques. The fraction of somatotropes forming plaques was increased to $40 \pm 6\%$ ($P<0.05$; Fig. 2A) and the MPA was 1.92-fold greater than controls ($3641 \pm 189 \mu m^2$; $P<0.01$; Fig. 2B). L-585 also caused a significant increase in TSI ($\times 10^{-2}$) (vehicle 5 ± 1 vs 13 ± 2 ; $P<0.01$; Fig. 2C).

The stimulatory effect of hGHRH and L-585 is receptor mediated

To study the nature of the GHS effect on somatotropes, ligands were applied in succession. The second application of 10 μ M hGHRH caused an increased $[Ca^{2+}]_i$ in 63% of the cells (60 of 96) that responded to the first application of hGHRH, and 59% of cells (57 of 96) that responded to the first application of 10 μ M L-585 responded to repeated application of L-585. A second application of 10 μ M hGHRH and 10 μ M L-585 did not have an additive effect on the increase in $[Ca^{2+}]_i$ (32 ± 2 vs 20 ± 1 nM; 58 ± 4 vs 25 ± 3 nM; Fig. 3B). Similarly, simultaneous application of hGHRH and L-585 in a concentration of 10 μ M also did not have an additive effect on the increase of $[Ca^{2+}]_i$ (57 ± 4 nM; $n=77$; Fig. 3C) but a sustained plateau phase of calcium increase was prolonged compared with either agonist alone. In parallel run controls, average increase in $[Ca^{2+}]_i$ after application of 10 μ M GHRH and 10 μ M L-585 alone was 56 ± 3 nM and 66 ± 5 nM respectively ($n=74$; Fig. 3A).

To determine whether the effects of hGHRH and L-585 were receptor mediated we utilized a GHRH-receptor antagonist and an SP analogue with GHRH antagonistic properties. The application of 10 μ M GHRH-receptor antagonist (Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹)-GRF (1–29) amide (human) for 10 min significantly decreased the rise in $[Ca^{2+}]_i$ evoked by hGHRH compared with controls run in parallel (68 ± 8 nM, $n=27$ vs 47 ± 3 nM, $n=68$; $P<0.01$; Fig. 4A and B). In contrast, the application of GHRH-receptor antagonist did not influence the stimulatory effect of L-585 (80 ± 11 nM, $n=27$ vs 80 ± 8 nM, $n=70$; Fig. 4B). SP analogue ([D-Arg¹, D-Phe⁵, D-Trp^{7,11}]-SP), at a concentration of 10 μ M, blocked the stimulatory effect of hGHRH (53 ± 2 nM, $n=48$ vs 0 nM, $n=46$) but it did not influence the stimulatory effect of L-585 (75 ± 4 nM, $n=48$ vs 71 ± 4 nM, $n=44$; Fig. 4C). These findings

Figure 1 Morphological and functional identification of cultured porcine somatotropes. (A) Cells stained with anti-GH antibody in cultured porcine pituitary cells. Magnification $40\times$. (B) The sequence of color images and kinetics of the $[Ca^{2+}]_i$ changes illustrate the response of isolated pituitary cells to application of 10 μ M hGHRH, 10 μ M L-585 and 50 mM K^+ . The GH-secreting cells (red cross and red line in the time-course histogram) responded to the application of hGHRH, L-585, and 50 mM K^+ . The non-GH-secreting cells responded to non-specific stimulation of 50 mM K^+ (green cross and green line in the time-course histogram) and non-excitable cells (possible stellate cells) failed to respond to any of stimuli (blue cross and blue line in the time-course histogram). (C) Calcium transient induced by L-585 coincides with release of GH. Bath application of L-585 induced calcium transient in one of two cells illustrated in (A) and (B) images and in the time-course histogram in (D). Phase contrast images in (C) illustrate formation of plaque only around the cell that responded with calcium transient to L-585 application in (B).

demonstrated that hGHRH and L-585 increase intracellular calcium concentration by acting through different receptors in cultured porcine somatotropes.

SS dose-dependently decreased the stimulatory effect of hGHRH and L-585

Applications of SS at concentrations of 5 and 10 μM decreased intracellular calcium levels by 15 ± 2 and 26 ± 3 nM respectively (Fig. 4D). The blocking effect of SS on the hGHRH- and L-585-evoked increase in $[\text{Ca}^{2+}]_i$ was dose dependent. Perfusion application of 10 μM SS for 5 min before the application of 10 μM hGHRH and 10 μM L-585 abolished the stimulatory effect of both agonists (Fig. 4D). After pretreatment of the cultures with 5 μM SS for 5 min, 59% of cells (26 of 44) responded to 10 μM hGHRH, and 57% of cells (25 of 44) responded to 10 μM L-585, while in parallel run controls 94% of cells (47 of 50) responded to the application of the agonists. In the presence of 5 μM SS, the increase in $[\text{Ca}^{2+}]_i$ evoked by hGHRH was significantly smaller compared with controls (44 ± 3 vs 25 ± 4 nM; $P < 0.01$), while the peak increase of $[\text{Ca}^{2+}]_i$ produced by L-585 was not significantly decreased (70 ± 6 vs 59 ± 6 nM).

Role of membrane depolarization and calcium channels in the hGHRH- and L-585-stimulatory effect

To investigate the role of extracellular calcium in hGHRH- and L-585-induced calcium transient in cultured somatotropes, cells were bathed in calcium-depleted HEPES with the addition of 1 mM EGTA to yield an estimated free extracellular calcium level of 26 nM. We found that it was necessary to have some calcium in the bathing medium, otherwise cells detached from the culture substrate. Consistent with the ability of ligands to

Figure 2 Treatment with L-585 increases GH secretion from isolated porcine somatotropes determined by RHPA. (A) % PFC in dispersed anterior pituitary cells when exposed for 3 h to vehicle (control) or 100 nM L-585. The results are expressed as the mean $\pm$ S.E.M. for three separate plaque assays. Two hundred pituitary cells were counted per assay ($*P < 0.05$ vs vehicle). (B) The MPA of somatotropes in RHPA described in (A) are shown on phase contrast images (a) and as histograms (b). (a) Monolayers prepared from the same protein-A-labeled ovine erythrocytes (small cells) and somatotropes (large cells) derived from 2-day-old pigs. The cultures were treated with control medium and L-585 100 nM. The cultures were incubated with GH antibody (1:60) for 3 h, exposed to complement (1:40) for 50 min, then fixed in 1% glutaraldehyde for 50 min. Phase contrast images show the increase in size of plaques (pale areas in the monolayer of red blood cells) formed by somatotropes (oval cells in the center of each plaque) treated with L-585. (b) The MPA of somatotropes are expressed as the mean $\pm$ S.E.M. of the MPA of 100 plaque-forming cells from three separate plaque assays ($**P < 0.01$ vs vehicle). (C) The TSI of somatotropes in RHPA described in (A) is calculated by multiplying PFC and MPA ($**P < 0.01$ vs vehicle).

Figure 3 hGHRH and L-585 induce calcium transient in isolated porcine somatotropes. (A) Increase in $[Ca^{2+}]_i$ after application of 10 μ M hGHRH, 10 μ M L-585, and 50 mM K^+ ($n=51$). (B) The second application of 10 μ M hGHRH and 10 μ M L-585 did not have an additive effect on the $[Ca^{2+}]_i$ increase ($n=57$). (C) Simultaneous application of 10 μ M hGHRH and 10 μ M L-585 did not cause an additive effect on the increase in $[Ca^{2+}]_i$ ($n=58$).

activate calcium channels, the stimulatory effect of hGHRH and L-585 was significantly decreased in calcium-depleted saline (Fig. 5A and B). In parallel run control experiments in normal Ca^{2+} HEPES, 88% of cells (70 of 80) responded to 10 μ M hGHRH and L-585. Only 11% (9 of 81) of cells responded to 10 μ M hGHRH in low Ca^{2+} HEPES. The average increase of $[Ca^{2+}]_i$ was 22 ± 7 nM, which is significantly less ($P<0.05$) than that evoked with hGHRH in normal Ca^{2+} HEPES (52 ± 4 nM). In contrast, 52% (42 from 81) of the cells

responded to 10 μ M L-585 in low Ca^{2+} HEPES (Fig. 5B). The amplitude of L-585-evoked transient increase in $[Ca^{2+}]_i$ in low calcium HEPES was significantly smaller than in normal Ca^{2+} HEPES (67 ± 5 vs 34 ± 2 nM; $P<0.01$), and the second, sustained phase of calcium increase was almost abolished (Fig. 5B). These results suggested that extracellular calcium has an important role in the effect of hGHRH and L-585 on GH-secreting cells.

It has been demonstrated that GHRP-induced depolarization of somatotropes can activate voltage-dependent calcium channels and result in an increase of $[Ca^{2+}]_i$ (Herrington & Hille 1994). To investigate the contribution of voltage-dependent Ca^{2+} channels in calcium influx evoked by hGHRH and L-585, experiments were performed in the presence of nifedipine, an antagonist of the L-type calcium channel. In control cultures, 10 μ M hGHRH and 10 μ M L-585 evoked a response in 94% of the cells (136 of 144). Application of 10 μ M hGHRH, in the presence of nifedipine, evoked a response in 88% of the cells (134 of 152) and Ca^{2+} transient in those cells was significantly decreased (55 ± 2 vs 25 ± 1 nM; $P<0.01$; Fig. 5C). In the presence of 10 μ M nifedipine, 91% of the cells (139 of 152) responded to 10 μ M L-585. The average increase in $[Ca^{2+}]_i$ was significantly smaller (70 ± 3 vs 47 ± 2 nM; $P<0.01$; Fig. 5C), and the duration of the sustained phase of calcium increase was brief.

To determine the role of Na^+ in hGHRH- and L-585-induced calcium transients, cultures were bathed in zero Na^+ solution (sodium was replaced with choline). Application of 10 μ M hGHRH in normal HEPES solution in parallel run control experiments evoked an increase in $[Ca^{2+}]_i$ in 92% of the cells (126 of 137). In zero Na^+ HEPES, 71% of the cells (112 of 157) responded to 10 μ M hGHRH, and the average amplitude of calcium increase was 19 ± 1 nM, a significantly smaller response compared with parallel run controls of 58 ± 2 nM ($P<0.01$; Fig. 5D). Similarly, 84% of the cells (132 of 157) responded to 10 μ M L-585 in zero Na^+ HEPES with 49 ± 3 nM increase in $[Ca^{2+}]_i$; the effect was significantly smaller compared with parallel run controls (61 ± 3 nM; $P<0.01$; Fig. 5D). In zero Na^+ HEPES, the effect of 50 mM K^+ on calcium transient was 40% of that recorded in normal HEPES (51 ± 2 vs 21 ± 1 nM; $P<0.01$; Fig. 5D). These results further suggest an involvement of Na^+ -dependent depolarization in calcium transients induced by both hGHRH and L-585.

Signal transduction pathways, PLC, and adenylate cyclase-cyclic adenosine 3,5-monophosphate (AC-cAMP) are activated by GH secretagogues

Results with the application of L-585 in low Ca^{2+} solution suggest that in L-585-induced GH secretion, extracellular Ca^{2+} has an important role but that intracellular Ca^{2+} has some role too. The involvement of intracellular Ca^{2+} stores in hGHRH and L-585 action was further investigated in

Figure 4 hGHRH and L-585 increased $[Ca^{2+}]_i$ in isolated porcine somatotropes acting through different receptors. (A) Control effect of 10 μ M hGHRH, 10 μ M L-585, and 50 mM K^+ on calcium transient in isolated porcine somatotropes ($n=21$). (B) Application of GHRH receptor antagonist, Phenylac-Tyr¹, D-Arg², p-chloro-Phe⁶, Homoarg⁹, Tyr (Me)¹⁰, Abu¹⁵, Nle²⁷, D-Arg²⁸, Homoarg²⁹-GRF (1–29) amide (human), decreased the stimulatory effect of 10 μ M hGHRH, while it had no influence on the L-585 effect ($n=30$). (C) SP analogue abolished only the effect of hGHRH, but not that of L-585 ($n=44$). (D) Application of 10 μ M SS decreased $[Ca^{2+}]_i$ and blocked the stimulatory effect of hGHRH and L-585 ($n=10$).

experiments with U73122, a selective inhibitor of PLC (Smith *et al.* 1990). Application of 10 μ M U73122 for 10 min significantly decreased the effect of hGHRH on calcium transient (53 ± 3 nM, $n=75$ vs 14 ± 1 nM, $n=62$; $P<0.01$). Inhibition of PLC by 10 μ M U73122 also significantly reduced the effect of L-585 (77 ± 5 nM, $n=75$ vs 12 ± 1 nM, $n=110$; $P<0.01$), implying an involvement of the PLC–inositol triphosphate (IP_3) pathway (Fig. 6).

It has been shown that GHRH activates the cyclic AMP pathway (Cheng *et al.* 1989). To determine whether L-585 activates this pathway, cell cultures were pretreated with SQ-22536, an adenylyl cyclase inhibitor (Tamaoki *et al.* 1993). SQ-22536 blocked the stimulatory effect of hGHRH and L-585 in a dose-dependent manner. After a 10-min application of SQ-22536 in concentrations of 50, 100, and 200 μ M, the amplitude of $[Ca^{2+}]_i$ increase evoked by 10 μ M hGHRH was decreased 51% ($P<0.01$), 68% ($P<0.01$), and 72% ($P<0.01$) respectively (Fig. 7A).

Pretreatment with SQ-22536 at a concentration of 50 μ M decreased the stimulatory effect of L-585 to 41% ($P<0.01$), while SQ-22536 in concentrations of 100 and 200 μ M had a more prominent effect and decreased the stimulatory effect of L-585 to 55% ($P<0.01$) (Fig. 7B).

Discussion

Porcine pituitary cells have a regulated secretory pathway utilized by secretory vesicles that fuse with the plasma membrane in response to a physiological stimulus such as GHRH or L-585 (Cho *et al.* 2002). Results from our experiments with simultaneous measurements of RHPA and calcium transients have shown that L-585 evoked an increase in $[Ca^{2+}]_i$ that coincided with GH release. The amplitude of the $[Ca^{2+}]_i$ increase was greater after exposure to L-585 compared with hGHRH at the same micromolar concentration. GH secretion appears to be

Figure 6 PLC-IP₃ pathway is involved in the stimulatory effect of hGHRH and L-585 on cultured porcine somatotopes. (A) Control effect of 10 μM hGHRH, 10 μM L-585, and 50 mM K⁺ on calcium transient in isolated porcine somatotopes ($n=36$). (B) Pretreatment of the cultured porcine pituitary cells with 10 μM U73122 significantly decreased the stimulatory effect of hGHRH and L-585 ($P<0.01$; $n=18$).

directly related to intracellular free calcium concentration (Lussier *et al.* 1991a) and our calcium-imaging experiments indicate that L-585 was more potent than hGHRH in the rapid release of GH. Our earlier *in vivo* experiments in the pig have shown that L-585 was more efficacious than GHRH in releasing significantly greater amounts of

Figure 5 Influx of calcium is involved in the hGHRH- and L-585-induced calcium transient in isolated porcine somatotopes. (A) Control effect of 10 μM hGHRH, 10 μM L-585, and 50 mM K⁺ on calcium transient in isolated porcine somatotopes ($n=40$). (B) Depletion of Ca²⁺ from the bathing solution dramatically decreased the stimulatory effect of hGHRH and L-585 on isolated somatotopes ($n=5$). (C) Perfusion of culture with 10 μM nifedipine, a blocker of L-type Ca channels, significantly decreased the stimulatory effect of hGHRH, L-585, and 50 mM K⁺ on isolated somatotopes ($n=70$). (D) Removal of Na⁺ ions from the bathing solution significantly decreased the effect of 10 μM hGHRH, 10 μM L-585, and 50 mM K⁺ on calcium transient ($n=37$).

Figure 7 Application of SQ-22536 (SQ), an adenylyl cyclase inhibitor, dose dependently blocked the stimulatory effect of hGHRH and L-585. (A) Dose-dependent effect of SQ on hGHRH-induced calcium transient in isolated porcine somatotropes (* $P < 0.01$ control (hGHRH) vs hGHRH+SQ). (B) Dose-dependent effect of SQ on L-585-induced calcium transient in isolated porcine somatotropes (* $P < 0.01$ control (L-585) vs L-585+SQ).

circulating GH (Hickey *et al.* 1996). These *in vivo* experiments also showed that GHRH and L-585 have a synergistic effect on GH secretion (Hickey *et al.* 1996). Simultaneous application of hGHRH and L-585 in the present study, however, did not have an additive effect on intracellular calcium concentration in cultured porcine somatotropes.

SS abolished the effect of hGHRH and L-585, but at a lower concentration it was more effective in suppressing the stimulatory effect of hGHRH compared with L-585. The effect of SS can be mediated by an inhibition of cAMP formation (Chen *et al.* 1996) or via decreased Ca^{2+} influx as a result of an increase in K^+ conductance and hyperpolarization of somatotropes (Lussier *et al.* 1991b). GHS depolarize the plasma membrane of somatotropes by

inhibiting K^+ channels, and have been suggested to behave as functional antagonists of SS (Smith *et al.* 1997).

GHRH receptor antagonist and SP analogue with GHRH receptor antagonist properties did not influence the stimulatory effect of L-585, while they decreased and abolished the stimulatory effect of hGHRH on calcium transient, suggesting that L-585 and hGHRH act via different receptors on porcine somatotropes. Similarly, Smith *et al.* (1993) have shown that activity of L-692,429 can be blocked by both peptide and non-peptidyl antagonists of GHRP-6, but not by an antagonist of GHRH. Contrary to our results on cultured porcine somatotropes, the SP analogue in rats significantly reduced both GHRP-6- and L-692,429-stimulated GH response but had no effect in inhibiting the GHRH-stimulated GH response (Cheng *et al.* 1994).

The depletion of extracellular calcium greatly diminished but did not abolish the stimulatory effect of hGHRH and L-585 on porcine somatotropes. This suggests that both GHRH and L-585 mobilize Ca^{2+} from intracellular stores, but Ca^{2+} influx has a major contribution to calcium transient. In somatotropes, the major Ca^{2+} channels are the voltage-gated T- and L-types (Chen *et al.* 1990). Perfusion of porcine cells with nifedipine significantly decreased the effect of hGHRH and L-585. This indicates an involvement of L-type Ca^{2+} channels in calcium influx induced by hGHRH and L-585, and that influx of calcium is a crucial step in the action of GHS.

The stimulatory effect of hGHRH and L-585 was greatly attenuated in a zero Na^+ environment, suggesting that both ligands can depolarize somatotropes, at least in part, through sodium channels. GHRH transiently increases Na^+ and Ca^{2+} current while it decreases membrane K^+ conductance, which leads to depolarization and an influx of extracellular Ca^{2+} (Chen *et al.* 1994). Potassium channels may also be involved in the action of L-585, because electrophysiology studies showed that peptidomimetics blocked K^+ currents in somatotropes, resulting in a depolarization and electrical spiking to enhance Ca^{2+} entry through voltage-gated channels (Smith *et al.* 1997). In our experiments, the increase in $[Ca^{2+}]_i$ after applying K^+ was significantly decreased by nifedipine and in sodium-free solution, indicating that calcium and sodium channels were involved in the depolarization of porcine somatotropes.

In this study, the few cells that responded to hGHRH in low calcium HEPES likely were low density (LD) cells. The first phase of L-585-evoked calcium increase resulted from intracellular Ca^{2+} mobilization, whereas the second phase represents calcium influx, because in low calcium HEPES, in nifedipine and in zero sodium HEPES, this second, sustained phase was almost abolished. In rats and pigs, it has been demonstrated that two morphologically and functionally distinct somatotrope subpopulations exist with LD and high density (HD) cells (Lindstrom & Savendahl 1996, Ramirez *et al.* 1999). In pigs, blockade of

Ca^{2+} influx with CoCl_2 reduced the GHRH-stimulated GH secretion in both LD and HD somatotropes, while depletion of thapsigargin-sensitive intracellular calcium stores only decreased the secretory response to GHRH in LD cells (Ramirez *et al.* 1999).

Pretreatment of porcine somatotropes with SQ-22536, an adenylyl cyclase inhibitor, decreased the stimulatory effect of hGHRH and L-585 on calcium transient in a dose-dependent manner, implying that the binding of hGHRH and L-585 to their receptors activates adenylyl cyclase. GHRP-2 applied to ovine somatotropes dose dependently increased intracellular cAMP levels, whereas the GHRP-2-stimulated GH secretion was blocked by the inhibitor of adenylyl cyclase (MDL 12,330A) and by a cAMP-binding antagonist (Rp-cAMP). GHRP-2 did not increase cAMP levels in rat somatotropes, suggesting the existence of several subtypes of GHS receptor that are variably expressed in different species (Wu *et al.* 1996).

In our experiments, U73122, a PLC inhibitor, decreased the effect of hGHRH on calcium transient, indicating the involvement of the PLC/IP₃ pathway in the hGHRH action on porcine somatotropes. Ramirez *et al.* (1999) demonstrated that the adenylyl cyclase inhibitor (MDL-12,330A) abolished GHRH-stimulated GH release in both LD and HD subpopulations of porcine somatotropes, whereas U73122 only partially reduced this effect in LD cells. In the present investigation, U73122 decreased the transient increase in $[\text{Ca}^{2+}]_i$ evoked by L-585. Consistent with activation of this pathway were the observations that GHRP-6 and non-peptidergic GHS increased phosphoinositide turnover, and caused translocation of protein kinase C (Adams *et al.* 1995, Mau *et al.* 1995). The activation and interplay of several pathways of signal transduction mediate the effect of GHS in mobilizing Ca^{2+} , cAMP, protein kinase A and C, and phospholipase C (Smith *et al.* 1997, Muller *et al.* 1999).

In summary, L-585 in porcine somatotropes activates adenylyl cyclase and PLC pathways, suggesting that it may act through different receptors that are coupled to G_s and G_q . Activation of these different signal transduction pathways mobilize calcium from internal stores during the first phase of the L-585-induced increase in $[\text{Ca}^{2+}]_i$. A second prolonged phase due to calcium influx results from somatotrope depolarization by L-585 acting on Na^+ and K^+ channels, and the activation of calcium channels through different second messengers. RHPA of cultured porcine somatotropes confirmed a connection between the L-585-evoked mobilization of calcium and GH release.

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