

Subpopulations of Chicken Somatotropes with Differing Intracellular Calcium Concentrations Responses to Secretagogues

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Summary and Implications

Multiple secretagogues stimulate the release of growth hormone (GH). The present studies examined the ability of chicken somatotropes to respond to GH secretagogues with increased intracellular calcium concentrations ($[Ca^{2+}]_i$). It was hypothesized that there are subsets of the somatotrope population with different responsiveness to the various secretagogues. Avian somatotropes were identified and distinguished from other anterior pituitary cells, by their unique ability to respond to GH-releasing hormone with increased $[Ca^{2+}]_i$ with immunocytochemistry used as a post-hoc confirmatory test. Large increases in $[Ca^{2+}]_i$ (222 ± 16 nm) were evoked by thyrotropin-releasing hormone in only 73% of the somatotropes. Similarly, $[Ca^{2+}]_i$ was increased by perfusion with pituitary adenylate cyclase-activating peptide in 85% and by leptin but only in 51% of somatotropes. Ghrelin acutely increased $[Ca^{2+}]_i$ in only 21% of somatotropes. Perfusion with gonadotropin-releasing hormone elevated $[Ca^{2+}]_i$, but in only 40% of somatotropes. The kinetics of calcium transients and the magnitude of the response differed from those observed in the presumptive gonadotropes. It is concluded that there are subsets of the somatotrope population in the anterior pituitary gland with differences in their ability to respond to various secretagogues.

Introduction

Multiple secretagogues stimulate the secretion of growth hormone (GH) from the anterior pituitary gland including GH-releasing hormone (GHRH), ghrelin and pituitary adenylate cyclase-activating peptide (PACAP) together with, at least in some species: leptin (rat in vivo, pig in vitro), thyrotropin-releasing hormone (TRH) (cattle) and gonadotropin-releasing hormone (GnRH) particularly in fish (goldfish, tilapia). The present studies examine the response of individual somatotropes to secretagogues. The technique of loading pituitary cells with fura-2/AM followed by ratiometric imaging allows determination of secretagogue-induced changes in

intracellular calcium concentrations ($[Ca^{2+}]_i$) in individual cells.

What is unknown is the extent to which individual somatotropes can respond to multiple secretagogues. It was hypothesized that there are subpopulations of somatotropes that respond to some but not to all secretagogues. There is evidence in support of this. For instance, it has been reported that TRH-induced increases in $[Ca^{2+}]_i$ occur in only 34% of neonatal rat somatotropes while leptin increased $[Ca^{2+}]_i$ in 54% of pig somatotropes. The young chicken was employed as the model to examine this in view of the ability of its pituitary gland to respond to multiple GH secretagogues including GHRH, ghrelin, TRH and PACAP and that effects of secretagogues on the chicken somatotrope $[Ca^{2+}]_i$ have not been previously reported. These secretagogues increase GH expression by chicken pituitary cells in vitro. The effect of GnRH was examined in view of the presence of GnRH receptor expression in 30–60% of rat somatotropes.

Materials and Methods

Experimental animals

Chickens were used for these experiments. Broiler chickens were raised at the Iowa State University Poultry Center. 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

Chickens (2–4 weeks old) were killed by decapitation. The anterior pituitary glands were immediately removed and collected in cold sterile EBSS solution (4°C). The tissue was transferred to a sterile cold (4°C) DMEM-0.1% BSA medium. Primary cell cultures from anterior pituitary glands were established using a method previously employed in our laboratory with neonatal pig anterior pituitary tissue. 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). The cells were allowed to attach, and then after 3–4 h, DMEM-0.1% BSA medium was exchanged with DMEM medium supplemented with 10% horse serum and 1 ml penicillin-streptomycin solution per 100 ml medium. Cultures were maintained at 37°C in a humidified 5% CO₂/95% air atmosphere. Studies examining the effects of secretagogues on changes in the $[Ca^{2+}]_i$ in somatotropes were carried out after 2–4 days in culture.

Perfusion of Anterior Pituitary Cells

The coverslips with pituitary cultures were mounted into a 50- μ l perfusion chamber and washed in Hepes saline

containing: NaCl 140 mM, KCl 5 mM, MgCl₂ 2 mM, CaCl₂ 2 mM and Hepes 10 mM (pH 7.4).

Intracellular Calcium Imaging

The effect of secretagogues on the [Ca²⁺]_i in somatotropes was evaluated by ratiometric imaging techniques. The cells were loaded with fura-2/AM for 60 min at 37°C in the dark. After removing growing medium, cultures were incubated in medium prepared by adding 2 mM fura-2/AM (Molecular Probes) (2 μl) and 25% (w/w) Pluronic F-127 (Molecular Probes) (1 μl) to Hepes imaging saline containing NaCl 140 mM, KCl 5 mM, MgCl₂ 2 mM, CaCl₂ 2 mM, glucose 5 mM and Hepes 10 mM (pH 7.35) (1 ml). Image processing and analysis was 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. Data are expressed as the increase in [Ca²⁺]_i or peak height.

Immunocytochemistry

Immunocytochemistry was performed using rabbit antibodies raised against recombinant chicken GH. This was kindly provided by American Cyanamid Co. (Princeton, N.J., USA). Negative controls were processed as above but with the omission of the specific antiserum but the presence of normal rabbit serum.

Results

Identification of GH-Producing Cells/Somatotropes

Approximately 25% of anterior pituitary cells in culture responded to application of GHRH (10 μM) for 2 min with an increase in [Ca²⁺]_i. These cells were functionally identified as somatotropes, and distinguished from other pituitary cells by their unique ability to respond to GHRH with increased [Ca²⁺]_i. Somatotropes in pituitary cell culture were confirmed by immunocytochemical staining. Somatotropes were approximately 10 μm in diameter.

The effect of GHRH on calcium transient in isolated chicken somatotropes was dose-dependent. Application of 10 μM GHRH for 2 min increased [Ca²⁺]_i above baseline by 120 ± SEM. 6 nM (n = 137; p < 0.01). Perfusion of cells with 1 μM GHRH for 2 min increased [Ca²⁺]_i by 47 ± 6 nM (n = 55; p < 0.01); this being smaller (p < 0.01) than the response after application of 10 μM GHRH.

Changes in Intracellular Calcium in Response to TRH

Perfusion of the anterior pituitary cells with TRH (1 μM) for 2 min evoked large increases in [Ca²⁺]_i, both in cells that did not respond to GHRH and in somatotropes. Of the cells that responded to GHRH, 73% (69 of 95) responded to perfusion with TRH (1 μM) for 2 min with average increases in [Ca²⁺]_i by 222 ± 16 nM (p < 0.01; fig. 1a).

The second application of TRH, 10 min after the first application, caused an increased [Ca²⁺]_i in 28% of the somatotropes (6 of 21) that responded to the first application of TRH. Moreover, the magnitude of increase in [Ca²⁺]_i evoked by the second application of TRH was also markedly lower than that evoked with the first application, 92 ± 15 vs. 216 ± 26 nM (p < 0.05; fig. 1b).

Changes in Intracellular Calcium in Isolated Chicken Pituitary Cells in Response to GnRH

Similar to the situation with TRH perfusion, perfusion of isolated chicken anterior pituitary cells with GnRH resulted in increases of [Ca²⁺]_i both in cells that did not respond to GHRH and in somatotropes. Application of GnRH (1 μM) for 1 min increased [Ca²⁺]_i in 21% of the anterior pituitary cells (45 of 213) that did not respond to GHRH. The magnitude of the increase in [Ca²⁺]_i in cells that did not respond to GHRH was 267 ± 23 nM (p < 0.01). It is presumed that this represents stimulation of gonadotropes. Unexpectedly, GnRH stimulated an increase in [Ca²⁺]_i in 40% of the anterior pituitary cells (21 of 53) that responded to GHRH. The magnitude of increase in [Ca²⁺]_i in response to GnRH in somatotropes was 129 ± 12 nM (n = 21; p < 0.01), which was lower (p < 0.01) than the response in cells that did not respond to GHRH. In somatotropes, application of GnRH evoked a slower increase in [Ca²⁺]_i and there was delay of a few seconds between the peak response to GnRH in gonadotropes and somatotropes.

In somatotropes, the repeated application of GnRH 10 min after the first application produced an increase in [Ca²⁺]_i in all cells (12 of 12) that responded to the first application of GnRH. The average increase in [Ca²⁺]_i produced by the second application was significantly smaller than the increase evoked by the first application (132 ± 19 vs. 70 ± 9 nM; p < 0.01).

Changes in Intracellular Calcium in Response to PACAP

Perfusion of anterior pituitary cells with PACAP (1 μM) for 2 min increased [Ca²⁺]_i in 85% of the cells (17 of 20) that responded to GHRH. The magnitude of the [Ca²⁺]_i increase evoked by PACAP in somatotropes was 90.0 ± 16.0 nM (n = 17; p < 0.01; fig. 2).

Changes in Intracellular Calcium in Response to Ghrelin

Ghrelin perfused at 1 μM for 2 min increased [Ca²⁺]_i in 21% of the isolated chicken anterior pituitary cells (9 of 43) that responded to GHRH. The magnitude of the increase in [Ca²⁺]_i in cells that responded to GHRH (somatotropes) was 94 ± 12 nM (n = 9; p < 0.01). Ghrelin had no effect on any of the cells that did not respond to GHRH.

Changes in Intracellular Calcium in Response to Leptin

When leptin (1 μM) was perfused for 3 min, there was an increase in the [Ca²⁺]_i in 58% of the anterior pituitary cells (11 of 19) that responded to GHRH. The average increase in [Ca²⁺]_i evoked by leptin in cells that responded to GHRH was 83 ± 10 nM (n = 11; p < 0.01). Leptin had no effect on the cells that did not respond to GHRH.

Discussion

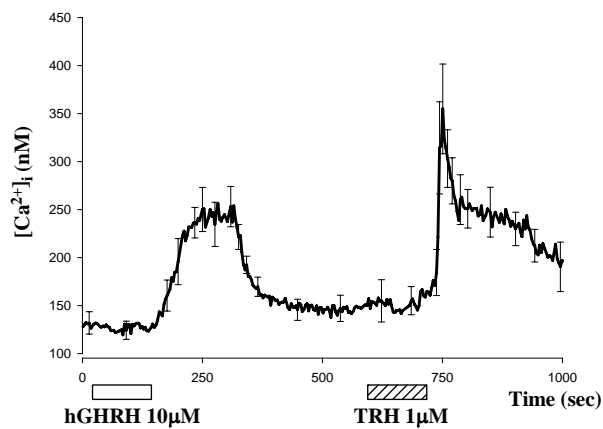
In the present studies, we have demonstrated that most, but not all, chicken GHRH-responding cells (somatotropes) also respond to other GH secretagogues, namely TRH, PACAP and leptin with increased $[Ca^{2+}]_i$. Of the anterior pituitary cells responding to GHRH, 73% also responded to TRH, 85% to PACAP and 58% to leptin. In contrast to the situation with TRH, PACAP and leptin, there was an increase in $[Ca^{2+}]_i$ in response to ghrelin in only 21% of the GHRH-responsive cells. The only cells that responded to PACAP, leptin or ghrelin also responded to GHRH, providing evidence for the response being somatotrope-specific. This is the first study to examine the ability of chicken somatotropes to exhibit changes in response to GH secretagogues with increases in $[Ca^{2+}]_i$.

The effect of GnRH on $[Ca^{2+}]_i$ in the somatotrope has not been previously examined. The ability of GnRH to evoke an increase $[Ca^{2+}]_i$ in somatotropes might have been unexpected in view of the paradigm that GnRH does not influence GH secretion in mammals.

It was noted that the increased $[Ca^{2+}]_i$ response to GnRH was delayed in some but not all somatotropes as opposed to the immediate response to GHRH, TRH, ghrelin and PACAP. The response may reflect communication between different cell types. There is an example of an analogous situation with stimulation of astrocytes by a glutamate agonist with increases in $[Ca^{2+}]_i$ in astrocytes being propagated from one cell to another. This may reflect Ca^{2+} -permeable gap junctions between pituitary cells and/or the release of paracrine factors.

In conclusion, the present studies provide evidence for the existence of several subsets of somatotropes with markedly different abilities to respond to secretagogues, particularly ghrelin. The physiological significance of subpopulations of somatotropes remains to be elucidated.

A



B

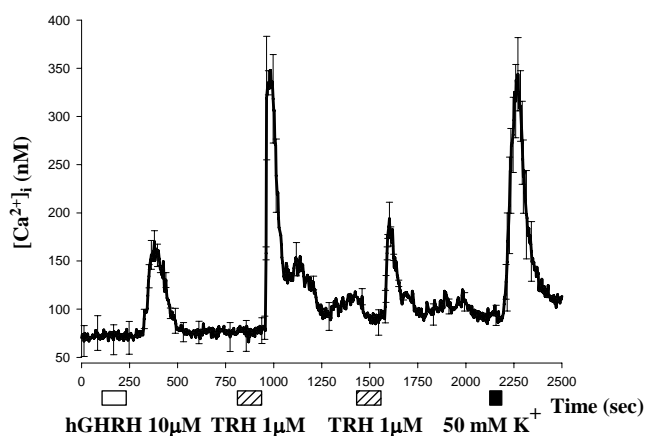


Figure 1. Stimulatory effect of TRH on $[Ca^{2+}]_i$ in chicken somatotropes. a Kinetics of the $[Ca^{2+}]_i$ changes illustrate the average response of isolated chicken pituitary cells to the 2-min perfusion application of 10 μ M GHRH and 2-min perfusion application of 1 μ M TRH. Cells that were functionally identified as somatotropes responded with calcium increase to the application of TRH ($n = 8$; $p < 0.01$). b Effects of GHRH (10 μ M), two sequential applications TRH (1 μ M), and 50 mM K^+ on calcium transient in isolated chicken somatotropes ($n=3$). Somatotropes were functionally identified by 2-min application of 10 μ M GHRH. Subsequent administration of 1 μ M TRH for 2 min evoked prompt increase in $[Ca^{2+}]_i$ in somatotropes. The repeated administration of TRH, 10 min after the first application of TRH, did not have effect on $[Ca^{2+}]_i$ in majority of somatotropes. In somatotropes that responded to the second application of 1 μ M TRH, the average increase in $[Ca^{2+}]_i$ was smaller ($P < 0.05$) than the response evoked by the first application of TRH.

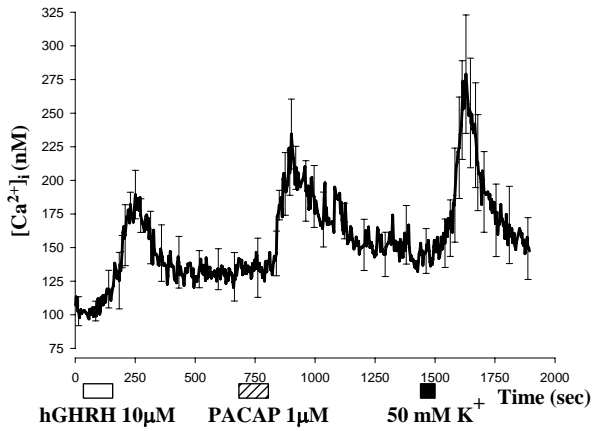


Figure 2. Stimulatory effect of PACAP on $[Ca^{2+}]_i$ in chicken somatotropes. Transient elevation of $[Ca^{2+}]_i$ after application of 10 µM GHRH, 1 µM PACAP and 50 mM K⁺. Cells that first were identified as somatotropes by $[Ca^{2+}]_i$ increase after the 2-min application of 10 µM GHRH, responded with increase in $[Ca^{2+}]_i$ to the 2-min perfusion application of 1 µM PACAP (n = 5; p < 0.01). All cells that responded to GHRH and PACAP also responded to 50 mM K⁺ (n = 5; p < 0.01).