A novel combination of two single cell assays allowed the simultaneous measurement of intracellular calcium concentration and hormone secretion in normal pituitary cells. ([Ca$$^{2+}$$]) was recorded using the fluorescent Ca$$^{2+}$$ indicator fura-2 and digital imaging microscopy. This technique was combined with a reverse hemolytic plaque assay for growth hormone in order to identify somatotropes and quantitate the amount of hormone released.

A dynamic profile of rhythmic calcium oscillations was found in spontaneously secreting somatotropes. Each somatotrope displayed a distinct frequency (one pulse every 5-30 s) and amplitude (range 50-450 nM) generated asynchronously from cell to cell. The amount of growth hormone (GH) released correlated directly with both the frequency and amplitude of calcium oscillations at the level of single GH cells. Furthermore, calcium excursions in somatotropes were rapidly suppressed by either (i) removal of extracellular calcium, (ii) somatostatin (1 nM), or (iii) the calcium channel blockers cobalt (2 mM) and verapamil (100 nM).

These observations demonstrate that spontaneous calcium oscillations are characteristic for normal somatotropes. These oscillations are related to spontaneous hormone secretion and due to influx through calcium channels in the membrane. Somatostatin, the physiologic inhibitor of GH secretion, suppresses calcium transients. These findings suggest that the intracellular signaling information may be encoded both in the frequency and amplitude of calcium oscillations.

Growth hormone secretion is under dual hypothalamic regulation by the stimulatory peptide, GHRH, and the inhibitory peptide, somatostatin (1). GHRH increases the generation of cAMP in somatotropes (2, 3); however, this second messenger alone cannot account for all cellular responses to stimulation or inhibition (4). There is indirect evidence for the additional involvement of calcium ions as a second messenger in these cells. For example, GH secretion is stimulated by calcium ionophores (5, 6) and inhibited by calcium channel blockers (7, 8). Calcium fluxes are affected by GHRH and somatostatin release-inhibiting hormone (somatostatin) (9, 10).

The precise role of the intracellular calcium concentration, ([Ca$$^{2+}$$]$$^i$$), in the somatocyte is not yet understood. In previous studies relatively homogeneous tumor cell lines have been used (11-13); however, major alterations of intracellular signaling pathways are recognized during oncogenesis (14-16). Furthermore, studies on whole populations are hindered by the mixture of at least five different cell types in the pituitary and average the response from many cells. Therefore they do not reflect the heterogeneity that has been found among single somatotropes (17, 18).

In order to investigate the role of calcium in normal somatotropes, calcium ions and hormone release were measured simultaneously in single pituitary cells obtained from normal tissue. [Ca$$^{2+}$$]$$^i$$ was monitored with high temporal resolution using the calcium-sensitive fluorescent dye fura-2 (19) and a digital imaging microscope (20). Somatotropes were identified using a reverse hemolytic plaque assay (RHPA), which determines the cumulative amount of GH secreted by single cells in culture (21). Therefore, in addition to identifying somatotropes, the RHPA quantitates hormone secretion, which can be compared directly with intracellular calcium determinations.

**EXPERIMENTAL PROCEDURES**

The methodology for the RHPA has been previously published in detail (22); this technique was modified to allow the simultaneous quantitation of intracellular calcium with fura-2 (23). Briefly, dispersed anterior pituitary cells from male rats were mixed with ovine red blood cells and plated as a monolayer in Cunningham chambers (24). Cells were loaded with fura-2, and GH antiseraum (25) was added. On an upright fluorescence microscope, simultaneous recordings from 8-15 pituitary cells were obtained and stored on videotape for subsequent digital image analysis. We found no evidence for intracellular compartmentalization or a calcium-insensitive form of the dye (26).

For dual-wavelength recordings, the ratio of the fluorescence intensities obtained with excitation at 340 and at 380 nm was calculated. This 340/380 ratio measures calcium in single cells essentially independent of dye content or cell thickness. Paired recordings could be made every 5 s. After background subtraction and spatial correction, 340/380 ratios were used to calculate [Ca$$^{2+}$$]$$^i$$. Portions of "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
A considerably higher temporal resolution, at the expense of absolute quantitation, could be achieved by continuous recordings with single-wavelength excitation (540 nm) and subsequent analysis at 150-300-ms intervals.

After calcium recordings were completed, complement was added which develops lysis zones around those cells that secreted GH. These plaques therefore retrospectively identify the somatotropes.

RESULTS AND DISCUSSION

Two subpopulations exist among somatotropes. 29% (range 27.2-30.6) of all pituitary cells spontaneously secreted GH. A further 20% of the cell population secreted GH after stimulation with a supramaximal dose (10 nM) of human GHRH 1-40, accompanied by a 4-fold increase in mean plaque size (Table I). Somatostatin greatly reduced the number of plaque-forming cells without affecting plaque size. Cytosolic calcium measurements in spontaneously active somatotropes revealed distinctive features when compared to control cells (cells not spontaneously secreting GH).

Rhythmic transients of [Ca\textsuperscript{2+}], with high amplitude were spontaneously generated in about 35% of all pituitary cells (Figs. 1 and 2). This pattern was predominantly found in the subgroup of spontaneously secreting GH cells; 81% of spontaneously plaque-forming somatotropes oscillated, compared to only 17% of control cells (p < 0.001, n = 118, X\textsuperscript{2} test). These control cells include both nonplaque-forming somatotropes (secretory rate below the detection limit of the RHPA) and cells belonging to the other cell types of the pituitary.

TABLE I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of secretory cells</th>
<th>Mean plaque size</th>
<th>Total secretion index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10\textsuperscript{6} pm\textsuperscript{2}</td>
<td>10\textsuperscript{6}</td>
<td></td>
</tr>
<tr>
<td>A) Basal</td>
<td>28.8 ± 1.1</td>
<td>5.9 ± 1.2</td>
<td>17.1 ± 3.5</td>
</tr>
<tr>
<td>B) GHRH (10 nM)</td>
<td>49.1 ± 5.5</td>
<td>24.9 ± 5.8</td>
<td>122.6 ± 41.1*</td>
</tr>
<tr>
<td>C) SRIF (1 nM)</td>
<td>5.0 ± 0.6</td>
<td>6.3 ± 1.5</td>
<td>3.1 ± 0.6*</td>
</tr>
<tr>
<td>D) Ca\textsuperscript{2+}-free medium</td>
<td>3.3 ± 0.3</td>
<td>6.3 ± 2.6</td>
<td>2.1 ± 1.0*</td>
</tr>
</tbody>
</table>

*Different from basal (Duncan's multiple range test, α = 0.01).

The sensitivity of the assay is adjusted so that both stimulation and inhibition of hormone secretion can be measured.

Future studies using RHPAs for different pituitary hormones will clarify the nature of these oscillating cells.

Further analysis showed that the frequency of these oscillations in the somatotrope ranged between 2 and 13 pulses/min (single wavelength monitoring from 27 somatotropes; Fig. 3A). Amplitudes between 50 and 450 nM were found (340/380 ratios measured every 5 s in 42 somatotropes). Additional evidence against technical artifacts is the finding that transients were timed asynchronously among GH cells and were still detected when cells had been cultured for up to 4 days (data not shown).

These results were further confirmed by the observation that the mean cytosolic calcium concentration under unstimulated conditions (mean ± S.E., nM) was significantly higher in the group of secreting somatotropes (238 ± 18; n = 42) compared to control cells (111 ± 8; n = 188, p < 0.01, Student's t test).

We next asked if there was a relationship between the calcium oscillations and the amount of GH released. Previous RHPA studies have demonstrated a remarkable heterogeneity in the amount of hormone secreted by apparently similar GH.
cells (17, 18). Three parameters (GH plaque area, frequency, and amplitude of calcium oscillations) were determined from a total of 27 secretory cells. GH plaque size directly correlated with the number of oscillations/min (Fig. 3A) as well as the magnitude of these calcium transients (Fig. 3B; p < 0.01). These findings suggest that the temporal organization of calcium rises may play an important role in GH exocytosis.

These calcium transients in the somatotropes were due to the influx of extracellular calcium, rather than mobilization from internal stores. Calcium oscillations were rapidly abolished when extracellular calcium was removed (n = 13, Fig. 4A). Spontaneous GH release is significantly inhibited in calcium-free medium (Table I). Furthermore, the effect was reproduced with inorganic or organic calcium channel blockers. Both cobalt chloride (2 mM, n = 16; Fig. 4B) and verapamil (100 μM, n = 15; data not shown) depressed spontaneous calcium oscillations. This finding provides further evidence for a central role of intracellular calcium in the GH secretory process, suggesting a link between calcium entry through ion channels in the membrane and hormone exocytosis.

Calcium transients were suppressed with a similar time course after exposing somatotropes to 1 mM somatostatin (Fig. 4C), a dose that significantly inhibited hormone secretion (Table I). We propose that the mechanism by which somatostatin inhibits GH secretion is to close those calcium channels responsible for spontaneous calcium transients. Further studies are needed to clarify whether this is a direct effect on calcium channels (27, 28) or indirectly mediated by hyperpolarization due to increased potassium conductance (29). This might explain results by numerous investigators that the action of somatostatin is not confined to the inhibition of hormone release (30, 31).

Oscillations in cytosolic free calcium have previously been reported in stimulated hepatocytes using aequorin as an indicator (33) and in mouse oocytes following fertilization or treatment with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (34). In contrast to these systems, where a stimulatory input induces intracellular oscillations, we report calcium oscillations that occur spontaneously, unless suppressed by somatostatin.

Repetitive spontaneous action potentials were found in both normal (35, 36) and neoplastic pituitary cells (37, 38). A recent study combining whole-cell patch-clamp recordings with fura-2 measurement of intracellular calcium demonstrated that action potentials precede spontaneous calcium rises in the cytosol (39). These results in the prolactin secretory GH3 tumor cell line cannot directly be extrapolated to normal tissue. We demonstrate here that (at least in male rats) calcium oscillations are primarily found in the somatotropes, a cell type that displays high spontaneous secretory rates after acute dispersion. The question whether spontaneous action potentials (40, 41) and calcium oscillations (42) are also found in lactotropes is complicated by the necessity for reliable cell identification, the occurrence of mammom- somatotropes (43), and possibly gender differences. Further investigation is therefore necessary.

Interestingly, pulsatile events are found not only at the level of single pituitary cells but also at the whole gland level; pulsatile hormone release has been reported in vivo in perfused pituitaries (44, 45) and in hypophysectomized animals with adenohypophyses grafted under the kidney capsule (46). In vivo, GH is secreted into the circulation in a pulsatile manner in all mammals studied so far (47, 48). These secretory episodes are under hypothalamic control.

Our findings provide evidence for a link between calcium oscillations and the secretory rate of individual cells. It has been proposed that the biological purpose of frequency-modulated signaling is to make the signal more resistant to noise (49). Our findings raise the possibility that components of cellular responses (e.g., synthesis, release) may be triggered by messenger signals coded in either analog or digital form.

Acknowledgments—We thank Drs. Wylie Vale and Jean Rivier, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA for their gift of human GHRH-(1–40)-OH. We appreciate the help of Dr. D. L. Kaiser with the statistical analysis.

REFERENCES


Fig. 4. Effect of calcium-free medium (A), calcium channel blocker (B), or somatostatin (C) on intracellular calcium in somatotropes. Single-wavelength excitation at 340 nm was used, and the medium surrounding the cells was exchanged after 2 min. The oscillatory pattern during base line was abruptly stopped and the mean cytosolic calcium level was reduced when (A) extracellular calcium was removed (Spinner's minimum essential medium + 1.5 mM EGTA), (B) the calcium channel blocker cobalt chloride (2 mM) or (C) somatostatin (1 nM) was delivered. Recordings are representative for more than 10 cells in each group. When assay medium alone was exchanged in the chamber, no change of the oscillatory pattern occurred (data not shown). SRIF, somatostatin release-inhibiting hormone (somatostatin).
Calum Oscillations in Growth Hormone Cells

Supplemental Material to

EXPERIMENTAL PROCEDURES

Antisera

Polyclonal antibodies were generated using a rabbit, and then purified using affinity chromatography. Upon elution from the column, the antibodies were dialyzed against phosphate-buffered saline. The antibody specificity was confirmed using immunoblotting and immunoprecipitation assays.

Preparation of Cells and Reagents

Rat pituitary cells were isolated from 10-week-old Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.). The cells were maintained in a mixture of DMEM/F12 media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). The cells were seeded at a density of 10^5 cells per well in 24-well plates and allowed to attach for 24 hours. After attachment, the cells were washed with PBS and incubated with 1% FBS-containing media for an additional 24 hours.

Modification of the calcium stress signal was performed as follows: Cells were treated with 10 nM of a calcium ionophore A23187 (Calbiochem) for 1 hour. After treatment, the cells were washed with PBS and loaded with 5 mM of fluo-3/AM (Molecular Probes) for 30 minutes at 37°C. The cells were then washed with PBS and imaged using a confocal microscope.

Videoimicroscopy and Digital Image Analysis

Fluorescence video-imicroscopy equipment consisted of a Nikon Diaphot microscope equipped with an Nikon 010 digital camera. Images were analyzed using the Nikon NIS-Elements software. The calcium transients were measured using a custom-written software to detect the peak fluorescence intensity of each cell. The calcium transients were then averaged over all cells in the field of view to determine the average calcium signal. The calcium transients were further analyzed using a custom-written software to determine the calcium signal strength, calcium signal duration, and calcium signal amplitude.

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