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THE SOMATOTROPE: AN ENDOCRINE CELL WITH FUNCTIONAL CALCIUM TRANSIENTS

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Summary

Growth hormone (GH) secretion by the somatotrope is under dual regulation by the hypothalamic peptides, somatostatin (SS) and GH-releasing hormone (GHRH). Cytosolic free calcium concentration and cumulative GH release were measured simultaneously in anterior pituitary cells from adult male rats. This was made possible using a combination of digital imaging video microscopy with the fluorescent calcium indicator Fura-2 and the reverse haemolytic plaque assay (RHPA) to identify the cell type and measure hormone secretion from the cells under study. This technique allows calcium measurements to be made at very short time intervals (<150 ms) in single cells. Spontaneous calcium transients were demonstrated in 85 % of GH plaque-forming cells. These occurred at a frequency of 2–13 min^{-1} and had an amplitude of 50–500 $nmol\,l^{-1}$. The somatotropes with the largest calcium fluctuations produced the largest plaques; thus, the calcium transients appeared to correlate with hormone release. Since the somatotrope alone shows these fluctuations, the mean intracellular calcium concentration is $238 \pm 18\,nmol\,l^{-1}$ in somatotropes and $113 \pm 8\,nmol\,l^{-1}$ in non-somatotropes. Upon exposure to SS (1 $nmol\,l^{-1}$) intracellular calcium fell from 200–250 $nmol\,l^{-1}$ to 50–100 $nmol\,l^{-1}$ with an apparent reduction in oscillations. Withdrawal of SS increased the intracellular calcium level. GHRH increased intracellular calcium but 10 $nmol\,l^{-1}$ GHRH given simultaneously with 1 $nmol\,l^{-1}$ SS reduced intracellular calcium to that level observed during SS alone. Thus, the SS effect on intracellular calcium predominates. The effects of SS can be mimicked by removal of extracellular calcium, or by the addition of $CoCl_2$ (2 $nmol\,l^{-1}$) or by verapamil (100 $\mu mol\,l^{-1}$), two agents which block calcium channels.

The hormone secretion index (indicated by the area of the plaque formed in RHPA) enables us to demonstrate that GHRH in this system increases GH secretion, and SS inhibits it. In combination, GHRH and SS oppose one another.

Spontaneous calcium oscillations are characteristic for normal somatotropes. These oscillations are related to spontaneous hormone secretion and due to influx of calcium through ion channels in the membrane. Intracellular signalling information may be encoded in both frequency and amplitude of calcium oscillations. The actions of GHRH and SS on regulation of GH secretion are

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proposed to be mediated, at least in part, by regulation of intracellular cytosolic free calcium. This modulation is dependent on extracellular calcium concentrations. We are now investigating the molecular mechanisms involved in this process.

Introduction

It is accepted that neuronal cells and cardiac pacemaker cells have spontaneous functional oscillations. Traditionally, endocrine cells have been considered as slaves to multiple, different inputs including hormonal, paracrine, autocrine and neuronal inputs. Whether these inputs are in the form of neurotransmitters or hormones, they act through binding to specific receptors on the cell surface membrane before their signal is transduced within the cell through second messengers. Until recently it has been generally accepted that a receptor is linked through a particular transduction pathway and only one second messenger or one cascade pathway is involved (e.g. cyclic AMP, InsP_3, calcium, diacylglycerol/protein kinase C pathway). This is reminiscent of the now disproved concept that a neurone only contains one neurotransmitter; it is widely accepted that neurones contain different peptide neurotransmitters in addition to catecholamines (Hokfelt *et al.* 1986). Thus, the complexity of regulation is much greater than had hitherto been thought.

It became evident, after several years of studying the somatotrope, that some characteristics of this cell type have a direct bearing on the above problems, notably: (1) the cell has separate receptors for stimulatory (GHRH) and inhibitory (somatostatin) hypothalamic regulatory hormones; (2) these two hormones act through multiple intracellular regulators; and (3) somatotropes spontaneously secrete GH after dispersion.

In vivo, growth hormone secretion is regulated by the growth-hormone-releasing hormone (GHRH) and the inhibitory peptide somatostatin (Tannenbaum & Ling, 1984). At the cellular level, GHRH acts in part by increasing the generation of cyclic AMP in the somatotrope (Brazeau *et al.* 1982; Cronin *et al.* 1983). This second messenger alone does not account for all the effects of GHRH (Spence, Sheppard & Kraicer, 1980). There is much evidence that calcium ions play a role as second messenger in the somatotrope. In addition, growth hormone secretion is stimulated by calcium ionophores and inhibited by calcium channel blockers (Cronin *et al.* 1985; Kracier & Chow, 1982; Bilezikjian & Vale, 1983; Szabo, 1986). Calcium fluxes are affected by GHRH and somatostatin (Login & Judd, 1986; Milligan, Kraicer, Fawcett & Illner, 1972).

There have been several problems in studying the precise role of intracellular calcium concentration in somatotrope function. Some studies have utilized homogeneous tumour cell lines, but major alterations of intracellular signalling pathways are known during oncogenesis (Berridge & Irvine, 1984; Hanley & Jackson, 1987; Michell, 1984). Studies utilizing cell lines cannot necessarily extrapolated to normal somatotrope functions (Gershengorn & Thaw, 1983;

Kruskal, Keith & Maxfield, 1984; Tan & Tashjian, 1981). For example, no GHRH receptors are found on GH_3 cells (J. Zysk & M. O. Thorner, personal observations). The mixture of at least five different pituitary cell types in the pituitary gland hinders the study of whole populations of anterior pituitary cells. Thus, the average response from many cells does not reflect what is going on at the level of the somatotrope. In addition, since somatotropes are heterogeneous in their secretory capacities, studies at any level higher than that of the single cell are likely to mask important observations in single somatotropes.

We have measured $[Ca^{2+}]_i$ and hormone release simultaneously in single pituitary cells obtained from normal tissue. Intracellular calcium concentration was monitored with high temporal resolution using the calcium-sensitive fluorescent dye Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) and a digital imaging microscope (Williams, Fogarty, Tsien & Fay, 1985). A reverse haemolytic plaque assay (RHPA) was used to identify somatotropes. This assay determines the cumulative amount of growth hormone secreted by single cells in culture (Neill & Frawley, 1983); a technique which not only identifies somatotropes, but also allows the quantification of hormone secretion that can then be compared with intracellular calcium determinations.

Calcium oscillations in somatotropes

49 % of pituitary cells from normal, adult, male rats are somatotropes. Two-thirds of these spontaneously secrete growth hormone, whereas the rest secrete growth hormone only after stimulation with GHRH ($10\,nmol\,l^{-1}$). Also, after treatment with GHRH, each cell secretes more hormone, as demonstrated by a four-fold increase in mean plaque size. This contrasts with the effect of somatostatin which greatly reduces the number of plaque-forming cells without affecting plaque size.

35 % of all pituitary cells demonstrate high-amplitude rhythmic transients of intracellular calcium (Fig. 1). This pattern was found mainly in the subgroup of cells spontaneously secreting growth hormone: 81 % of spontaneously plaque-forming somatotropes oscillated, compared with only 17 % of control cells. The frequency of these oscillations in the somatotrope ranges from 2 to $13\,pulses\,min^{-1}$. The amplitudes of these Ca^{2+} oscillations vary between 50 and $500\,nmol\,l^{-1}$.

The presence of these oscillations is confirmed by the observation that the mean cytosolic calcium concentration under unstimulated conditions (mean ± S.E.M., $nmol\,l^{-1}$) was significantly higher in the group of secreting somatotropes (238 ± 18; $N = 122$) than in control cells (111 ± 8; $N = 189$). To investigate the relationship between the calcium oscillations and growth hormone release, growth hormone plaque area and calcium oscillations (frequency and amplitude) were determined from 27 secretory cells. The size of the growth hormone plaque correlated directly with the frequency of oscillations and with the magnitude of the calcium transients. These observations suggest that the temporal organization of calcium

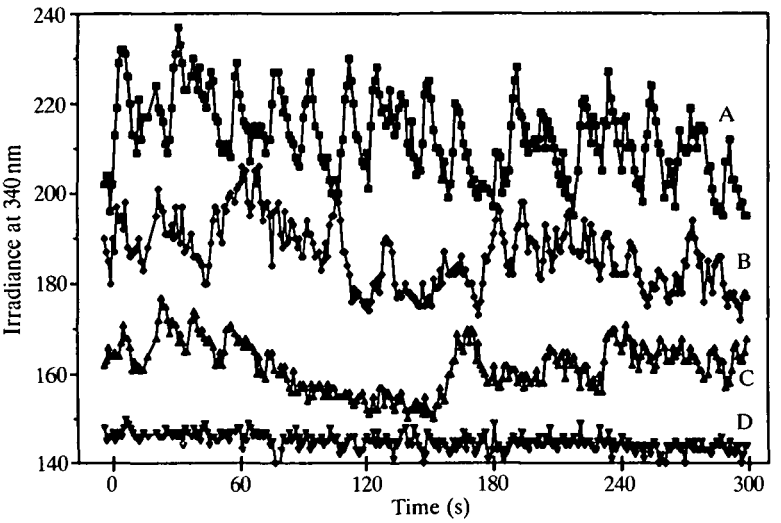

Fig. 1. Comparison of calcium oscillations in cells secreting different amounts of growth hormone (GH). Simultaneous recordings in real time from four cells in the same field. (A–C) Spontaneously active somatotropes; (D) control cell. The relative amount of GH secreted was determined by the area of each haemolytic plaque (μm^2): 6751 (A), 2257 (B) and 1142 (C). A computer program for pulse analysis objectively calculated 8·7 (A), 6·3 (B) and 3 (C) pulses min^{-1} for the somatotropes. No significant pulse was detected for the control cell. A direct relationship between both frequency and amplitude of calcium oscillations and the relative amount of GH secreted by each cell was established from 22 somatotropes in nine independent experiments.

rises may play an important role in growth hormone exocytosis. Studies in which cells were exposed either to reduced extracellular calcium concentration or to inorganic and organic calcium channel blockers indicate that these calcium transients in the somatotrope are due to influx of extracellular calcium. Furthermore, removal of extracellular calcium inhibits growth hormone secretion.

Repetitive spontaneous action potentials have been found in both normal (Israel, Denef & Vincent, 1983; Ozawa & Sand, 1977) and neoplastic pituitary cells (Biales, Dichter & Tischler, 1977; Kidokoro, 1975). Schlegel and colleagues have combined whole-cell patch-clamp recordings with Fura-2 measurement of intracellular calcium concentration (Schlegel *et al.* 1987). They demonstrated that action potentials precede spontaneous calcium rises in the cytosol. Unfortunately, these studies were performed in the GH_3 tumour cell line and cannot directly be extrapolated to normal tissue. We have demonstrated that calcium oscillations are primarily found in somatotropes from male rats. This cell type displays high spontaneous secretory rates after acute dispersion. These oscillations presumably have important functions. The biological purpose of frequency-modulated signalling is to make the signal more resistant to noise (Rapp, Mees & Sparrow, 1981). Our findings raise the possibility that components of cellular responses may be

triggered by messenger signals coded in either analogue or digital form or in a combination of the two.

Effects of GHRH and somatostatin

When cells were stimulated with 10 nmol l^{-1} GHRH, $[Ca^{2+}]_i$ increased from a basal level of 234 ± 17 to a peak concentration of 480 ± 61 nmol l^{-1} (Fig. 2).

This is a supramaximal dose of GHRH to stimulate growth hormone secretion, as demonstrated by the plaque assay. As with plaque areas, individual responses to GHRH were heterogeneous: calcium rises in single somatotropes varied from 50 to 800 nmol l^{-1}. On average, intracellular calcium concentration after GHRH stimulation gradually fell towards basal levels over 10 min and reached 50 % of peak levels at that time. When GHRH was removed 10 min after stimulation, $[Ca^{2+}]_i$ was reduced to the basal level. As with the spontaneous Ca^{2+} transients, the GHRH-induced increase in free intracellular calcium depended on calcium influx rather than mobilization of calcium from internal stores. The calcium rise

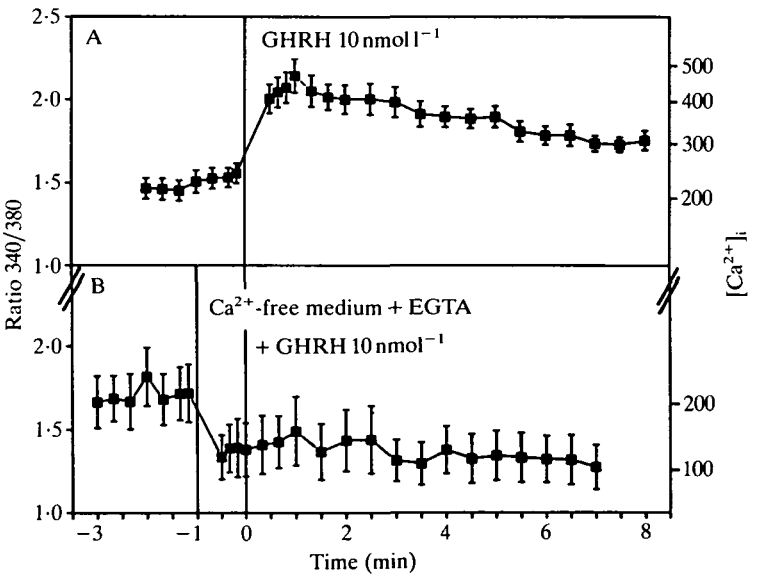

Fig. 2. (A) Growth-hormone-releasing hormone (GHRH) increases the free cytosolic calcium concentration in normal somatotropes. Baseline recording for 2 min, followed by addition of 10 nmol l^{-1} GHRH. Left axis, ratio of fluorescence intensities with excitation at 340 and 380 nm; right axis, free intracellular calcium concentration, $[Ca^{2+}]_i$. The vertical line indicates the replacement of fresh medium inside the chamber. Reproduced with permission from Holl, Thorner & Leong (1988) ($N = 21$). (B) GHRH-induced increase in $[Ca^{2+}]_i$ is dependent on extracellular calcium. Recording in normal medium for 2 min, followed by exchange to 'calcium-free' medium (+1·5 mmol l^{-1} EGTA). One minute later, GHRH in 'calcium-free' medium is delivered. Reproduced with permission from Holl, Thorner & Leong (1988). Values are means; bars indicate ±S.E. ($N = 11$).

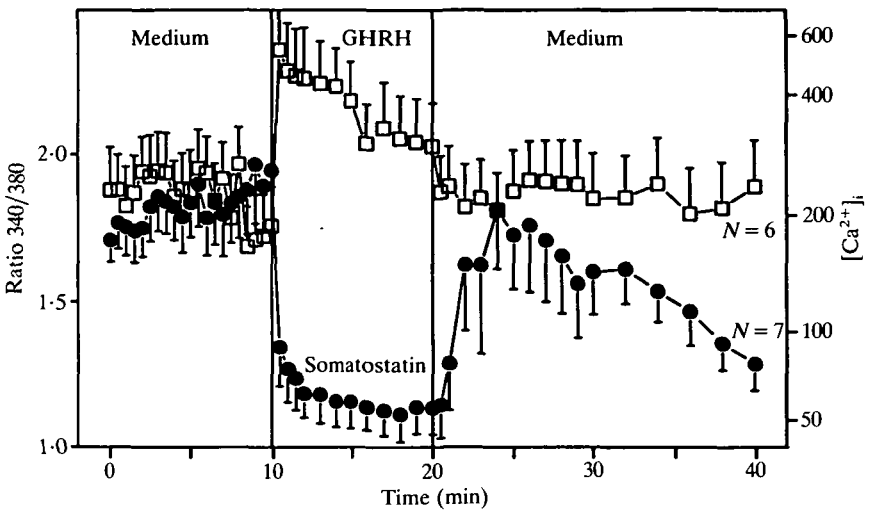

Fig. 3. Effect of growth-hormone-releasing hormone (GHRH) and somatostatin on cytosolic free calcium concentration in somatotropes. Recordings of $[Ca^{2+}]_i$ were made every 30 s. Basal recordings for 10 min, followed by the addition of either 10 nmol l^{-1} of GHRH or 1 nmol l^{-1} of somatostatin. After a further 10 min, the regulatory peptides were replaced with fresh medium. Data points represent the mean ± S.E. of a group of six or seven somatotropes, respectively, as identified by the reverse haemolytic plaque assay. The ratio of fluorescence with excitation at 340 and 380 nm is given on the left axis, the corresponding free intracellular calcium concentration is shown on the right axis. Reproduced with permission from Holl, Thorner & Leong (1988).

was completely abolished in the absence of extracellular calcium. Within 1 min of adding calcium-free medium (containing 1·5 mmol l^{-1} EGTA), no increase in $[Ca^{2+}]_i$ could be detected in 11 cells treated with GHRH (Fig. 2). These observations were substantiated with experiments using the calcium channel blockers cobalt chloride and verapamil.

When somatotropes were exposed to somatostatin for up to 30 min, a rapid decrease in $[Ca^{2+}]_i$ to 50 ± 100 nmol l^{-1} was observed and was sustained for as long as the somatostatin treatment was maintained (Fig. 3). 1 nmol l^{-1} somatostatin inhibited spontaneous growth hormone secretion and also decreased $[Ca^{2+}]_i$ to 50 ± 100 nmol l^{-1}. This response of intracellular calcium to somatostatin is more uniform in timing and extent, in contrast to the effects of GHRH. The results are reminiscent of those, described above, when cells were exposed to calcium-free medium or to calcium channel blocking agents. Upon removal of somatostatin, intracellular calcium level returned to baseline. If the same cells were treated with GHRH, the increase in calcium concentration was large and sustained. When cells were exposed to GHRH and somatostatin simultaneously, the effects of GHRH on growth hormone secretion were inhibited. The intracellular calcium measurements demonstrated a decrease in calcium concentration, identical to that in the presence of somatostatin alone (Fig. 4). 1 nmol l^{-1} somatostatin was, therefore,

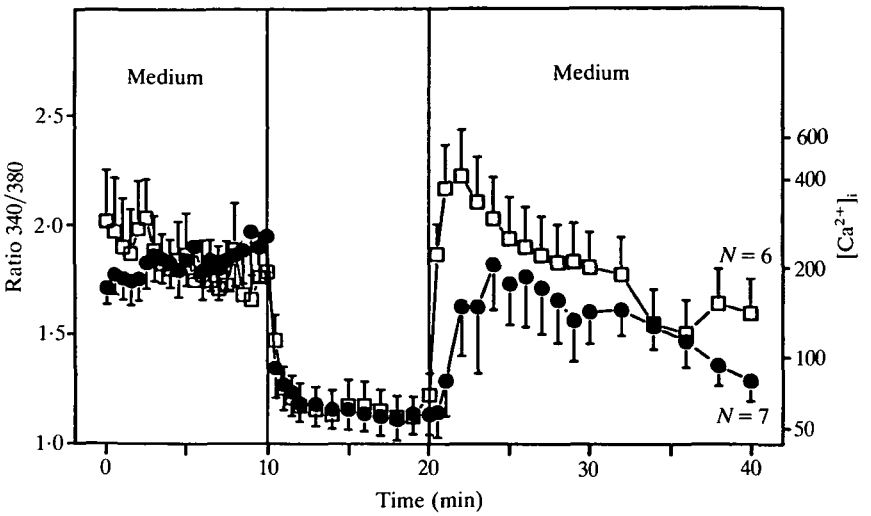

Fig. 4. Effect of somatostatin + growth-hormone-releasing hormone (GHRH) (□) compared with somatostatin alone (●) on intracellular calcium concentration in normal somatotropes. The experimental format is identical to that of Fig. 3. Somatostatin causes a similar decrease in $[Ca^{2+}]_i$ in the presence or absence of GHRH, but the calcium rebound after withdrawal is greater in cells previously exposed to GHRH. Reproduced with permission from Holl, Thorner & Leong (1988).

able to overcome completely the increases in $[Ca^{2+}]_i$ and growth hormone secretion induced by 10 nmol l^{-1} GHRH. After removal of the hypothalamic hormones, the calcium rebound was greater in those cells that had been exposed to both GHRH and somatostatin than in those exposed to somatostatin alone.

Intracellular pathways

In the third series of experiments, three non-physiological experimental manipulations were performed to evaluate the effects of stimulation of adenylate cyclase, protein kinase C, and alteration of membrane potential. The agents used were forskolin, which activates adenylate cyclase, phorbol dibutyrate (PDB), which activates protein kinase C, and high concentrations of potassium. Forskolin (5 μmol l^{-1}) increased growth hormone secretion and intracellular calcium concentration in a manner similar to GHRH. In cells exposed to forskolin and somatostatin simultaneously, the $[Ca^{2+}]_i$ decreased to the same level as observed after somatostatin alone. Cells exposed to PDB (100 nmol l^{-1}) demonstrated an increase in growth hormone release and an increase in $[Ca^{2+}]_i$. Co-incubation with PDB and somatostatin decreased $[Ca^{2+}]_i$. Cells exposed to increasing concentrations of extracellular potassium (from 5 to 60 mmol l^{-1}) showed a dose-dependent increase in $[Ca^{2+}]_i$. Somatostatin, however, could not overcome the effect of high potassium (60 mmol l^{-1}).

These observations can all be formulated into a hypothesis. The GHRH-induced increase of $[Ca^{2+}]_i$ in the somatotrope may be mediated by a cyclic-AMP-dependent phosphorylation of calcium channels which is a prerequisite for channel opening. In cardiac muscle cells, cyclic AMP has been shown to modulate calcium channels in the membrane and thereby increase calcium influx (Irisawa & Kokubun, 1983; Osterrieder *et al.* 1982; Reuter, Stevens, Tsien & Yellen, 1982). A similar observation has been made with pituitary cells (Armstrong & Eckert, 1987). The calcium channels in the pituitary cells belong to the same dihydropyridine-sensitive subclass (L-type). We therefore suggest that cyclic AMP generated in the somatotrope in response to GHRH leads to phosphorylation of calcium channels which allows their opening and thereby increases calcium influx. Our studies showing a requirement for extracellular calcium to sustain the increases in calcium level are fully consistent with this scheme.

The mechanism by which somatostatin reduces intracellular calcium concentration is less well understood. In preliminary studies, we have shown that the effect of somatostatin can be blocked by pretreatment of the cells with pertussis toxin (Holl, Hewlett & Leong, 1988). This indicates that an inhibitory G protein is involved in the mechanism of action of somatostatin. In both pituitary and pancreatic beta cells, somatostatin has been shown to increase potassium conductance, thereby leading to a hyperpolarization of the cell membrane, which in turn closes voltage-dependent calcium channels. This leads to a reduction of intracellular calcium concentration (Yatani *et al.* 1987; Koch, Blalock & Schonbrunn, 1988; Yamashita, Kojima, Shibuya & Ogata, 1987; Pace & Tarvin, 1981).

Cyclic AMP has been shown to be important in the regulation of transcription of the growth hormone gene (Barinaga *et al.* 1983; Simard, Labrie & Gossard, 1986). Somatostatin can only partially inhibit GHRH-induced cyclic AMP production and does not reduce growth hormone transcription (Harwood, Grewe & Aguilera, 1984; Barinaga *et al.* 1985). Thus, the GHRH-induced increase in cyclic AMP level in somatotropes presumably performs two functions: (1) an activation of protein kinase A, which phosphorylates calcium channels and thereby increases calcium influx into the cell to sustain release of hormone; and (2) an increase in the transcription of the growth hormone gene through cyclic AMP. Somatostatin primarily regulates the timing of secretory events using intracellular calcium as a second messenger. The GHRH-induced increase in intracellular calcium concentration and the somatostatin-induced inhibition of adenylate cyclase represent 'cross-talk' between the two intracellular messenger systems, thus integrating biologically useful responses: the acute onset of hormone exocytosis after stimulation and the limitation of hormone accumulation in the cell during prolonged inhibition. Although the precise mechanisms are still unknown, somatostatin regulates GH secretion at a distal site by affecting ion conductances across the membrane. The spontaneous oscillations of calcium concentration in the somatotrope may represent cross-talk between several intracellular regulators interacting at the membrane level. These oscillations presumably have important functions.

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