Differential secretion of gonadotrophins: investigation of the role of secretogranin II and chromogranin A in the release of LH and FSH in L β T2 cells

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Abstract

This study investigated the role of the secretory granule proteins, secretogranin II (SgII) and chromogranin A (CgA), in the differential secretion of FSH and LH from L β T2 mouse gonadotroph cells. Exogenous activin, which synergises with GnRH, is essential for the release of FSH from these cells, but also has stimulatory effects on LH and enhances GnRH-induced LH secretion. Two experiments are reported. In experiment 1, cultures were supplemented with activin (0–50 ng/ml), with and without a daily 1 h treatment of 10 nM GnRH, for 3 days. Protein secretion and mRNA levels were measured. In experiment 2, cells were treated with activin (50 ng/ml) alone, a daily 1 h treatment of 10 nM GnRH, or a combination of both for 6 days. In addition, cells exposed to activin+GnRH for 3 days were subsequently left untreated or given activin or GnRH alone for a further 3 days for comparison with cells maintained in activin+GnRH for 6 days. Protein secretion, intracellular protein and mRNA levels were measured. FSH secretion was stimulated, dose dependently, by activin and this effect increased synergistically in the presence of GnRH. The close correlation between secreted and intracellular FSH and FSH β mRNA levels was maintained in cells that had undergone treatment withdrawal after previous exposure to activin+GnRH, but there was no correlation between FSH and the granins. These results are consistent with the view that FSH released in response to activin/GnRH is constitutively secreted via a granin-independent pathway. SgII secretion mirrored the GnRH-induced secretion of LH, but was unaffected by activin, which stimulated LH secretion and had a detrimental effect on CgA mRNA transcription. This confirms previous observations that the LH released in response to GnRH is co-released with SgII via a regulated, granin-dependent pathway, and, in addition, suggests that activin may stimulate LH secretion through a constitutive, granin-independent pathway.

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Introduction

The control of reproduction requires that the secretion of the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is tightly controlled, particularly in the female to allow selection and maturation of the preovulatory follicles in the ovary. LH and FSH are produced in bihormonal gonadotroph cells in the anterior pituitary (Liu *et al.* 1988, Currie & McNeilly 1995, Thomas & Clarke 1997, Crawford *et al.* 2002), but have distinctly different patterns of secretion (Childs *et al.* 1987, Lloyd & Childs 1988). While

there is some basal, non-pulsatile secretion of LH (McNeilly *et al.* 1991, Crawford *et al.* 2002), this hormone is released predominantly from intracellular stores in discrete pulses under the influence of pulsatile hypothalamic gonadotrophin-releasing hormone (GnRH), a regulated pathway of secretion (Clarke & Cummins 1982, Burgess & Kelly 1987). In contrast, while there is limited pulsatile secretion of FSH, often independent of pulsatile GnRH (Farnworth 1995, Padmanabhan *et al.* 1997, Crawford *et al.* 2002), the bulk of this hormone appears to be constitutively secreted and thus the amount released is directly related to the rate of

synthesis (Muyan *et al.* 1994, Farnworth 1995). Principally, regulation of LH release is via regulation of the pattern of pulsatile GnRH input (Clarke & Cummins 1982), while that of FSH is through negative feedback of gonadal steroids, particularly oestradiol and testosterone, and inhibins, which act directly at the pituitary (Vale *et al.* 1988, Gharib *et al.* 1990, McNeilly *et al.* 1991, Besecke *et al.* 1996, Padmanabhan & McNeilly 2001).

Since both LH and FSH are produced in, and released from, the same gonadotrophs, there must be an intracellular mechanism controlling discrete release of the gonadotrophins, which both consist of a common α -subunit combined with a hormone-specific β -subunit and have a deduced similar tertiary structure (Laphorn *et al.* 1994). A potential mechanism could involve differential intracellular packaging of LH and FSH within the gonadotroph, leading to different pathways of secretion. The principal cargo proteins in secretory granules are the granins, chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (SgII). These proteins aggregate at low pH and high Ca^{2+} concentrations (Yoo 1996), as found within the trans-Golgi network where granule formation is initiated, and thus have been proposed to function as helper proteins in packaging other proteins into secretory granules (Huttner & Natori 1995). Indeed it has been suggested that CgA specifically acts as the master 'on/off' switch regulating secretory granule formation in neuroendocrine cells (Kim *et al.* 2002). We (Crawford & McNeilly 2002, Crawford *et al.* 2002) and others (Watanabe *et al.* 1991, 1993) have shown that LH is co-packaged with SgII into electron-dense secretory granules in gonadotrophs and that pulsatile secretion of LH requires the presence of these granules (Crawford *et al.* 2002). Furthermore, basal secretion of LH *in vivo* is associated with granules containing only LH and no SgII (Crawford *et al.* 2002). Thus, packaging of LH into two different types of granule appears to determine the mode of secretion of LH.

While there is an association between levels of FSH β mRNA, synthesis and release of FSH, and plasma FSH levels, little is known about the role that packaging of FSH may play in this type of secretion (McNeilly 1988, Farnworth 1995, McNeilly *et al.* 2003). FSH appears to be localised in different regions of the gonadotrophs compared with LH (Thomas & Clarke 1997) and preliminary

results suggest that FSH is associated with electron-lucent bodies and is not present within the LH+ve/SgII+ve electron-dense granules (Crawford *et al.* 2002). Indeed, the differential pattern of LH and FSH secretion would predict that the two hormones should be in different storage granules prior to release, or such different patterns of secretion could not occur.

Previously, studies using the L β T2 gonadotroph cell line showed that *in vitro* LH release in response to GnRH was closely associated with a parallel release of SgII (Nicol *et al.* 2002). While no clear correlation was observed between the release or storage of LH and CgA, this granin has been localised to the periphery of LH+ve/SgII+ve granules in the sheep pituitary (Crawford & McNeilly 2002) as well as in light-dense vesicles containing FSH in the rat pituitary (Watanabe *et al.* 1991). Furthermore, we have previously shown that mRNA levels for CgA increased in response to GnRH, suggesting a possible role in the regulated release of LH (Nicol *et al.* 2002).

Early reports utilising L β T2 cells had suggested that they were unable to synthesise and secrete FSH (Turgeon *et al.* 1996), so the relationship between the granins and FSH secretion was not evaluated. However, combined activin and GnRH treatment has recently been shown to stimulate FSH β mRNA levels and subsequent secretion of FSH in these cells (Graham *et al.* 1999). Thus, in the present study we have utilised this finding to manipulate the patterns of synthesis and secretion of FSH, as well as LH, in order to evaluate interactions between the gonadotrophins and the granins. The results support the concept that FSH secretion is via a distinct intracellular pathway, independent of LH and granin release.

Materials and methods

Cell culture

L β T2 cells were kindly provided by Dr P. Mellon (University of California, San Diego, CA, USA) and cultures were maintained as described previously (Nicol *et al.* 2002). For experimental purposes cells were cultured on six-well plates coated with phenol-red-free Matrigel (1:3; BD Biosciences Clontech, Cowley, Oxford, UK), in phenol-red-free Dulbecco's modified Eagle's medium (DMEM) (1 g/l glucose) supplemented

with 10% charcoal-treated fetal calf serum, 3.5 g/l glucose, 4 mM L-glutamine, 1 $\times$ non-essential amino acids, 20 nM dexamethasone, 50 U/ml penicillin and 50 μ g/ml streptomycin. Cell culture reagents were obtained from Sigma unless otherwise indicated.

Experiment 1

To confirm the appropriate concentration of activin for use in subsequent experiments, an activin dose response was carried out in the presence and absence of GnRH. L β T2 cells were seeded at a density of 2×10^6 cells per well (three wells per treatment group), allowed to attach overnight, then exposed to recombinant human activin A (R&D Systems, Abingdon, Oxon, UK) at 0, 10, 20 or 50 ng/ml, with and without a daily 1 h treatment of 10 nM GnRH (Peninsula Laboratories Europe Ltd, St Helens, Merseyside, UK), for 3 days, as described by Graham *et al.* (1999). Daily protein secretion and day 4 mRNA levels were measured.

Experiment 2

There were two parts to this experiment. (A) In an attempt to further stimulate levels of FSH/LH production and secretion, observed as a result of the treatment regime described above, the duration of treatment was extended. Cells were plated at a density of 1.25×10^6 cells/well (six wells per treatment group) and treated with activin/GnRH as described above, except that activin was used at 50 ng/ml only and the treatment period was extended to 6 days. (B) The effects of withdrawal of activin, GnRH or activin+GnRH from cells previously exposed to activin+GnRH were assessed. Cells were plated at 1.25×10^6 cells per well (six wells per group), treated with activin (50 ng/ml)/GnRH as described above for 3 days, then transferred to untreated or activin-only conditions or given daily 1 h GnRH treatments only for a further 3 days and compared with cells maintained in activin+GnRH for 6 days. For both parts of this experiment daily protein secretion was measured (six wells per group). On day 7 each group was split into two: three wells for measurement of mRNA levels and three wells for measurement of intracellular protein.

Media samples were collected at each medium change for RIA to measure protein secretion. In

experiment 1 all cells (day 4), and in experiment 2 half of each group (day 7) were suspended in RNazol B (Tel-Test, Inc., TX, USA) and total RNA prepared, according to the manufacturer's protocol, for quantitative reverse transcription (RT)-PCR analysis of mRNA levels. In experiment 2 the remaining cells on day 7 were used to prepare intracellular protein extracts as described previously (Nicol *et al.* 2002) for analysis by RIA.

Radioimmunoassays

Gonadotrophins

Secreted and intracellular FSH and LH were measured by RIA as previously described (McNeilly *et al.* 1996), using agents supplied by Dr A Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA). The reference preparations were AFP-5308D and rLH-RP-1, and the minimum detectable concentrations were 1 ng/ml and 200 pg/ml, for FSH and LH respectively. The intra- and inter-assay coefficients of variation were < 10% for both assays.

Granins

SgII was measured as described previously (Stridsberg *et al.* 1995, Nicol *et al.* 2002), using an antibody against the human SgII amino acid sequence 154–165 (hSgII154–165). CgA was measured using an antibody to human CgA amino acid sequence 324–337 (hCgA324–337), which detects the WE-14 region of CgA, as described previously (Portela Gomes & Stridsberg 2001, Nicol *et al.* 2002). The detection limits were < 10 fmol and the total assay variation was < 10% for both assays.

Quantitative RT-PCR

Levels of mRNA for LH β , FSH β , SgII, CgA, GnRH receptor (GnRH-R), inhibin/activin β B-subunit and the activin receptors IIA (ARIIA), IIB (ARIIB) and IB (ARIB) were measured using quantitative Taqman RT-PCR. This was performed as described previously for LH β (Nicol *et al.* 2002). Primer and probe sequences were designed using Primer Express software (PE Biosystems, Warrington, UK) and were synthesized either by PE Biosystems or by Biosource Europe S.A.,

Table 1 Sequences of primers and probes used for Taqman RT-PCR analysis

Gene	Forward Primer	Reverse primer	Probe (FAM labelled)
FSHβ	ggagagcaatctgctgccata	gcagaaacggcactcttct	ctgtgaattgaccaacatcaccatctcagtaga
LHβ	tgtcctagcatggtccgagtact	agggctacaggaaaggagactatg	cggctgcttgcctcctgtgc
GnRH-R	tgtgtgaccactgcagctt	tgaagaggcagcgcaaggt	cagtgtgtggcatcaggccttctacaacttt
βB-subunit	atgtcccctgccagttca	ctcaggaggggaaagtgcattg	ttgccctcccgcctgtctcc
ARIIA	aagttcagaggtggcaagtc	caacacctctggagccatatactt	cgggtaccaacctgcatggg
ARIIB	ggcagagtgaacgggaaatc	gcagcaatgaactgaacaag	cacacccggcatgaagcacgaa
ARIB	ccatgaagactatcaactgccgtat	caaccttcgcactctctcaa	acgacttagtgccctccgaccttcc

(Nivelles, Belgium). The probe and primer sets used for all sequences are shown in Table 1.

Statistical analysis

Data are reported as means ± S.E.M. Comparisons between experimental groups were determined using one-way ANOVA, and where a significant interaction was found, *post-hoc* analysis was performed using Tukey’s test. Analysis of interaction between activin and GnRH in the dose–response experiment was carried out using two-way ANOVA and the Bonferroni method. All statistical analyses were performed using GraphPad Prism version 4 (GraphPad software Inc., San Diego, CA, USA). *P* < 0.05 was considered significant.

Results

Experiment 1: Activin dose response

This experiment examined the effects of constant exposure to 0, 10, 20 and 50 ng/ml activin A for 3 days, in the presence and absence of daily GnRH treatment, on the secretion of FSH, LH, SgII and CgA and on mRNA levels for FSHβ, LHβ, SgII and CgA. Secretion profiles for each protein, representing the total protein secreted during the 1 h GnRH treatment and the subsequent overnight incubation, and the corresponding day 4 mRNA levels, are depicted in Fig. 1.

FSH

Activin alone (20 and 50 ng/ml) increased FSH secretion (*P* < 0.01) on day 3, whereas GnRH alone had no effect (Fig. 1A(i)). While activin+GnRH had a small stimulatory effect (*P* < 0.01) on

FSH secretion even on day 1, by days 2 and 3 the combined treatment markedly stimulated (*P* < 0.001) FSH release, in a dose-dependent manner, compared with untreated controls and individual treatments (Fig. 1A(i)). FSHβ mRNA was unaffected by individual treatments, but was up-regulated (*P* < 0.001) by activin (50 ng/ml)+GnRH (Fig. 1B(i)).

LH

All concentrations of activin stimulated (*P* < 0.001) LH secretion on days 1 and 2, but on day 3 only 50 ng/ml had a stimulatory (*P* < 0.01) effect (Fig. 1A(ii)). GnRH alone also stimulated (*P* < 0.001) daily LH secretion (Fig. 1A(ii)). Combined activin+GnRH further increased LH secretion (*P* < 0.001) beyond levels observed with individual treatments, but this effect was similar at all concentrations of activin (Fig. 1A(ii)). Activin (50 ng/ml)+GnRH increased (*P* < 0.01) LHβ mRNA transcription (Fig. 1B(ii)).

SgII

Activin alone had little effect on SgII secretion overall, with only a slight increase obtained in response to 50 ng/ml (*P* < 0.05) on day 2, whereas GnRH alone markedly increased (*P* < 0.001) SgII secretion each day (Fig. 1A(iii)). On day 3 activin (20 and 50 ng/ml)+GnRH increased SgII secretion (*P* < 0.01 and *P* < 0.001 respectively) compared with GnRH alone (Fig. 1A(iii)). SgII mRNA levels were not significantly affected by any treatment (Fig. 1B(iii)).

CgA

Activin alone had no effect on CgA secretion, whereas GnRH stimulated (day 1, *P* < 0.05; days 2

Figure 1 Activin dose response. (A) Total daily secretion of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA from L β T2 cell cultures supplemented with human recombinant activin A (0, 10, 20 and 50 ng/ml), with and without a daily 1 h treatment of 10 nM GnRH, for 3 days. Each value represents the total protein secreted during the 1 h GnRH (or corresponding control) treatment and the subsequent overnight incubation. (B) mRNA levels for (i) FSH β , (ii) LH β , (iii) SgII and (iv) CgA from L β T2 cells cultured as described above and harvested on day 4. Different letters indicate significant differences between treatment groups. Values represent means $\pm$ S.E.M.; $n=3$.

and 3, $P < 0.001$) release of this protein (Fig. 1A(iv)). However, when GnRH was combined with activin, a decrease in CgA release ($P < 0.001$), compared with the GnRH-stimulated levels was observed, although the amounts of CgA released remained higher ($P < 0.05$) than those obtained with activin alone (Fig. 1A(iv)). Activin (50 ng/ml)+GnRH increased ($P < 0.05$) CgA mRNA compared with activin alone (Fig. 1B(iv)).

The results from this experiment indicated that 50 ng/ml activin, in the presence of GnRH, was required to have an effect on FSHβ mRNA levels in LβT2 cells. This concentration of activin also produced the greatest stimulation of FSH release and was effective in enhancing the stimulatory effects of GnRH on LH secretion. Consequently, 50 ng/ml activin was used in the subsequent experiments.

Experiment 2(A): effects of 6 days treatment with activin, GnRH and activin+GnRH

The effects of constant exposure to 50 ng/ml activin A, in the presence and absence of a daily 1 h treatment of 10 nM GnRH, for 6 days, on the secretion (day 3 and day 6 results are reported) and intracellular storage (on day 7) of FSH, LH, SgII and CgA were determined. In addition, day 7 FSHβ, LHβ, SgII, CgA, GnRH-R, βB-subunit and activin receptor (IB, IIA and IIB) mRNA levels were measured.

FSH

Activin+GnRH increased ($P < 0.001$) FSH secretion on days 3 (Fig. 2A(i)) and 6 (Fig. 2B(i)), whereas individual treatments had no effect. Similarly, intracellular FSH (Fig. 2C(i)) and FSHβ mRNA (Fig. 2D(i)) levels increased ($P < 0.001$) in response to activin+GnRH only.

LH

On day 3, activin alone ($P < 0.01$) and GnRH alone ($P < 0.001$) both increased LH secretion, with GnRH producing the higher ($P < 0.01$) response (Fig. 2A(ii)). Combined activin+GnRH enhanced ($P < 0.001$) LH secretion approximately 2-fold compared with individual treatments. Activin alone ($P < 0.01$) and GnRH alone ($P < 0.05$) increased

LH secretion on day 6 also, but there was no difference between these two responses (Fig. 2B(ii)). Combined activin+GnRH again enhanced ($P < 0.001$) LH secretion compared with individual treatments (Fig. 2B(ii)). Intracellular LH and LHβ mRNA were increased ($P < 0.01$, Fig. 2C(ii) and $P < 0.05$, Fig. 2D(ii) respectively) by activin+GnRH.

SgII

GnRH alone increased ($P < 0.001$) SgII secretion on days 3 and 6 (Fig. 2A (iii) and 2B (iii) respectively). Activin had no effect on SgII release. However, activin increased ($P < 0.05$) intracellular SgII (Fig. 2C(iii)), whereas GnRH and activin+GnRH reduced ($P < 0.01$ and $P < 0.05$ respectively) intracellular levels of this protein (Fig. 2C(iii)).

CgA

GnRH increased ($P < 0.001$) CgA secretion on day 3. However, when GnRH was combined with activin, CgA secretion was similar to control levels (Fig. 2A(iv)). After 6 days of treatment there were no differences in secretion or intracellular protein levels (Fig. 2B(iv)). While CgA mRNA from all treatment groups was unchanged compared with controls, GnRH-stimulated cells had significantly higher ($P < 0.05$) levels of this mRNA compared with cells treated with activin alone (Fig. 2D(iv)).

GnRH receptor, βB-subunit and activin receptor mRNAs

GnRH-R mRNA levels were increased ($P < 0.05$) by activin+GnRH (Fig. 3A(i)). βB-subunit mRNA from all treatment groups was unchanged compared with controls, but was lower ($P < 0.05$) in GnRH-treated cells compared with activin-treated cells (Fig. 3A(iii)). Activin receptor mRNA levels were unchanged by the treatments given (data not shown).

Experiment 2(B): effects of activin, GnRH or activin+GnRH withdrawal after prior exposure to activin+GnRH

After exposure to activin plus daily GnRH treatment, for 3 days, cells were transferred to either untreated medium (AG/C) or given activin

Figure 2 Effects of continual exposure to activin, GnRH and activin+GnRH for 6 days. (A) Day 3 secretion of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA from L β T2 cell cultures supplemented with human recombinant activin A (50 ng/ml), in the presence (AG) and absence (A) of a daily 1 h treatment of 10 nM GnRH, or with GnRH alone (G) for 6 days. Each value represents the total protein secreted during the 1 h GnRH (or corresponding control) treatment and the subsequent overnight incubation. (B) Day 6 secretion of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA from L β T2 cells cultured as described. (C) Intracellular levels of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA in L β T2 cells cultured as described and harvested on day 7. (D) mRNA levels for (i) FSH β , (ii) LH β , (iii) SgII and (iv) CgA in L β T2 cells cultured as described and harvested on day 7. Different letters indicate significant differences between treatment groups. Values represent means $\pm$ S.E.M.; $n=6$ (A and B), $n=3$ (C and D).

(AG/A) or GnRH (AG/G) alone and cultured for a further 3 days (see Fig. 4A). Results were compared with those from cells maintained in activin+GnRH (AG) for the duration of the experiment.

To demonstrate the immediate effects of the treatment changes Fig. 4B depicts protein secretion in the transition period from day 3, with all cells still under the effects of activin+GnRH, over to day 4, the first day of treatment withdrawal. Day 3 secretion for all four groups treated with activin+GnRH was similar so results for one group only are shown. Compared with cells maintained in activin+GnRH, FSH secretion on day 4 (Fig. 4B(i)) was reduced (AG/C and AG/G, $P < 0.001$; AG/A, $P < 0.05$) in all groups where individual or combined treatments were withdrawn. However, cells transferred to activin only continued to secrete more FSH than cells transferred to control medium ($P < 0.05$) or given GnRH only ($P < 0.01$). LH secretion in cells transferred to untreated or activin-only conditions was slightly reduced on day 4, compared with activin+GnRH-treated cells, but this did not reach significance (Fig. 4B(ii)). Cells transferred to GnRH only had unchanged LH secretion compared with activin+GnRH-treated cells, but secreted significantly more ($P < 0.01$) LH compared with cells cultured in the absence of GnRH (Fig. 4B(ii)). SgII secretion was reduced ($P < 0.001$) in cells transferred to untreated or activin-only conditions, compared with cells maintained in activin+GnRH or those given GnRH only (Fig. 4B(iii)). Day 4 CgA secretion was unchanged by withdrawal of GnRH and activin+GnRH (Fig. 4B(iv)). However, withdrawal of activin, but continued GnRH, resulted in increased CgA compared with all other groups (AG, $P < 0.05$; AG/C and AG/A, $P < 0.001$).

Day 6 protein secretion and day 7 intracellular protein and mRNA levels are shown in Fig. 5.

FSH

Withdrawal of GnRH; but continued activin-reduced ($P < 0.001$) FSH secretion to approximately half that from cells maintained in activin+GnRH, whereas withdrawal of activin $\pm$ GnRH resulted in basal levels (see Fig. 2B(i)) of FSH secretion (Fig. 5A(i)). Similar results were obtained for intracellular protein (Fig. 5B(i)) and FSH β mRNA levels (Fig. 5C(i)), but in the latter case levels were maintained closer to those in

Figure 3 (A) Effects of continual exposure to activin, GnRH and activin+GnRH for 6 days; mRNA levels for (i) GnRH-R and (ii) activin/inhibin β B-subunit in L β T2 cell cultures supplemented with human recombinant activin A (50 ng/ml), in the presence (AG) and absence (A) of a daily 1 h treatment of 10 nM GnRH, or with GnRH alone (G) for 6 days. (B) Effects of individual or combined treatment withdrawal in cells previously exposed to activin+GnRH for 3 days; mRNA levels are shown for (i) GnRH-R and (ii) activin/inhibin β B-subunit in L β T2 cell cultures supplemented with human recombinant activin A (50 ng/ml) in the presence of a daily 1 h treatment of 10 nM GnRH for 3 days, then transferred to untreated (AG/C), activin only (AG/A) or GnRH only (AG/G) conditions, and cultured for a further 3 days and compared with cells maintained in activin+GnRH (AG) for 6 days. All cells were harvested on day 7. Different letters indicate significant differences between treatment groups. Values represent means $\pm$ S.E.M.; $n=3$.

activin+GnRH-treated cells by continued activin treatment.

LH

LH secretion was reduced ($P < 0.01$), compared with activin+GnRH-treated cultures, in cells transferred to untreated or activin-only conditions (Fig. 5A(ii)), whereas there were no significant changes in intracellular LH levels in response to any of the treatment regimes (Fig. 5B(ii)). LH β

Figure 4 Effects of individual or combined treatment withdrawal in cells previously exposed to activin+GnRH: protein secretion on days 3 and 4. (A) Treatment protocol. LβT2 cells were cultured in human recombinant activin A (50 ng/ml), in the presence of a daily 1 h treatment of 10 nM GnRH (AG), for 3 days, then transferred to untreated (AG/C), activin only (AG/A) or GnRH only (AG/G) conditions and cultured for a further 3 days, and compared with cells maintained in activin+GnRH (AG) for 6 days. (B) Day 3 (prior to treatment withdrawal) and day 4 (after treatment withdrawal) secretion of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA from LβT2 cells cultured as described above. Each value represents the total protein secreted during the 1 h GnRH (or corresponding control) treatment and the subsequent overnight incubation. Different letters indicate significant differences between treatment groups. Values represent means ± S.E.M.; *n*=6.

Figure 5 Effects of individual or combined treatment withdrawal in cells previously exposed to activin+GnRH. (A) Day 6 secretion of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA from LβT2 cells cultured in human recombinant activin A (50 ng/ml), in the presence of a daily 1 h treatment of 10 nM GnRH, for 3 days, then transferred to untreated (AG/C), activin-only (AG/A) or GnRH-only (AG/G) conditions and cultured for a further 3 days, and compared with cells maintained in activin+GnRH (AG) for 6 days. (B) Intracellular levels of (i) FSH, (ii) LH, (iii) SgII and (iv) CgA in LβT2 cells cultured as described above and harvested on day 7. (C) mRNA levels for (i) FSHβ, (ii) LHβ, (iii) SgII and (iv) CgA in LβT2 cells cultured as described above and harvested on day 7. Different letters indicate significant differences between treatment groups. Values represent means±S.E.M.; $n=6$ (A), $n=3$ (B and C).

mRNA levels were reduced ($P<0.05$) after transfer to untreated or GnRH-only conditions, and, while there was a small reduction after transfer to activin only, this did not reach significance (Fig. 5C(ii)).

SgII

Withdrawal of all treatments reduced ($P<0.001$) SgII secretion compared with activin+GnRH-treated cells. However, activin-stimulated cells secreted more ($P<0.001$) SgII than cells trans-

ferred to untreated conditions, and GnRH-treated cells released more ($P < 0.001$) SgII compared with cells transferred to activin only (Fig. 5A(iii)).

CgA

Withdrawal of GnRH and activin+GnRH reduced ($P < 0.001$) CgA secretion compared with activin+GnRH-treated cells, whereas cells that continued to receive daily GnRH secreted similar amounts of CgA as activin+GnRH-treated cells (Fig. 5A(iv)).

GnRH receptor, activin β B-subunit and activin receptor mRNAs

Activin and activin+GnRH withdrawal reduced ($P < 0.05$) GnRH-R mRNA levels, compared with activin+GnRH-treated cells (Fig. 3B(i)), but had no significant effects on β B-subunit mRNA levels (Fig. 3B(ii)) or on activin receptor mRNA (data not shown).

Discussion

Previously we have demonstrated a close correlation between the GnRH-stimulated release of both LH and SgII *in vitro* (Nicol *et al.* 2002), and the co-localisation and co-release of LH and SgII *in vivo* (Crawford *et al.* 2002), suggesting that SgII plays an important role in the regulated secretion of LH. In the current report we have extended these studies to examine in detail the relationship between the synthesis, storage and secretion of FSH, as well as LH and the granins in L β T2 cells, to investigate further the role of granin proteins in the differential secretion of the gonadotrophins.

Early studies on L β T2 cells indicated that they did not express FSH β mRNA or secrete FSH (Turgeon *et al.* 1996). The ability of activin ($\pm$ GnRH) to stimulate FSH production in these cells has since been reported (Graham *et al.* 1999, Pernasetti *et al.* 2001), which has further elevated the importance of the L β T2 cell line as a model for investigating gonadotroph function. It must be recognised, however, that the use of these immortalised cell lines can only give an indication of potential mechanisms involved in differential LH and FSH secretion and may not completely, or accurately, reflect gonadotroph function *in vivo*.

Nevertheless, they do provide a reproducible model gonadotroph system with which to explore potential *in vivo* mechanisms.

While FSH secretion in L β T2 cells was stimulated, in a dose-dependent manner, by activin alone, and was unaffected by GnRH alone, there was a synergistic effect between the two treatments. In contrast to the previous report (Graham *et al.* 1999), we observed a significantly higher maximal FSH response (approx 11-fold after 72 h) to activin in the presence of GnRH compared with activin alone. Very similar response patterns were obtained for secreted and intracellular FSH and FSH β mRNA levels. This correlation was maintained in cells subjected to treatment withdrawal after previous exposure to activin+GnRH. The close correlation between stored and secreted FSH, and FSH β mRNA levels, and the lack of any correlation between the secretion of FSH and that of the granins, lead to the conclusion that the FSH produced in L β T2 cells under activin/GnRH stimulation is constitutively secreted via a granin-independent pathway. Since GnRH is ineffective in stimulating or maintaining FSH production in the absence of exogenous activin, endogenous activin would appear to be absent or at very low levels in L β T2 cells, despite the fact that they do express β B-subunit mRNA (see Fig. 3), and this presumably is one of the reasons why FSH was reportedly absent from these cells in earlier experiments (Turgeon *et al.* 1996).

While the effect of the interaction between activin and GnRH on FSH production and secretion has been previously noted (Weiss *et al.* 1992, 1993), the exact nature of the interaction is still unclear. While activin alone stimulated FSH secretion as described previously (Graham *et al.* 1999) it appears that GnRH requires the presence of activin in order to exert its actions. Furthermore activin alone was able to maintain FSH β mRNA at the levels induced by activin+GnRH when GnRH stimulation has been removed, possibly due to post-transcriptional effects on FSH β mRNA stability (Carroll *et al.* 1991). In perfused rat pituitary cultures GnRH at least doubled the increase in FSH β mRNA induced by exogenous activin (Weiss *et al.* 1993), and it is estimated that GnRH controls about 50–67% of FSH β mRNA levels at a particular concentration of activin (Miller *et al.* 2002). Studies in GH3 cells engineered

to express the rat GnRH-R (rGnRH-R) and in L β T2 cells have indicated the presence of GnRH-responsive elements (enhancers) on the rat and ovine FSH β (oFSH β) promoters (Kaiser *et al.* 1995, Pernasetti *et al.* 2001) and it has been reported that two activating protein-1-like enhancers on the oFSH β proximal promoter are required for GnRH induction of oFSH β -Luc transcription (Strahl *et al.* 1998), suggesting a mechanism whereby GnRH can directly influence FSH β transcription (Miller *et al.* 2002). Regardless of the mechanisms by which activin and GnRH increase or maintain FSH β mRNA levels, the present results, showing a close relationship between FSH β mRNA levels and the secretion of FSH, are compatible with the concept of release of FSH mainly through a constitutive, granin-independent pathway.

Activin also had a stimulatory effect on LH secretion, although this response was not dose dependent. After 6 days of treatment the amount of LH secreted in response to activin was similar to that released in response to GnRH. Combined activin+GnRH induced a further increase over individual treatment levels, which appeared to be additive rather than synergistic. Activin A has previously been shown to stimulate the synthesis of GnRH receptors, through a different mechanism from GnRH (Braden & Conn 1992) and, although neither activin nor GnRH alone appeared to have any effect on GnRH-R mRNA levels in this study, there was a significant increase after the combined treatment, and this may be one mechanism whereby the activin/GnRH-stimulated secretion of LH and FSH is regulated.

SgII secretion, in contrast to that of LH, was stimulated only by GnRH, and combined activin/GnRH produced no additive or synergistic effects. The present results suggest that the LH released in response to GnRH is co-released with SgII via a regulated secretory pathway and that the activin-stimulated LH release is probably via a constitutive, SgII-granule-independent pathway. It is likely that this pathway of release is also CgA independent, as activin had a detrimental effect on CgA mRNA levels and CgA secretion. Studies in mice have indicated that LH+ve/SgII-ve storage granules are released constitutively to maintain basal LH concentrations (Crawford *et al.* 2002) and it is possible that activin may be influencing the amount of LH which is directed into this type of granule. While intracellular SgII was reduced in GnRH-

and activin+GnRH-stimulated cells, presumably reflecting the ongoing secretion of LH+ve/SgII+ve granules in response to GnRH, levels were increased in activin-stimulated cells. Intracellular LH was also raised in activin-treated cells. This raises the possibility that, due to the lack of GnRH input and the associated regulatory pathway stimulation and release, there was an increase in the number of LH+ve/SgII+ve granules in activin-treated cells as seen *in vivo* after GnRH immunoneutralisation in mice (Crawford *et al.* 2002).

The aggregative properties of SgII within the trans-Golgi network are considered to be important in facilitating the packaging of LH into LH+ve/SgII+ve secretory granules, thus controlling the LH available for release through the regulated pathway, in response to GnRH stimulation (Gerdes *et al.* 1989, Watanabe *et al.* 1991, Crawford & McNeilly 2002, Crawford *et al.* 2002). However the lack of evidence for the presence of SgII-only granules in mouse or sheep gonadotrophs (Crawford & McNeilly 2002, Crawford *et al.* 2002) suggests that LH may have more influence on the formation of LH+ve/SgII+ve gonadotroph secretory granules than was previously predicted and may actually have an active role in the co-aggregation with SgII during granule formation. A role for SgII in the GnRH responsiveness of secretory granules at the time of granule exocytosis has also been envisaged (Crawford *et al.* 2002), but it may be that CgA contributes more to this effect than SgII (see below).

CgA secretion was maintained by GnRH and reduced below control levels by transfer to activin only, confirming the effects of GnRH and activin on secretion of CgA that were observed in the other experiments described here and in our previous study (Nicol *et al.* 2002). With the concomitant up-regulation of FSH β and down-regulation of CgA gene expression in response to activin, it would appear counter-intuitive to conclude that CgA played a major role in the constitutive secretion of FSH. However, there is evidence to suggest that CgA and FSH are found within the same type of granule within gonadotrophs (Watanabe *et al.* 1991) and that CgA+ve granules increase in number shortly after the LH surge, at a time of higher plasma FSH concentrations (Watanabe *et al.* 1998). Thus a role for CgA in exocytosis associated with the constitutive pathway

cannot be ruled out and will require further investigation.

While CgA has been proposed as the master 'on/off' switch regulating the formation of dense-core secretory granules (Kim *et al.* 2002), the precise role of CgA in the regulated pathway of secretion remains to be fully explained. CgA within gonadotrophs appears punctate, thought to be due to compartmentalisation of this granin at the granule periphery (Crawford & McNeilly 2002, Crawford *et al.* 2002). In addition, inositol triphosphate receptors (IP₃Rs) on secretory granule membranes have been shown to interact with IP₃R binding sites on CgA (Yoo & Jeon 2000), thus contributing to intracellular Ca²⁺ control mechanisms and influencing regulated secretion. Together, these findings suggest that CgA may have an important role in granule exocytosis, but results in the current study, and in our previous report (Nicol *et al.* 2002), indicate that the release of CgA in response to GnRH does not correlate with that of LH, which suggests that CgA may be retained within the cell along with the associated granule membrane (Crawford *et al.* 2002).

In summary, the results reported in this study demonstrate that activin-/GnRH-induced FSH secretion in L β T2 cells is directly related to the rate of FSH synthesis and shows no correlation with either SgII or CgA, and thus appears to be released via a constitutive, granin-independent pathway. The activin-stimulated release of LH also appears to be granin-independent, suggesting constitutive release. In contrast, GnRH-induced secretion of LH correlates with that of SgII, and appears to involve CgA, suggesting release via a granin-related, regulated pathway. Further studies, involving the direct manipulation of granin levels, will be required to determine the precise role of these proteins in gonadotrophin secretion. However, the results presented here contribute further evidence for the importance of the specialised secretory granule proteins, SgII and CgA, in the differential secretion of the gonadotrophins.

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