Influence of steroids and GnRH on biosynthesis and secretion of secretogranin II and chromogranin A in relation to LH release in L β T2 gonadotroph cells

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Abstract

The granin proteins secretogranin II (SgII) and chromogranin A (CgA) are commonly found associated with LH and/or FSH within specialised secretory granules in gonadotroph cells, and it is possible that they play an important role in the differential secretion of the gonadotrophins. In this study we have examined the regulation of the biosynthesis and secretion of SgII and CgA, in relation to LH secretion, in the L β T2 mouse pituitary gonadotroph cell line.

Three experiments were carried out to investigate the effects of oestradiol (E2) and dexamethasone (Dex) in the presence and absence of GnRH (experiment 1), differing GnRH concentrations (experiment 2) and alterations in GnRH pulse frequency (experiment 3). In experiment 1, exposure to E2, Dex or E2+Dex, either with or without GnRH treatment, resulted in increased LH secretion. Steroids alone had no effect on LH β mRNA levels, but in the presence of GnRH LH β mRNA levels were increased in Dex- and E2+Dex-treated cells. GnRH receptor (GnRH-R) mRNA levels were up-regulated by Dex and E2+Dex, but were unaffected by GnRH. There were no steroid-induced changes in SgII or CgA mRNA, but increased levels of CgA mRNA were observed after GnRH treatment in cells cultured in the presence of Dex. In experiment 2, increasing concentrations of GnRH resulted in increases in LH secretion that were inversely dose-dependent. No changes in LH β , GnRH-R or SgII mRNA levels were observed, but there were dose-dependent increases in CgA mRNA levels. In experiment 3, GnRH was given as either 1 pulse/day or 4 pulses/day

for 3 days. Both pulse regimes resulted in increased LH, SgII and CgA secretion compared with controls during the first 15 min pulse on day 3. Exposure to GnRH at 4 pulses/day increased LH and SgII secretion compared with controls during all 4 pulses, but secretion of both proteins was reduced during pulses 2–4 compared with pulse 1. CgA secretion also increased due to GnRH in pulse 1, but was decreased by GnRH treatment during pulse 2, and unchanged by GnRH during pulses 3 and 4. Total daily secretion of LH and SgII from cells given 1 pulse/day of GnRH increased compared with controls on all three treatment days, while total CgA secretion increased in response to GnRH on days 2 and 3 only. Intracellular levels of SgII, but not LH, decreased after GnRH treatment. In contrast, intracellular CgA was increased, but only after 4 pulses/day of GnRH. Levels of LH β , but not SgII, mRNA were increased by both pulse regimes, while CgA mRNA levels increased after 1 pulse/day of GnRH.

These results indicate that there is a close correlation between the GnRH-stimulated release of LH and SgII from L β T2 cells, suggesting that SgII may have an influential role in the regulated secretion of LH, possibly by inducing LH aggregation to facilitate trafficking into secretory granules. CgA secretion does not appear to be closely associated with that of LH, but CgA expression does appear to be regulated by GnRH, which may indicate involvement in the control of LH secretion, possibly by influencing the proportion of LH in the different types of secretory granules.

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Introduction

Normal reproductive function is dependent on the production of the gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), from specialised

gonadotroph cells in the anterior pituitary. Synthesis and secretion of the gonadotrophins are regulated by pulsatile release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus (Clarke & Cummins 1982), by the negative feedback effects of gonadal steroids, acting

either directly at the pituitary or indirectly by regulating GnRH release from the hypothalamus (Gharib *et al.* 1990, McNeilly *et al.* 1991, Padmanabhan & McNeilly 2001), and by the gonadal peptides inhibin, activin and follistatin (Vale *et al.* 1988). Gonadotrophs are reported to be bihormonal (Liu *et al.* 1988, Currie & McNeilly 1995, Thomas & Clarke 1997), but under certain physiological and experimental conditions, monohormonal cells have been identified (Childs *et al.* 1994, Taragnat *et al.* 1998). Despite the bihormonal nature of gonadotrophs, these cells are capable of differentially regulating the secretion of LH and FSH. LH is stored intracellularly and released mainly in response to pulses of GnRH via a regulated secretory pathway (Burgess & Kelly 1987), although minimal release can occur constitutively (McNeilly *et al.* 1991). FSH is largely secreted via a 'constitutive-like' pathway, although there is evidence that this hormone may also be released by an alternative 'regulated' pathway (Farnworth 1995). At the sub-cellular level specialised secretory granules, containing either one or both gonadotrophins, have been observed (Childs *et al.* 1987, Lloyd & Childs 1988, Watanabe *et al.* 1991, Tougard & Tixier-Vidal 1994, Farnworth 1995), and the differential secretion of LH and FSH has been attributed to changes in the total number of granules and/or in the proportion of each type of granule within a cell (Childs *et al.* 1987, Watanabe *et al.* 1991). Secretory granules within neuroendocrine cells typically also contain acidic soluble proteins from the granin family (Huttner *et al.* 1991, Ozawa & Takata 1995). These proteins aggregate at low pH and high calcium concentrations, conditions found in the trans-Golgi network (TGN), and it has been suggested that they function as 'aggregation-inducers', involved in the packaging of peptide hormones and neuropeptides into secretory granules formed within the TGN (Chanat & Huttner 1991, Watanabe *et al.* 1991). The three major granin proteins, secretogranin II (SgII), chromogranin A (CgA) and chromogranin B (CgB), have all been detected within gonadotrophs in the rat anterior pituitary (Rundle *et al.* 1986, Watanabe *et al.* 1991). Immunocytochemical studies revealed an association between SgII and LH in small, electron-dense granules and between CgA, FSH, and to a lesser extent LH, in larger, moderately electron-dense granules. It has been suggested that these subsets of secretory granules involve different sorting mechanisms, which may reflect two regulated pathways of secretion in gonadotrophs (Watanabe *et al.* 1991, 1993). In GnRH-stimulated pituitary cell aggregates SgII has been shown to be co-released with LH (Sion *et al.* 1988), reflecting the close proximity of these proteins within secretory granules. A third, intermediate type of secretory granule, which is normally observed only sporadically, appears more abundant after acute GnRH treatment of gonadotrophs. These are immunopositive for both gonadotrophins and both granins, but SgII and LH are localised together in an electron-dense core and CgA and FSH are observed in the

electron-lucent area peripheral to this (Watanabe *et al.* 1993, Crawford *et al.* 2002b). It is postulated that hyperstimulation of the gonadotrophs results in a dramatic increase in the flow of secretory proteins through the TGN, and that the processes involved in partitioning different secretory proteins/granins fail, increasing the number of intermediate granules (Watanabe *et al.* 1993). It is still unclear how the formation of these granules relates to the increased secretion of the gonadotrophins in response to GnRH. It is interesting to note that the synthesis and secretion of SgII and CgA within the pituitary appear to be regulated by gonadal steroids and by glucocorticoids. Oestradiol (E2) down-regulated both SgII and CgA mRNA in female rat pituitaries (Anouar & Duval 1992). SgII release, along with that of LH, was suppressed after E2 treatment, leading to an increase in pituitary content for these two proteins. CgA cell content, on the other hand, was reduced by E2 treatment (Anouar & Duval 1991), which could be attributed to either increased secretion and/or increased degradation of CgA. In contrast, CgA is reported to be up-regulated by dexamethasone (Dex) (Fischer-Colbrie *et al.* 1989). Sequence analysis of the bovine CgA promoter revealed the presence of sequences similar to the consensus glucocorticoid and oestrogen response elements (Iacangelo *et al.* 1991).

The L β T2 mouse gonadotroph cell line has previously been shown to exhibit structural and functional characteristics of normal pituitary gonadotrophs (Turgeon *et al.* 1996) and, as shown in the present study, these cells also express the granins. We have utilised this cell line to investigate the effects of E2, Dex and GnRH on the expression, storage and secretion of the granin proteins SgII and CgA in relation to the regulated secretion of LH.

Materials and Methods

This study was divided into three experiments whereby the effects of E2 and/or Dex in the presence and absence of GnRH (experiment 1), differing GnRH concentrations (experiment 2) and alterations in GnRH pulse frequency (experiment 3) were investigated. LH secretion and mRNA levels for LH β , GnRH receptor (GnRH-R), SgII and CgA were measured in experiments 1 and 2. LH, SgII and CgA secretion and intracellular content, as well as LH β , GnRH-R, SgII and CgA mRNA levels were investigated in experiment 3.

Cell culture

L β T2 cells were kindly provided by Dr P Mellon (University of California, San Diego, CA, USA). They were grown in DMEM, 10% foetal calf serum (FCS), 50 U/ml penicillin and 50 μ g/ml streptomycin at 37 °C in 5% CO₂, on flasks coated with Matrigel (Becton

Dickinson Labware, Oxford, Oxon, UK) diluted 1:29 in PBS. For experimental evaluation cells were plated, at a density of 3.5×10^6 cells/well, on six-well plates coated with Matrigel (diluted 1:3), in phenol red-free DMEM (1 g/l glucose) supplemented with 10% charcoal-treated FCS, 3.5 g/l glucose, 4 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. They were allowed to attach for approximately 3 h, then basal medium was replaced as required for each experiment. Cell culture reagents were obtained from Sigma (Poole, Dorset, UK) unless otherwise indicated.

To assess the effects of E2, Dex and GnRH (experiment 1), cells were grown in medium that was either steroid-free, or supplemented with 0.2 nM E2, 20 nM Dex or 0.2 nM E2+20 nM Dex. After overnight culture, medium was removed and replaced with fresh medium for 30 min to establish a baseline for secretion. This was then replaced with medium containing 10 nM GnRH (Peninsula Laboratories Europe Ltd, St Helens, Merseyside, UK) for 15 min, followed by GnRH-free medium for an inter-pulse interval of 75 min. Cells were given 4 pulses of GnRH per day over 3 days. Control cells had their medium changed daily. To investigate the effects of different GnRH concentrations (experiment 2), all cells were cultured in medium supplemented with E2+Dex, and GnRH was administered as 4 pulses/day for 3 days as described above, using concentrations of 0, 10, 50 and 200 nM. To determine the effects of different GnRH pulse frequencies (experiment 3), cells were again cultured in E2+Dex, and 0 or 10 nM GnRH was given as either one 15 min pulse/day or four 15 min pulses/day over 3 days as described above.

Media samples were collected at each medium change and stored at -20°C for RIA. For all experiments, on day 4 cells were suspended in RNazolB (AMS Biotechnology (Europe) Ltd, Abingdon, Oxon, UK) and total RNA prepared, according to the manufacturer's protocol, for Northern analysis or quantitative RT-PCR. In experiment 3 some samples were suspended in 100 mM sodium carbonate pH 8.4 containing Complete Protease Inhibitors (Roche), incubated at room temperature for 1 h, and then subjected to three cycles of rapid freeze-thawing to release intracellular proteins. After centrifugation at 12 000 g for 5 min supernatants were stored at -20°C for RIA.

RIA

LH assay The concentration of LH in cell culture media and intracellular protein extracts was measured by RIA using reagents supplied by Dr A Parlow (NIDDK, Torrance, CA, USA), with all samples from each experiment being assayed in duplicate in the same assay as previously described (McNeilly *et al.* 1996). The reference preparation used was rLH-RP-1 and the minimum detectable concentration was 200 pg/ml. The intra- and inter-assay coefficients of variation were <10%.

Granins SgII was measured as secretoneurin. A peptide covering the human SgII amino acid sequence 154–165 (hSgII154–165), with an additional cysteine residue in the C-terminal, was synthesised using Fmoc techniques. Before injection into rabbits, the peptide was coupled to Inject maleimide-activated keyhole limpet cyanogen according to the manufacturer's instructions (Pierce, Rockford, IL, USA). For preparation of tracer, the peptide was labelled with ^{125}I (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Bucks, UK) using the chloramine-T method as described previously (Stridsberg *et al.* 1995). The assay was constructed as follows: standards and unknown samples were incubated with tracer (30 000 c.p.m./tube) and primary antibody (1/105 000) for 3 days at 4°C . All standards and samples were assayed in duplicate. Antibody-bound radioactivity was separated from free tracer by addition of a secondary antibody, goat anti-rabbit IgG, coupled to a solid phase (Decanting suspension 3; Pharmacia Biotech, Uppsala, Sweden). The antibody-bound radioactivity was measured in a gamma counter (Auto gamma; Wallac, Pharmacia Biotech) and the data were calculated using a logit-log transformation program (Multicalc; Wallac). CgA was measured using an antibody to either human CgA amino acid sequence 324–337 (hCgA324–337), which detects the WE-14 region of CgA (Portela-Gomes & Stridsberg 2001), or to human CgA amino acid sequence 17–38 (hCgA17–38), which detects vasostatin (Stridsberg *et al.* 2000, Portela-Gomes & Stridsberg 2001). For hCgA324–337 the assay method was performed as described above with a primary antibody dilution of 1/90 000. The hCgA17–38 assay was also performed as described above, with a primary antibody dilution of 1/120 000 (Stridsberg *et al.* 2000). All granin assays showed sufficient interspecies cross-reaction (Stridsberg *et al.* 2000) to allow measurement in samples from the mouse-derived L β T2 cell line. The detection limits were <10 fmol and the total assay variation was <10% for all assays.

Northern blots

The mouse GnRH-R (1.2 kb) cDNA (Tsutsumi *et al.* 1992) was obtained in house from Mr R Sellar (MRC Human Reproductive Sciences Unit, Edinburgh, UK), the bovine SgII (2.3 kb) cDNA from Dr R Fischer-Colbrie (University of Innsbruck, Innsbruck, Austria) and the human CgA (1.8 kb) cDNA from ATCC (Manassas, VA, USA). The rat 18S rRNA cDNA was from Dr K Turner (MRC Human Reproductive Sciences Unit). Radiolabelled cDNA probes were prepared to high specific activity ($>1 \times 10^9$ c.p.m./ μ g) with [α - ^{32}P]dCTP using a Rediprime kit (Amersham Pharmacia Biotech).

Samples of total RNA (20 μ g) were separated on 1.5% agarose/0.66 M formaldehyde gels and transferred to Hybond N (Amersham Pharmacia Biotech). After UV cross-linking for 2 min, membranes were pre-hybridised

for 4 h at 65 °C in buffer containing 0.2 M sodium phosphate, pH 7.2, 1 mM EDTA, pH 8, 7% (w/v) SDS, 1% (w/v) BSA and 15% (v/v) deionised formamide. Hybridisation was carried out overnight at 65 °C in fresh buffer containing radiolabelled cDNA probe at $2\text{--}3 \times 10^6$ c.p.m./ml. Membranes were washed 3×20 min in 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, pH 8 and 1% (w/v) SDS. Intensity of bands was quantified using a Storm 860 phosphorimager (Molecular Dynamics, Chesham, Bucks, UK) and changes in mRNA levels were assessed in relation to 18S rRNA levels.

Quantitative RT-PCR

Our Northern blot signals for the LH β transcript appeared to be quite low, making detection and subsequent analysis more difficult. Consequently, we have utilised the more sensitive Taqman quantitative PCR technique to assess changes in expression of this mRNA.

LH β primers and probe were designed using Primer Express software (PE Biosystems, Warrington, Cheshire, UK) and synthesised by PE Biosystems. Sequences were: forward primer: TGTCCTAGCATGGTCCGAGTACT; reverse primer: AGGGCTACAGGAAAGGAGACTATG; probe (FAM labelled): CGGCTGCTTTGCCTCCTGTGC. Ribosomal 18S primers and probe were from a Taqman Ribosomal RNA Control Reagents kit (VIC labelled probe; PE Biosystems).

Aliquots of total RNA were treated with DNase I using DNA-free (Ambion (Europe) Ltd, Huntingdon, Cambs, UK) according to manufacturer's protocol. Samples were then reverse transcribed, using oligo d(T)16, with Taqman Reverse Transcription Reagents (PE Biosystems) according to the manufacturer's protocol, using a programme of 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. DNase-treated RNA was added at 10 ng/ μ l. For PCR a reaction mix was prepared consisting of Taqman Universal PCR Master Mix ($1 \times$), LH β forward and reverse primers (300 nM each), LH β probe (200 nM), ribosomal 18S forward and reverse primers (50 nM each) and ribosomal 18S probe (200 nM). This was aliquoted into separate tubes for each sample, then cDNA was added at 1 μ l/25 μ l reaction mix (equivalent to 10 ng total RNA/25 μ l), and mixed well. Twenty-five microlitre aliquots were transferred to wells in a 96-well PCR plate, with each sample added in duplicate. Optical caps were fixed onto the plates and the PCR reaction run on an ABI Prism 7700 PCR machine (PE Biosystems) using standard conditions. Controls included cDNA prepared with omission of Multiscribe reverse transcriptase enzyme to check for efficiency of DNase treatment. Prior to analysis, a validation assay was performed to demonstrate that amplification of target gene and the reference (18S) were approximately equal. Quantification was performed by the $\Delta\Delta C_t$ method (Bulletin #5; PE Biosystems). A standard PCR reaction

using the LH β primers was run on an agarose gel to confirm amplification of a single product of the correct size.

Statistical analysis

Data are reported as means $\pm$ S.E.M. Comparisons between controls and treatment groups were analysed by an unpaired *t*-test. Differences between multiple groups were determined by one-way ANOVA, followed by Fisher's protected least square differences test using Statview version 4.02 (Abacus Concepts Ltd, Berkeley, CA, USA). $P < 0.05$ was considered significant.

Results

Experiment 1: effects of E2, Dex and GnRH

A preliminary experiment, performed to establish the optimum culture conditions for further studies, examined the effects of 0.2 nM E2, 20 nM Dex and 0.2 nM E2 + 20 nM Dex, with and without GnRH treatment, on LH secretion and LH β , GnRH-R, SgII and CgA mRNA levels. LH secretion results for each pulse of GnRH represent the total LH secreted during the 15 min GnRH exposure and the 75 min inter-pulse interval. By the third day of culture all steroid treatments resulted in increased ($P < 0.001$) LH secretion in response to the first GnRH pulse (Fig. 1a). Dex and E2+Dex treatments increased ($P < 0.001$) LH secretion compared with E2 alone. During pulses 2–4 LH secretion was reduced ($P < 0.05$) in cells given Dex and E2+Dex compared with control and E2-treated cells. More ($P < 0.001$) LH was released in response to pulse 1, compared with pulses 2–4. Total daily LH secretion from cells cultured in the absence of GnRH was also increased ($P < 0.01$) on days 2 and 3 in response to all steroid treatments (data not shown). In the absence of GnRH, LH β mRNA levels were unchanged by steroid treatment, but in the presence of GnRH, LH β mRNA expression was increased by Dex and E2+Dex, compared with GnRH-treated controls ($P < 0.01$) and with the corresponding treatment groups within non-GnRH-treated cells ($P < 0.05$, $P < 0.01$ respectively; Fig. 1b (i)). GnRH-R mRNA levels were increased by Dex and E2+Dex (no GnRH: $P < 0.01$, $P < 0.001$ respectively; +GnRH: $P < 0.001$), but GnRH had no effect on GnRH-R mRNA (Fig. 1b (ii)). There were no significant changes in SgII or CgA message levels as a result of steroid treatment. However, up-regulation ($P < 0.05$) of CgA mRNA in response to GnRH was observed in Dex-treated cells (Fig. 1b (iv)).

Experiment 2: effects of differing GnRH concentrations

Having established that LH secretion, and LH β and GnRH-R mRNA levels, were optimally increased in

Figure 1 (a) LH secretion on treatment day 3 from LβT2 cells cultured either in the absence of steroids or in the presence of 0.2 nM E2, 20 nM Dex or 0.2 nM E2+20 nM Dex and given 4 pulses of 10 nM GnRH per day for 3 days. Results for each pulse represent the total LH secreted during the 15 min GnRH exposure and the 75 min inter-pulse interval. (b) mRNA levels for (i) LHβ, (ii) GnRH-R, (iii) SgII and (iv) CgA in LβT2 cells cultured as described above, with and without GnRH. Different letters represent significant differences between treatment groups. The asterisk denotes the significant effect of GnRH within the Dex-treated group (* $P < 0.05$). Values represent means $\pm$ S.E.M., $n = 3$.

response to GnRH in the presence of E2+Dex, all subsequent experiments were conducted using this combination of steroids in the basal medium. A further preliminary experiment was carried out to investigate the effects of GnRH concentration on the regulation of LH secretion in LβT2 cells. Cultures were pulsed with GnRH as before, but this time using concentrations of 0, 10, 50 and 200 nM. LH secretion demonstrated a similar pattern to that shown in Fig. 1a, with less ($P < 0.001$) LH secreted in pulses 2–4 compared with pulse 1 (data not shown). Figure 2a shows the LH secretory response for the first pulse only on each day of treatment. LH secretion increased ($P < 0.001$) after GnRH treatment at all concentrations. On day 1 LH secretion was similar in all GnRH treatment groups. On days 2 and 3 LH secretion was reduced ($P < 0.001$) in 50 and 200 nM GnRH-treated cells, compared with 10 nM GnRH-treated cells. On day 3 decreased ($P < 0.001$) LH secretion was observed in 200 nM GnRH-treated cells compared with 50 nM

Figure 2 (a) LH secretion, in response to the first pulse of GnRH each day, from LβT2 cells cultured in the presence of E2+Dex and given 0, 10, 50 or 200 nM GnRH at 4 pulses/day for 3 days. Results for each pulse represent the total LH secreted during the 15 min GnRH exposure and the 75 min inter-pulse interval. (b) mRNA levels for (i) LHβ, (ii) GnRH-R, (iii) SgII and (iv) CgA in LβT2 cells cultured as described above. Different letters represent significant differences between treatment groups. Values represent means $\pm$ S.E.M., $n = 3$.

GnRH-treated cells. In this experiment, LHβ, GnRH-R and SgII mRNA levels were unaffected by GnRH at any concentration (Fig. 2b (i–iii)). CgA mRNA levels, however, demonstrated concentration-dependent increases in response to GnRH treatment, significant ($P < 0.05$) compared with controls at 50 and 200 nM (Fig. 2b (iv)). As stimulation of LH secretion was greatest when GnRH was applied at 10 nM it was decided to continue to use this concentration in subsequent experiments.

Experiment 3: effects of GnRH pulse frequency

The previous experiments in this study utilised a GnRH pulse protocol of four 15 min pulses per day with 75 min inter-pulse intervals, but it is possible that alternative frequencies of GnRH exposure may have different effects on the stimulation of LH production, storage and secretion in LβT2 cells, which may in turn be reflected in changes

Figure 3 (a) (i) LH, (ii) SgII and (iii) CgA324–337 secretion during the first 15 min pulse on day 3 from $L\beta T2$ cells cultured in the presence of E2+Dex and given 0 or 10 nM GnRH as either 4 pulses/day or as 1 pulse/day for 3 days. (b) (i) LH, (ii) SgII, (iii) CgA324–337 and (iv) CgA17–38 secretion during the 4 pulses on day 3 from cells cultured in the presence of E2+Dex and given 0 or 10 nM GnRH as 4 pulses/day for 3 days. Different letters represent significant differences within treatment groups. Asterisks represent significant differences between treatment groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Values represent means $\pm$ S.E.M., $n = 6$.

in the expression and secretion of the granins. This experiment looked in detail at the effects of giving only one 15 min pulse of 10 nM GnRH per day, compared with 4 pulses per day.

LH secretion during GnRH pulses over the 3 days of treatment for the 4 pulses/day group showed a similar pattern to that obtained in the first experiment, with more ($P < 0.001$) LH secreted during pulse 1 compared with subsequent pulses (data not shown). Figure 3a shows secretory responses of LH, SgII and CgA on treatment day 3 during the first 15 min pulse of GnRH in the 4 pulses/day group and during the single 15 min pulse for the 1 pulse/day cells. LH, SgII and CgA secretion were increased ($P < 0.001$) compared with controls after both GnRH treatments. There was a greater ($P < 0.001$) GnRH pulse response for all three proteins by the 1 pulse/day group compared with the 4 pulses/day group. However, whereas there was no difference in LH secretion from control cells in the two treatment groups, both SgII and CgA secretion were higher ($P < 0.05$, $P < 0.001$ respectively) in 1 pulse/day controls compared with 4 pulses/day controls (Fig. 3a (i–iii)).

The secretion of LH, SgII and CgA during all 4 pulses (15 min pulse and following 75 min inter-pulse interval)

on day 3 for the 4 pulses/day group is shown in Fig. 3b. LH and SgII appeared to be released in a very similar fashion, with higher ($P < 0.001$) levels of both released in response to GnRH in all pulses, and more ($P < 0.001$) LH and SgII released during pulse 1 compared with pulses 2–4 (Fig. 3b (i, ii)). CgA secretion was measured by two assays, each detecting different regions of the protein. CgA324–337 secretion was stimulated ($P = 0.001$) by GnRH during the first pulse on day 3, but was inhibited ($P < 0.05$) by GnRH during the second pulse and there were no significant GnRH-induced changes during pulses 3 and 4 (Fig. 3b (iii)). CgA17–38 secretion was similar in control and GnRH-treated cells and during all pulses (Fig. 3b (iv)).

Total daily secretion of LH, SgII and CgA over the three treatment days was measured in cells given 1 pulse/day of GnRH. This represented the total LH secreted during the 30 min baseline incubation, the 15 min pulse and the overnight incubation. Total LH secretion increased ($P < 0.001$) each day in response to GnRH, and each day there was an increase ($P < 0.001$) in LH secretion compared with the previous day, which could be attributed to an increase in cell number, since LH secretion from control cells on day 3 was also increased

Figure 4 Total daily secretion from L β T2 cells cultured in the presence of E2+Dex and given 0 or 10 nM GnRH as either 4 pulses/day or 1 pulse/day for 3 days. (a) Total LH secretion from cells given 4 pulses/day. (b) Total LH, (c) total SgII and (d) total CgA secretion from cells given 1 pulse/day. Different letters represent significant differences between control and treatment groups and between days. Values represent means $\pm$ S.E.M., $n=6$.

($P<0.05$) compared with day 1 (Fig. 4b). SgII secretion also increased ($P<0.001$) each day in response to GnRH. There was no difference in SgII secretion between days 1 and 2, but day 3 secretion was slightly increased ($P<0.05$, $P<0.01$ respectively) compared with days 1 and 2 (Fig. 4c). SgII secretion from control cells did not change over the treatment period. CgA secretion was increased ($P<0.001$) compared with controls on days 2 and 3 only. There was no change in control secretion of this protein over the 3 days of treatment (Fig. 4d). Total LH secreted during the two pulse protocols was also compared. LH secretion from cells given 1 pulse/day of GnRH was less on days 2 and 3 ($P<0.05$, $P<0.01$ respectively) than from cells given 4 pulses/day (Fig. 4a and b).

Intracellular levels of LH were unchanged by GnRH (Fig. 5a (i)), while SgII levels decreased after both GnRH treatments (4/day: $P<0.01$; 1/day: $P<0.05$; Fig. 5a (ii)). Conversely, intracellular levels of CgA increased ($P<0.05$) after GnRH treatment in the 4 pulses/day group only.

In this experiment, LH β mRNA levels increased significantly in response to both GnRH treatment protocols (4/day: $P<0.01$; 1/day: $P<0.001$; Fig. 5b (i)). As found previously, GnRH-R (not shown) and SgII mRNA levels did not change in response to GnRH (Fig. 5b (ii)). CgA mRNA expression increased ($P<0.05$) in response to GnRH at 1 pulse/day only (Fig. 5b (iii)).

Discussion

Specialised secretory granules, and their associated granin proteins, commonly found in neuroendocrine cells, are thought to be important mediators of the regulated secretory pathway (Ozawa & Takata 1995). However, the regulation of granin synthesis and secretion in relation to LH and FSH production, and the role of different types of

granules in the fundamental processes of normal reproduction, are still largely unclear. The present study used the L β T2 pituitary gonadotroph cell model to examine the regulation of the major granin proteins SgII and CgA, under conditions designed to stimulate secretion of LH. Ultrastructural examination of L β T2 cells has demonstrated the presence of secretory granules within the cytoplasm (Turgeon *et al.* 1996) and it has been confirmed that these cells are immunoreactive for both SgII and CgA (L Nicol, unpublished observation). CgB, the third major granin protein reported to be present in gonadotrophs (Rundle *et al.* 1986, Watanabe *et al.* 1991), is virtually undetectable in L β T2 cells by immunocytochemistry (L Nicol, unpublished observation) and by RIA (M Stridsberg, unpublished observation), which may indicate that this granin plays little part in the storage and secretion of the gonadotrophins in these cells.

LH secretion in L β T2 cells was stimulated by the presence of steroids, an effect which appeared to be mediated, at least partly, by up-regulation of both GnRH-R and LH β mRNAs. In contrast to a previous study (Turgeon *et al.* 1996), we did not observe any changes in steady state GnRH-R mRNA levels after GnRH treatment, only after steroid treatment, and we observed increased LH β mRNA levels, not only as a result of GnRH treatment, but also due to the presence of Dex and E2+Dex in the culture medium. The stimulatory effects of Dex on LH synthesis and secretion, and GnRH-R mRNA levels, in L β T2 cells are contrasted by the effects of glucocorticoids in normal pituitary cells. Dex blocked ovulation in cycling rats by inhibiting the normal preovulatory LH surge (Baldwin & Sawyer 1974), and substantially decreased GnRH-stimulated LH secretion in cultured bovine anterior pituitary cells (Padmanabhan *et al.* 1983). In addition, corticosterone inhibited LH release from male (Tibolt & Childs 1985) and female

Figure 5 (a) Intracellular protein content for (i) LH, (ii) SgII and (iii) CgA in LβT2 cells cultured in the presence of E2+Dex and given 0 or 10 nM GnRH as either 4 pulses/day or 1 pulse/day for 3 days. Values represent means $\pm$ S.E.M., $n=3$. (b) mRNA levels for (i) LHβ, (ii) SgII and (iii) CgA in cells treated as described above. Asterisks denote significant differences between treatment groups. Values represent means $\pm$ S.E.M., $n=6$.

(Baldwin *et al.* 1991) cultured rat anterior pituitary cells. Other studies have reported that LH release is unaffected by glucocorticoid treatment (Suter & Schwartz 1985a,b). Pituitary GnRH-R levels were also unchanged by glucocorticoid treatment (Ringstrom *et al.* 1992). While normal gonadotrophs are terminally differentiated, LβT2 cells are potentially capable of further differentiation and it is possible that genetic differences exist between the two cell types which could explain the different glucocorticoid responses. Dex also has a detrimental effect on LβT2 cell proliferation (Turgeon *et al.* 1996), but any direct relationship between frequency of cell division and the level of GnRH-mediated LH release has not yet been demonstrated.

There was no evidence for any steroid-induced changes in granin expression in these cells. The presence of Dex did, however, appear to enhance the up-regulation of CgA mRNA by GnRH. We did not investigate steroid concentration effects and it is possible that the concentrations used, or the duration of exposure, chosen as being effective in altering the expression of LHβ and GnRH-R mRNA, and stimulating LH secretion, as reported previously (Turgeon *et al.* 1996), were not sufficient to elicit effects on the expression of SgII or CgA. It has been shown that pituitary expression of the granins is affected by steroid hormones and by corticosteroids. For example, in female

rat pituitaries, ovariectomy increased SgII and CgA mRNA and protein levels, as well as increasing LHβ, FSHβ and gonadotrophin α -subunit mRNA levels and LH content (Anouar *et al.* 1991). Subsequent oestrogen replacement resulted in CgA message reverting to control levels with a very similar response to that of LHβ. Oestrogen also down-regulated SgII mRNA levels, but in this case a higher concentration of E2 was required to produce an effect. Pituitary CgA content was decreased after E2 treatment, but there was no effect on the post-ovariectomy rise in SgII protein levels (Anouar *et al.* 1991). Similar results have been observed *in vitro*, where E2 was shown to down-regulate steady state mRNA levels for both SgII and CgA in cultured pituitary cell aggregates, but reduced intracellular content of CgA only (Anouar & Duval 1992). Regulation of CgA expression by Dex has also been reported. CgA biosynthesis in rat pituitaries was stimulated by Dex treatment (Fischer-Colbrie *et al.* 1989) and it seems likely that this effect may be directly attributed to alterations in CgA gene transcription, as glucocorticoid response element-like sequences have been located on the CgA promoter (Iacangelo *et al.* 1991).

Exposure to GnRH resulted in up-regulation of the LH secretory response in LβT2 cells. This response was attenuated with increasing concentration of GnRH, to the point where the LH secreted on day 3 of treatment with

10 nM GnRH was nearly four times that secreted in response to 200 nM GnRH. This result may be due to increased desensitisation of GnRH-R at the higher concentrations of GnRH used, with concomitant loss of intracellular signalling events leading to the release of LH (Weiss *et al.* 1995), rather than to any decrease in GnRH-R numbers, as there were no changes in GnRH-R mRNA levels at any of the concentrations of GnRH used. In the experiment to examine the effects of GnRH concentration we did not detect any changes in LH β mRNA levels at any of the concentrations used. This was an unexpected result in the light of findings from the other experiments in this study, and of previously reported up-regulation of LH β mRNA after GnRH exposure (Turgeon *et al.* 1996). One explanation for this could be that GnRH may not have been adequately removed from the cells after the 15 min GnRH pulses, essentially leading to low levels of constant GnRH exposure during the 75 min inter-pulse intervals and the overnight incubations, resulting in decreased responsiveness of the intracellular pathways involved in GnRH-mediated signalling and subsequent down-regulation of LH β mRNA synthesis.

Stimulation of the cells with increasing concentrations of GnRH appeared to have no effect on the expression of SgII, but a concentration-dependent increase in CgA mRNA levels was observed. CgA mRNA levels were also increased in cells given the lower concentration (10 nM) of GnRH at a reduced pulse frequency, and these cells released less LH on a daily basis than cells given GnRH at the higher frequency. Intracellular levels of CgA also increased after exposure to 10 nM GnRH, but, in contrast to the mRNA results, this was significant only with the higher frequency of GnRH treatment.

Although there were no changes to SgII mRNA expression, intracellular levels of SgII were decreased after GnRH treatment. This presumably reflected the GnRH-induced stimulation of SgII secretion which was observed at both pulse frequencies. Although intracellular storage of LH was not changed significantly by GnRH treatment in this study, there was a trend towards a decrease, in line with that of SgII. The pattern of SgII secretion in cells given multiple pulses of GnRH was very similar to that of LH, and indicates probable co-secretion of the two proteins. The co-secretion of LH and SgII has been reported previously in perfused pituitary cell aggregates (Sion *et al.* 1988) and the two proteins are commonly found within the same granules in rat (Watanabe *et al.* 1991), sheep (Crawford *et al.* 2002a) and mouse (Crawford *et al.* 2002b) gonadotrophs. Although CgA secretion in L β T2 cells was also stimulated by GnRH treatment to a certain extent, release during the 4 pulses/day experiment did not show such a well-defined pattern as those of LH and SgII, with GnRH-induced stimulation of secretion only evident for pulse 1. Interestingly, the daily total secretion of SgII from cells given only 1 pulse/day of GnRH did not correlate with that of LH. SgII secretion

increased each day in response to GnRH, as did that of CgA, but whereas LH secretion from both control and GnRH-stimulated cells increased daily, probably as a result of cell proliferation, neither SgII nor CgA showed cell density-dependent increases in daily secretion. These results suggest that increasing cell density may have a detrimental effect on the basal secretion of both SgII and CgA.

It appears, therefore, that in L β T2 cells, there is a close correlation between LH and SgII storage and secretion and an inverse correlation between CgA production and LH secretion. It has been reported that higher levels of pituitary CgA coincide with greater numbers of large sized secretory granules, typically immunopositive for CgA, in gonadotrophs (Watanabe *et al.* 1998). As these granules are typically also immunopositive for LH, it is possible that more LH is being diverted, with or without SgII, into storage in these larger granules, rather than into the small LH/SgII immunopositive granules, which are released in response to GnRH stimulation. Further studies are required to determine the composition and distribution of granules within GnRH-stimulated L β T2 cells.

As well as being an integral component of secretory granules, the granins are known to function as precursors of biologically active peptides (Huttner *et al.* 1991). For CgA, the extent to which processing occurs appears to be highly tissue-dependent, with a correlation between the level of the prohormone convertase PC2 and the levels of processed forms of this protein (Arden *et al.* 1994). In the present study CgA levels have been quantified using two assays. The first used an antibody against amino acids hCgA324–337 which detects the WE-14 region of CgA (Portela-Gomes & Stridsberg 2001). It is not clear whether this assay detects mainly intact CgA, or also CgA-derived fragments, but it is thought that processing within this region is less common than in the terminal regions. The second assay utilised an antibody against amino acids hCgA17–38 which detects the CgA-derived peptide vasostatin. It has been shown previously that this assay is largely specific for vasostatin and generally does not detect the intact CgA molecule (Stridsberg *et al.* 2000). Processing does appear to occur in L β T2 cells as the hCgA17–38 vasostatin assay detected reasonable levels of this peptide in the samples analysed. However, vasostatin did not appear to be regulated by GnRH, and samples collected over the duration of the 4 pulses on day 3 of GnRH treatment showed no differences in levels of secretion indicating that this peptide is probably constitutively released from L β T2 cells. The function of vasostatin in L β T2 cells is unknown, but it has previously been shown to have a suppressive effect on vascular contractility and on parathyroid hormone secretion (Angeletti *et al.* 1994, 1996).

In conclusion, the results reported in this study indicate that there is a close correlation between the GnRH-stimulated release of LH and SgII from L β T2 cells, and

suggest that SgII may have an important role to play in the regulated secretion of LH, possibly by inducing the aggregation of LH to facilitate trafficking into specialised secretory granules. CgA secretion, on the other hand, does not appear to be closely associated with the release of LH, but evidence that CgA synthesis and storage are regulated by GnRH may indicate that it is also involved in the control of regulated LH secretion, perhaps by influencing the amount of LH sorted into the different types of secretory granules and thus altering the amount of LH available for release in response to GnRH stimulation.

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