Replenishment of LH stores of gonadotrophs in relation to gene expression, synthesis and secretion of LH after the preovulatory phase of the sheep oestrous cycle

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Abstract

The pattern of replenishment of LH secretory granule stores in sheep pituitary gonadotrophs, after an induced LH surge, was determined by immunogold localisation at the ultrastructural level by electron microscopy. Twenty-four Welsh Mountain ewes were initially synchronised with progestagen devices for 14 days before luteolysis was induced by a prostaglandin F2α analogue, cloprostenol. A further 24 h later, a preovulatory LH surge was induced by intravenous injection of a GnRH agonist, buserelin. Animals were divided into four groups (n=6) and blood sampled at 2 h intervals from 4 h prior, to 18 h after, buserelin administration and then at infrequent intervals (1 to 8 h) thereafter until death. Pulse profiles of LH were also obtained by an additional collection of blood samples within a 6 h window directly preceding death. Groups of animals were killed at 24, 48, 72 or 96 h after buserelin treatment. Pituitaries were dissected and processed for transmission electron microscopy and frozen for later molecular biological analysis. A characteristic preovulatory surge of LH was observed in all animals. The cytoplasm of gonadotrophs, in animals killed 24 h after buserelin treatment, was completely empty of secretory granules. This was associated with diminutive pituitary LH content, low pituitary GnRH binding levels and an almost complete absence (one pulse in one animal) of LH pulsatile secretion. Despite the lack of apparent secretory activity, clusters of exposed LHβ label present within the cytoplasm at this time and constant LHβ mRNA expression levels irrespective of tissue collection time, suggest that the cell is actively synthesising LHβ. The formation of sparse numbers of small LHβ immuno-labelled electron-dense secretory granules was apparent at 48 h after buserelin treatment, and replenishment of LHβ immuno-labelled granule stores continued until total granule numbers had increased two-fold (P<0.01) by 96 h post-treatment. Affiliated with granule replenishment was a significant increase in pituitary LH content (P<0.01), pituitary GnRH binding levels (P<0.01) and the restoration of LH pulsatile secretion. Despite the replenishment of granule stores with time, cytoplasmic area did not vary. These results suggest that restoration of pulsatile LH secretion after a preovulatory LH surge is related to replenishment of LHβ secretory granule stores and an increase in GnRH binding levels.


Introduction

Gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH) have long been recognised as a requisite in the regulation and overall success of reproductive function in most vertebrate species. Gonadotrophins are synthesised by, and secreted from, a highly specialised anterior pituitary gonadotroph cell. Populations of gonadotrophs are described as being both monohormonal and bihormonal secretory cells (Tixier-Vidal et al. 1975, Moriarty 1976, Lloyd & Childs 1988). In the rat, distinct populations of gonadotrophs have been characterised by size (Denef et al. 1980) and reportedly exhibit differences in their storage and secretory responses to gonadotrophin-releasing hormone (GnRH) (Lloyd & Childs 1988) as well as shift in proportions during the oestrous cycle (Childs et al. 1987). Limited studies of sheep pituitaries provide conflicting information whereby some studies report that there are 10–15% more LH-positive than FSH-positive gonadotrophs (Gross et al. 1984), while others observe a homogeneous population of bihormonal gonadotrophs (Currie & McNeilly 1995, Thomas & Clarke 1997). The proportion of monohormonal LH- and FSH-positive gonadotrophs may vary across the oestrous cycle (Taranget et al. 1998), and possibly in relation to changes in the pattern of pulsatile GnRH secretion (Molter-Gérard et al. 1999a, b).
Despite the fact that gonadotrophs are reported to be bi-hormonal, an ability which distinguishes them from other pituitary-derived endocrine cell types, gonadotrophins are not secreted in a parallel fashion (Chil
thesise and secrete both gonadotrophins, which have distinct but complementary actions exerting trophic effects on the gonads. It has been known for some time that LH secretion directly mimics the pulsatile release of GnRH (Clarke & Cummins 1982, Levine et al. 1985) while FSH is secreted in a tonic manner (Farnworth et al. 1988, Farnworth 1995). Previously, we have shown that LH secretion was not related to changes in LHβ mRNA levels and that the location of LHβ-immuno-labelled secretory granules within the gonadotroph changed during the oestrous cycle in ewes. The granules migrated from throughout the gonadotroph cytoplasm to an aspect abutting the nearest sinusoid (polarisation) as the oestrous cycle progressed (Currie & McNeilly 1995). This priming effect is thought to be facilitated through the actions of oestrogen in ovine gonadotrophs (Thomas & Clarke 1997), and GnRH in cultured mouse pituitaries (Lewis et al. 1986).

This study investigated the pattern of replenishment of LHβ immuno-labelled granule stores and of changes in ultrastructural morphology of gonadotrophs in sheep immediately after an induced preovulatory LH surge. These changes were related to levels of pituitary LHβ gene expression, LH content and GnRH binding.

Materials and Methods

Animals and experimental design

Twenty-four Welsh Mountain ewes with regular oestrous cycles were synchronised by withdrawal of progestagen-impregnated sponges (60 mg medroxyprogesterone acetate per sponge; Intervet Laboratories Ltd, Cambridge, Cambs, UK) and randomly assigned to four groups (n=6 per group). Luteolysis was induced 14 days later (approximately day 10 of following cycle) by administration of a synthetic analogue of prostaglandin F2α (100 mg cloprostenol; Estrumate, Coopers Animal Health Ltd, Crewe, Cheshire, UK). A further 24 h later, a preovula
tory LH surge was induced by intravenous injection of a GnRH agonist (50 mg buserelin; Hoechst, Frankfurt, Germany; Currie & McNeilly 1995). Each group of animals was killed at either 24, 48, 72 or 96 h after GnRH agonist administration. Blood samples were collected from all animals via jugular catheter at 2 h intervals from 4 h prior, to 18 h after, GnRH agonist administration and then at infrequent intervals (one sample per 1–8 h) thereafter until euthanasia. Pulse profiles of LH were also obtained by an additional collection of blood samples every 10 min for 6 h directly preceding death. Following euthanasia, pituitary glands were immediately removed, and a 1 mm tissue slice was removed and processed for immuno- cytochemistry. The remainder of the tissue was frozen in liquid nitrogen for analysis using molecular biology techniques.

Plasma concentrations of LH were measured by homologous radioimmunoassay as previously described (McNeilly et al. 1986). Within this study, the limit of detection for NIH ovine LH-S18 was 0.3 ng/ml, and the intra- and inter-assay coefficients of variation were 11·2 and 11·4% respectively.

Tissue preparation and immunocytochemistry

Pituitary glands were removed immediately following death and a small transverse section of pituitary tissue was removed from the adenohypophyseal mid-region in all animals for processing for TEM. The remainder of the pituitary gland was snap frozen in liquid nitrogen for analysis of pituitary LHβ mRNA expression and LH content, as well as GnRH binding.

Total RNA was extracted from frozen pituitary tissue and LHβ mRNA levels were quantified in 15 µg samples by Northern analysis using a 32P-labelled cDNA probe for LHβ (Brooks et al. 1992). Uniformity of loading was measured by reprobing the membrane with a rat 18S ribosomal RNA oligonucleotide probe. The intensity of the bands was quantified using a phosphorimaging system (Phosphorimagge 425; Molecular Dynamics, Sevenoaks, Kent, UK). Changes in specific mRNA levels were expressed as corrected values (expressed relative to 18S rRNA concentrations).

Pituitary LH content and GnRH binding levels were measured as previously described (Brooks et al. 1992). The sensitivity of the LH radioimmunoassay used to determine pituitary LH content was 2 ng/ml and the intra-assay coefficient of variation was 11·6%.

Tissue processing for TEM has been described in full in Currie & McNeilly 1995. In brief, tissue sections were immersion-fixed in 4% paraformaldehyde, 0·1% glutar
aldehyde in 0·1 M cacodylate buffer at 20 °C for no more than 18 h and then stored in fresh buffer until infiltration and embedding. Immediately prior to further processing, tissue sections were dissected into approximately 1 mm3 pieces and then subjected to a graduated acetone treatment regimen (50–100% Analar acetone; Scotlab, Coatbridge, Lanarkshire, UK). Specimens were infiltrated in Unicryl resin (British Biocell International, Cardiff, UK), embedded in gelatin capsules and heat polymerised at 60 °C for 48 h.

Thin sections (approximately 60 nm) were cut using a Reichert OMU4 ultramicrotome (Wild Leitz Ltd, Milton Keynes, Bucks, UK) and a Diatome diamond knife (Wild Leitz), and were mounted on 200 mesh nickel grids
Ultrathin sections were immunogold labelled for LHβ using a modification of a method described by Watanabe et al. 1991. Sections were blocked with 5% normal goat serum in Tris histochemical buffer (THB; 0·02 M Tris, 0·5 M sodium chloride, 0·1% BSA, pH 8·2) and washed briefly in THB. Gonadotrophs were identified using an immunogold labelling method whereby incubation with rabbit antisera against ovine LHβ (1:800; NIDDK-anti-oβLH-IC-1; AFP 69707P) was followed by a goat anti-rabbit IgG-gold particle conjugate (1:100; 5 or 15 nm diameter gold particles; British Biocell International) secondary antibody.

Stereological determination of LHβ immuno-labelled granule size within ovine gonadotrophs was conducted by systematic random selection (Lucocq 1993) of 20 cells per animal group (fully described in Currie & McNeilly 1995). TEM micrographs (magnifications=4600× and 7700×) of each cell were produced and then captured on a Sony video camera. Cells were selected indiscriminately until a minimum of 1000 granule profile diameters were measured by the Cue 2 Morphometric Analysis Software (Olympus Optical Company, London, UK) on an IBM computer. Granule profile diameter and distribution of granule size were calculated using the Schwartz–Saltykov diameter analysis (Underwood 1970), which requires the granules to be spherical in shape (i.e. axial ratio ≈ 1). The calculated axial ratio for 100 randomly selected granules in three different pituitaries from this study was 0·92 ± 0·05. Granule diameter measurements were divided into ten class intervals defined as equal percentage increments of the maximum observed granule diameter.

Statistical analysis

All values reported are means ± S.E.M. Data were analysed using one-way ANOVA followed by Fisher’s protected least significant difference test where a significant interaction (P<0·5) was found. The parameters of LH pulse secretion were determined using the Munro pulse analysis programme (Zaristow Software, Haddington, East Lothian, UK).

Results

Plasma LH concentration

A surge in plasma LH was induced immediately after GnRH agonist administration in every animal from all treatment groups (Fig. 1). Mean LH concentrations were at basal levels prior to GnRH agonist treatment. The maximum amplitude and duration of the LH surge were not significantly different between the groups and individual values ranged from 95–392 ng/ml and 10–14 h, respectively. After the preovulatory surge, mean plasma LH concentrations returned to basal levels for the rest of the sampling period. Plasma LH concentration was not significantly different between groups at time of death.

Mean plasma LH pulse frequency increased from only one pulse detected in one animal killed 24 h after GnRH agonist administration to multiple pulses detected in all

![Graph](https://example.com/graph.png)
other groups (Table 1). Representative profiles of LH pulses from all groups are shown in Fig. 2. Mean LH pulse amplitude (total pulse amplitude minus basal concentration) was not significantly different between animals killed at 48, 72 and 96 h after GnRH agonist treatment (Table 1). The amplitude of the single pulse measured in an animal killed at 24 h after treatment was lower than the amplitude of any pulses from animals in the remaining groups. Mean basal LH levels were not significantly different between any treatment groups (Table 1).

Pituitary mRNA LHβ expression, LH content and GnRH binding activity

Levels of expression of LHβ mRNA did not differ significantly between 24, 48, 72 or 96 h after GnRH agonist treatment (Fig. 3a). In contrast, pituitary LH content had significantly increased in animals killed at 48 (P<0.001), 72 (P<0.01) and 96 h (P<0.005) after GnRH agonist administration compared with animals killed at 24 h post-treatment (Fig. 3b). Although pituitary LH content appeared to continually increase after 48 h administration of GnRH agonist, this trend was not significant.Levels of GnRH binding in pituitary tissue also increased significantly in a constant manner after treatment (Fig. 3c).

Ultrastructural observations and immunocytochemistry

Pituitary gonadotrophs were immuno-identified using a LHβ antibody–gold conjugate complex. Gonadotrophs observed at 24 h after GnRH agonist administration were explicitly devoid of any visible secretory granules (Fig. 4a);
however, areas of cytoplasm were observed to be immuno-labelled for LHβ (Fig. 4b). The LHβ-gold label was only located within the cytoplasm of gonadotrophs and, although they were detected in clusters, they did not appear to be encased in a formal granule structure envelope. A small number of mitochondria were present throughout the cytoplasm at this time. At 48 h after GnRH agonist treatment, the formation of sparse numbers of small LHβ immuno-labelled electron-dense secretory granules was apparent (Fig. 4c, d). The number of mitochondria present appeared to have increased as compared with animals killed at 24 h post-surge. A high proportion of granules appeared to be located at the periphery of the cell, at an aspect juxtaposed to a blood vessel. At 72 h after GnRH agonist treatment, more granules appeared to be present within the cytoplasm as compared with the two previous time points, and these granules were again predominately located at a vascularity supported area of cell membrane (Fig. 4e, f). Large numbers of mitochondria appeared to be present. At 96 h after GnRH agonist treatment, gonadotrophs contained numerous LHβ-positive electron dense granules and they appeared to have increased in size (Fig. 4g, h). Granule re-population had progressed to the extent that morphology of some cells approached that of a classically non-polarised cell while others appeared to remain polarised.

**Stereological analysis of granule size and number, and gonadotroph cytoplasmic area**

The Schwartz–Saltykov analysis demonstrated the existence of a class of granules of approximately 61–90 nm in diameter (modal class 0.3). This class, with one exception (Fig. 5a (i)), was observed in all animals at 48 and 72 h after the induced LH surge (Fig. 5a, b), but was absent in animals killed at 96 h post-surge (Fig. 5c). In addition, the range of maximum granule diameter appeared to continually increase from animals killed at 48 h (range: modal class 0.7–0.8, granule diameter 181–240 nm) to 72 h (0.7–0.9, 181–270 nm) and 96 h (0.7–Dmax, 181–300 nm) after the induced LH surge. Irrespective of this apparent trend, the median granule diameter remained unchanged (with one exception, Fig. 5c (ii)) regardless of time of death (modal class 0.4, granule diameter approximately 91–120 nm).

The mean number of granules present within a gonadotroph cell had increased significantly (P<0.01) in animals killed 96 h after an induced LH surge as compared with animals killed 48 h post-surge (Table 1). However, the mean area of gonadotroph cytoplasm was not different between groups (Table 1).

**Discussion**

This study has depicted the cytoplasmic void present within gonadotrophs immediately after an exogenously
Figure 4 Transmission electron micrographs of immuno-identified gonadotrophs in sheep pituitary glands at varying times after induction of a LH surge with GnRH agonist, buserelin. (a) A gonadotroph and adjoining blood vessel at 24 h after the LH surge. Note that the cytoplasm is implicitly devoid of secretory granules. Scale represents 2 μm. (b) At higher magnifications, distinct, exposed clusters of LHβ-immunogold label (arrowheads) were observed in the cytoplasm of gonadotrophs at 24 h after the LH surge; however no formal granule structure was obvious. Scale represents 200 nm. (c) At 48 h after the LH surge, sparse numbers of secretory granules were evident within the cytoplasm and were located in a peripheral aspect of the gonadotroph (arrows). There appeared to be numerous mitochondria at this time compared with 24 h after the LH surge. Scale represents 2 μm. (d) At a higher magnification, newly formed distinct granules were immuno-positive for LHβ (arrowheads) at 48 h post-surge. Scale represents 200 nm.
Figure 4  (e) Gonadotrophs appeared to contain more secretory granules at 72 h than 48 h post-surge (c.f. Fig. 4c). Granules appeared to be less dispersed at this time and were located in close proximity to a vascularly supported area of cell membrane (arrows). Scale represents 2 μm. (f) At higher magnifications, the distinct secretory granules present at 72 h post-surge contained abundant LHβ label (arrowheads). Scale represents 200 nm. (g) At 96 h after the LH surge, gonadotrophs had numerous secretory granules that appeared to be evenly dispersed through the cytoplasm. Scale represents 2 μm. (h) Copious numbers of highly LHβ-labelled secretory granules (arrowheads) are evident at 96 h post-treatment. Scale represents 200 nm. G, gonadotroph; C, cytoplasm; M, mitochondria, and V, blood vessel.
induced LH surge, and its subsequent replenishment with secretory granules and cell constituents with time. The parameters of the induced LH surge, which every animal exhibited following administration of GnRH agonist, was similar to an endogenously regulated preovulatory LH surge (Brooks et al. 1993). At 24 h post-surge, all gonadotrophs were implicitly devoid of LH immuno-labelled secretory granules. In addition, few mitochondria were present at this time, suggesting these cells had a low energy requirement (Alberts et al. 1989) and therefore were in an inactive secretory state. The almost complete loss of detection of LH pulses in systemic plasma at this time supports this presumption. Despite depressed secretion, the synthesis of LH is evident in exposed 'premature' clusters of LH label within the cytoplasm. Furthermore, LH mRNA expression levels were consistent in all groups and low levels of pituitary LH content was detected 24 h after the surge. It should be noted that, due to the sensitive antigenic properties of FSHβ, tissue samples were not osmium-treated, a step that greatly enhances membrane contrast (Berryman et al. 1992). Therefore, although the 'premature' clusters of LHβ label did not appear to have a membranous envelope, it is possible these clusters were contained in small transitional vesicles and that the lack of detection of membrane structure was due to limitations of the TEM processing method. Alternatively, these clusters of LH label may represent peripheral Golgi-derived structures. In either case, future studies involving the ultrastructure of granules within gonadotrophs should employ a tissue-processing method that utilises a low concentration tannic acid post-fixation step, which has been shown to improve membrane contrast whilst preserving the antigenic properties of the tissue (Berryman et al. 1992).

The continuous formation of LHβ immuno-labelled secretory granules with time in this study was consistent with continuous augmentation of pituitary LH content and the return of pulsatile LH secretion. It is interesting to note that the newly synthesised secretory granules were predominantly located at a region of the cell adjacent to a sinusoid. The trafficking of granules in gonadotrophs to a vascularity supported area of cell membrane has previously been suggested to switch a cell from a non-releasable into a potentially releasable state (Currie & McNeilly 1995). This priming mechanism was suggested to be facilitated through the actions of either oestrogen (Thomas & Clarke 1997) or GnRH (Lewis et al. 1986). In this study, granulogenesis occurred either at a peripheral site adjacent

Figure 5 Comparison of gonadotroph granule diameter distribution, as determined by Schwartz-Saltykov analysis, in individual animals (i, ii, iii, iv) at (a) 48, (b) 72 and (c) 96 h after induction of a LH surge by GnRH agonist, buserelin. Class intervals are 10% decrements of the maximum granule diameter \( D_{\text{max}} = 300 \text{ nm} \).
to a blood vessel or elsewhere within the cytoplasm and the granules were actively redistributed to this potential exocytotic site. The latter hypothesis is favoured due to the apparent increase in number of mitochondria present as the post-surge interval increased. This increased energy requirement (Alberts et al. 1989) may be due to the transportation of secretory granules to their potential site of release.

Replenishment of secretory granules was so great that, by 96 h after the surge, some cells were no longer in a ‘polarised’ state. Perhaps, in the absence of a stored pool of granules, newly synthesised secretory granules containing LHβ are immediately positioned at the site of exocytosis to maintain basal levels of LH. Once sufficient numbers of granules are available at the cell–sinusoid interface, granules need no longer be actively synthesised in, or transported to, this area. However, an external signal, such as an increase in GnRH pulse frequency and/or oestrogen levels at impending ovulation, may stimulate active mobilisation of granules to this potential site of secretion (Currie & McNeilly 1995). Gonadotrophs would then be in a ‘releasable’ state for a further external signal to trigger a rapid and concomitant release of granule content resulting in the preovulatory LH surge.

Despite the extensive replenishment of secretory granules by 96 h after the surge, granules numbers appeared to have only reached half that observed in a previous study involving sheep killed on day 12 of the luteal stage. In addition, pituitary LH content of sheep killed 96 h post-ovulation in this study is also less than half that measured in day 12 luteal stage animals. Interestingly, GnRH-binding levels in day 12 luteal stage animals are similar to binding levels in animals killed at 24 h post-surge in this study (Currie & McNeilly 1995). This suggests that the increase in GnRH binding levels observed after the surge in this study may be due to an increase in oestriol levels which occur at this time due to follicle recruitment and maturation (Campbell et al. 1990). Future work should include tissue collected from animals killed later than 96 h post-surge to determine the time period required for gonadotrophs to fully replenish their gonadotrophin stores.

Cells contain a network of actin filaments, microtubules and intermediate filaments which comprise the dynamic cytoskeleton of the cell (Joshi 1998). A recent study has provided evidence for a direct linkage between vesicle transport and actin re-assembly (Rozelle et al. 2000), the latter directed by various actin–regulatory proteins (Carlier 1998, Puius et al. 1998). An increase in microfilament length and alterations in the orientation angle relative to the plasmalemma has been reported in gonadotrophs of female mice primed with GnRH (Lewis et al. 1985). Perhaps the actin fibres in unstimulated animals are arranged in a tightly packed bundle formation. This would allow for trafficking of smaller granules only to the potential site of release and maintenance of basal LH levels. External stimuli such as increased GnRH-pulse frequency or oestrogen levels that occur at impending ovulation, may modify actin formation into loose assemblies in gonadotrophs to allow trafficking of all granule sizes, and therefore become ‘polarised’ at impending ovulation as has been observed previously (Currie & McNeilly 1995, Thomas & Clarke 1997). The present study provided some inconclusive evidence that granules may increase in size with time. The upper limit of the range of maximum vesicle diameter increased from 240 nm at 48 h, to 300 nm at 96 h after the surge; however, median vesicle diameter (91–120 nm) remained unchanged with time and an overall shift in the number of larger granules present was not evident.

Studies in the rat have identified sex-related differences in the proportions of three distinct subsets of granules within gonadotrophs. In males, typical gonadotrophs contained both small granules immuno-labelled for LHβ and larger granules immuno-labelled for FSHβ and, to a lesser extent, LHβ. However, upon stimulation with GnRH treatment, a third type of bihormonal granule was identified called an ‘intermediate’ secretory granule which displayed distinct gonadotrophin compartmentalisation (Watanabe et al. 1993). Conversely, the principal granule type in female rat gonadotrophs was small, electron-dense and only immuno-labelled for LHβ. The ratio of intermediate secretory granules was dependent upon reproductive cycle stage (Childs et al. 1987, Watanabe et al. 1998). In this study, granules possessed a uniform appearance and no ‘intermediate’ form was observed. Double immuno-labelling for LHβ and FSHβ has identified segregation of gonadotrophins (FSHβ located at granule periphery) within granules in sheep gonadotrophs; however, there are no discernible compartmentalisation margins (personal observation).

An alteration in epithelial cell volume is not uncommon when tissue is in the process of remodelling (Crawford et al. 1999). Similarly, rat gonadotrophs undergo hypertrophy after ovariectomy, and subsequent destruction of GnRH neuron terminals countermands this effect (Cronin et al. 1982). In addition, gonadotrophs increase in both density and size as oestrus approaches in rats (Childs et al. 1982, 1987) and it was proposed that gonadotrophs expand at this time to provide a larger surface area to support increased numbers of GnRH receptors. In the present study, cytoplasmic area of gonadotrophs did not change significantly upon restoring LH reserves after an induced LH surge, despite significant alterations in the number of cytoplasmic constituents.

Pituitary GnRH-binding levels increase in ewes at impending ovulation due to GnRH receptor up-regulation caused by increased oestriol production from the dominant follicle(s) (Huang & Miller 1980, Moss & Nett 1980, Brooks & McNeilly 1994, 1996). Treatment with the GnRH agonist, buserelin, has been shown to actively reduce GnRH binding in the pituitary gland by
attenuating GnRH receptor mRNA expression levels (Brooks & McNeily 1994). In support, GnRH binding–levels in this study are low shortly after the induced LH surge; however, continually increase thereafter to peak 72 h post-treatment. This pattern of change in GnRH binding is probably due to recruitment of oestrogenic follicles, stimulated by the increasing FSH levels at this time (Baird & McNeily 1981).

In conclusion, gonadotrophs were completely devoid of secretory granules immediately after an induced LH surge. Cytoplasmic replenishment of granules was initially observed 48 h after an induced LH surge, and was associated with the return of pulsatile LH secretion and with augmented pituitary LH content. Newly synthesised secretory granules were located near the gonado- trophe periphery at a potential site of exocytosis, thereby exhibiting a ‘polarised’ distribution. By 96 h after induc- tion of a LH surge, granule replenishment had progressed to the extent that morphology of some cells approached that of a classically ‘non-polarised’ cell.

Acknowledgements

We should like to thank Mr Ian Swanston and Dr Julie Brooks for expert technical help; Mr Tom McFetters and Mr Ted Pinner for preparation of the figures; Mr Denis Doogan and staff at the Marshall building, University of Edinburgh for assistance with animals; and NIDDK for the provision of some assay reagents. J L Crawford was sup- ported by a Post-doctorate fellowship from the Journal of Reproduction and Fertility.

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Received 12 May 2000

Accepted 2 August 2000