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Expression of the oxytocin gene in the large cells of the bovine corpus luteum

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In the bovine ovary there is a delay of 4–6 days between the observed maximum of oxytocin mRNA and the peak in the luteal levels of oxytocin nonapeptide. This implies a maturation process involving components of the post-translational processing pathway. In situ hybridization shows the oxytocin gene to be transcribed exclusively in the large cells of the corpus luteum at the beginning of the estrous cycle.

Post-translational processing; Hybridization; Neuropeptide expression; Oxytocin; (Large luteal cell, Bovine corpus luteum)

1. INTRODUCTION

Although the nonapeptide oxytocin is primarily thought of as a neurohypophyseal hormone synthesized within the magnocellular nuclei of the hypothalamus, it has recently also been identified within several peripheral tissues, including the gonads. In particular, the ruminant corpus luteum has been shown to be especially rich in the hormone, releasing large quantities into the ovarian vein during the latter half of the estrous cycle, probably in response to the luteolytic secretion of prostaglandin $F_{2\alpha}$ by the uterus [1–3].

Sequence analysis showed that luteal oxytocin was the product of the same gene as in the hypothalamus, with an identical mRNA except that its polyadenyl tail was shorter [4]. Oxytocin mRNA levels measured through the estrous cycle indicated that the gene was transcribed predominantly in the first days after ovulation [5]. Yet oxytocin peptide levels measured in other studies [3,6] suggested peptide maxima at mid-cycle. Thus the preliminary luteal data point to a marked discrepancy between the appearance of mRNA and that of the processed peptide. This is in contrast to the hypothalamic situation where radiolabelled oxytocin or vasopressin appear in the neurohypophysis only hours, not days, after injection of [^{35}S]cysteine into the hypothalamic nuclei [7–9].

Using cows of known cycle date we have analysed both oxytocin mRNA and oxytocin peptide, within the same corpora lutea, to establish whether the bovine corpus luteum indeed exhibits a delay between transcription and appearance of the processed hormone. Transcription of the gene was measured either by dot-blot analysis or by in situ hybridization. The latter method allows precise localization of the cells where the respective gene is expressed.

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2. MATERIALS AND METHODS

2.1. *RNA preparation and assay*

Corpora lutea were collected from Fleckvieh cows of known estrous date within 20 min of death, and frozen immediately in liquid nitrogen. Later, RNA was prepared from a portion of these by the guanidinium isothiocyanate procedure as in [5]. 10 μg aliquots of this RNA were applied to a nitrocellulose filter using a slot manifold, and hybridized as described [5] with the oxytocin-specific probe (OT-3′ [4]), labelled by nick translation to a specific activity of 3×10^6 cpm/μg. Single-stranded m13 DNA into which the sense strand of the OT-3′ probe had been subcloned was used as calibration standard. After autoradiographic exposure for 4 h, exposed film was densitometrically scanned to quantitate the relative amounts of mRNA and calibration standard.

2.2. *Oxytocin and progesterone measurement*

Both oxytocin and progesterone were measured in extracted portions of the same corpora lutea, as above, using radioimmunoassays described elsewhere [3].

2.3. *In situ hybridization*

Oxytocin (OT-3′) and vasopressin (VP-3′) (see [4]) specific probes were subcloned into the plasmid pSP 64. Labelled single-stranded cRNA was obtained by transcription with [^{35}S]thio-GTP (Amersham Sp6 system) to give specific activities of 5×10^8 cpm/μg.

Corpora lutea of different days of the estrous cycle were obtained from Angus cross-bred cows of known cycle date, within 15 min of death, embedded in Tissue Tek mounting fluid and immediately frozen in liquid nitrogen. 6 μm cryostat-cut sections were mounted on cold 3′-aminopropyltriethoxysilane (Sigma) treated slides [10] and stored at −80°C. The tissue was not warmed at any stage of the preparation.

In situ hybridization was performed as described [11] with the following modifications: Slides were placed into freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, washed twice in PBS for 5 min each and then dehydrated by placing them for 5 min each into successively 60, 80, 95 and 100% ethanol. Slides were then dried in a vacuum desiccator. Prehybridization buffer contained 50% deionized formamide, 0.75 M NaCl, 0.025 M EDTA, 0.025 M Pipes, pH 6.8, 5 × Denhardt's, 0.2% SDS, 10 mM dithiothreitol, 250 μg/ml denatured herring sperm DNA, and 250 μg/ml yeast tRNA. Hybridization buffer contained in addition 10% dextran sulfate. 1 ml prehybridization buffer was then applied to each slide. These were placed flat into boxes lined with filter paper soaked with 50% formamide, and incubated for 3 h at 50°C. Prehybridization buffer was drained by tapping the edge of the slide against filter paper. 70 μl hybridization buffer containing 4 ng probe (1.7 ×

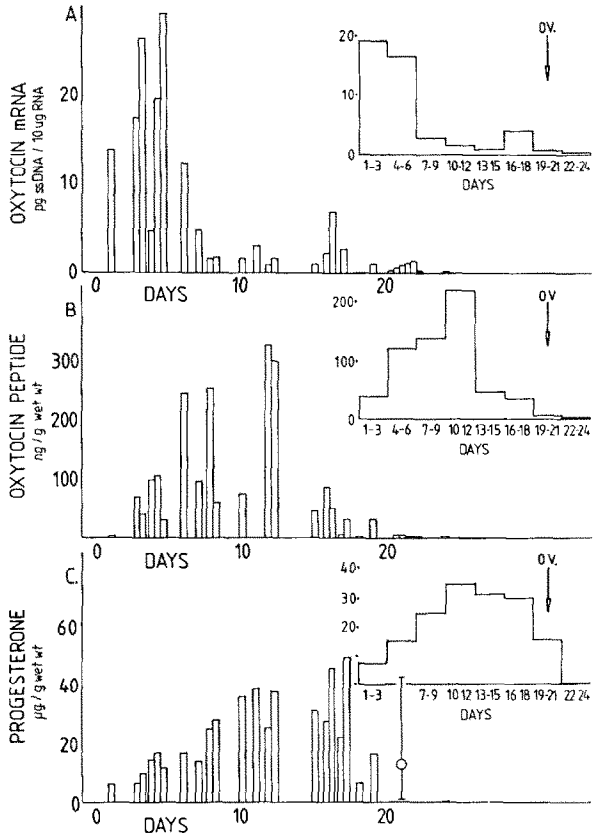

Fig.1. Levels of oxytocin mRNA (A), oxytocin peptide (B) and progesterone (C) in individual corpora lutea taken from cows of known estrous date. Individual values are shown as vertical bars in the main histograms. 3-day means are shown as insets. Because of the large number of samples collected for progesterone determination on day 21, these values are represented by a circle (mean) and a vertical (range). ov., day of new ovulation.

10^6 cpm) was applied to the tissue and coverslips were placed onto the slides and sealed with Pattex (Henkel KG, Düsseldorf). Hybridization was carried out at 50°C overnight in the tape-sealed filter-lined boxes.

After hybridization, coverslips were removed and the sections washed by dipping the slides into 4 × SSC containing 20 mM β-mercaptoethanol. After two such washes and two washes in 4 × SSC only, each of 5 min, the slides were placed into buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 40 μg/ml RNase A (Boehringer) and incubated for 30 min at 37°C. Slides were washed once in the same buffer without RNase A for 30 min at 37°C and then twice in 2 × SSC for 15 min at 50°C, dehydrated through 60, 80, 95 and 100% ethanol and dried in a vacuum desiccator.

The dried slides were exposed to X-ray film (Cronex) or dipped into Kodak NTB-3 nuclear track emulsion at 42°C. After exposure at 4°C for between 4 days and 2 weeks as indicated in the figure legends, autoradiograms were developed with Kodak chemicals, counterstained with hematoxylin eosin and coverslipped.

3. RESULTS

3.1. *Delay in the appearance of oxytocin peptide*

The bovine corpus luteum results from the massive tumor-like proliferation of the granulosa

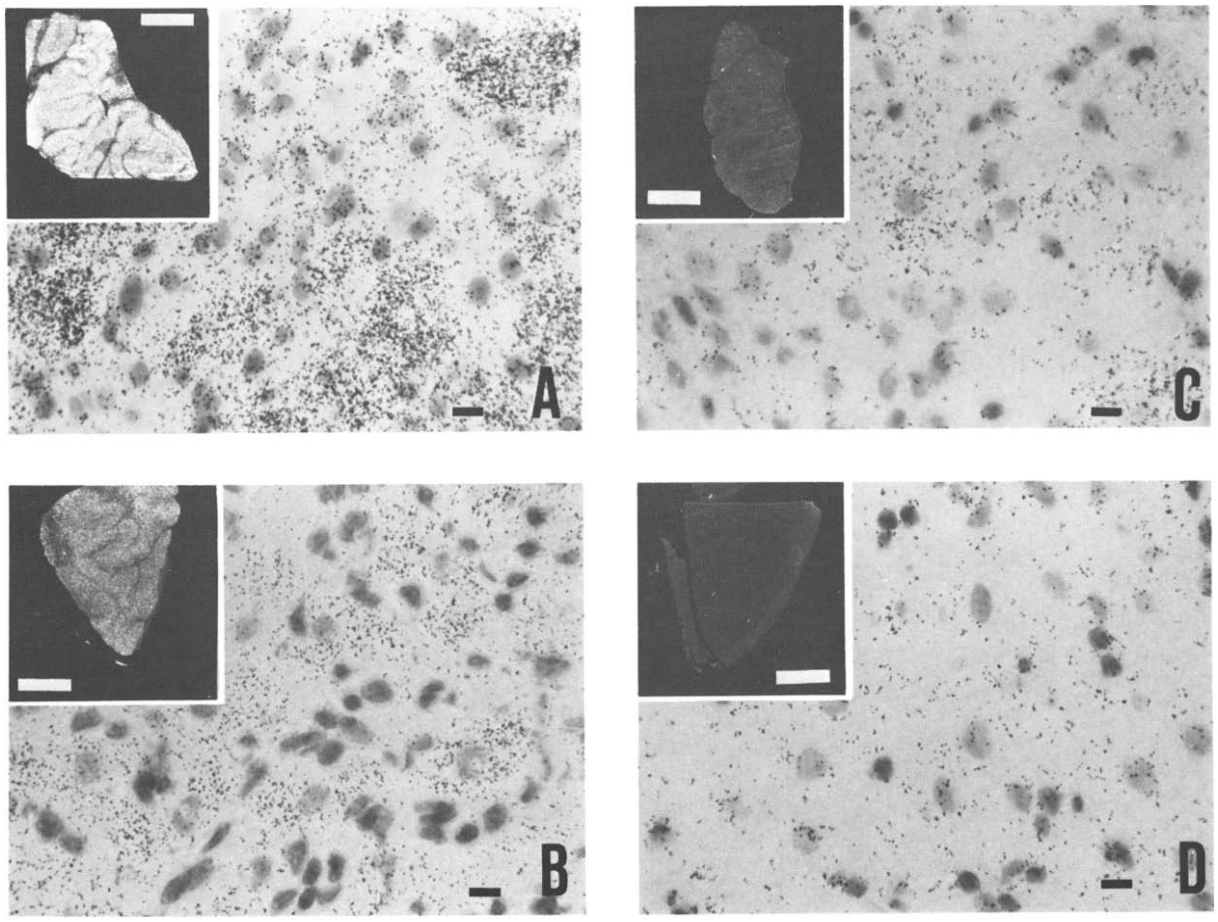

Fig.2. Oxytocin mRNA-specific in situ hybridization of bovine corpora lutea from various days of the non-pregnant cycle, and from pregnancy. A, day 3; B, day 11; C, day 19; D, pregnancy. Exposure for 2 weeks. Black bars, 1 μm; white bars, 3 mm.

and theca cell layers of the follicle from which the newly released oocyte has escaped. Necessarily this luteinization process is accompanied by considerable gene transcription [5]. One of these early genes is that for oxytocin (fig.1A) for which maximum mRNA levels are found in the first 6 days of the cycle, thereafter levels being reduced to basal amounts. The rapid decline from one day to the next around day 7 presumably reflects the relatively short half-life typical of most mRNAs.

It is a characteristic of the bovine corpus luteum that there is much variation between animals. This reflects the individual rates of biochemical activity within each animal, some cows having an mRNA or hormone production days in advance of others. For this reason, both individual data as well as pooled means are illustrated, to indicate the extent of variation and trends, respectively.

Fig.1B shows the accumulation of oxytocin peptide in the corpus luteum, using a radioimmunoassay system based on an antibody raised against the C-terminally amidated nonapeptide.

The histogram indicates that there is no correlation between the level of oxytocin mRNA and that of peptide within any one corpus luteum (cf. panels A and B). The trend is however evident: there is a marked delay in the maximum appearance of oxytocin peptide of some 6–7 days following the maximum appearance of oxytocin mRNA.

The principal steroidogenic function of the corpus luteum is indicated in the progesterone content (fig.1C), where maximum levels accumulate in the latter, luteolytic half of the cycle, offering a different profile from those for oxytocin and its mRNA.

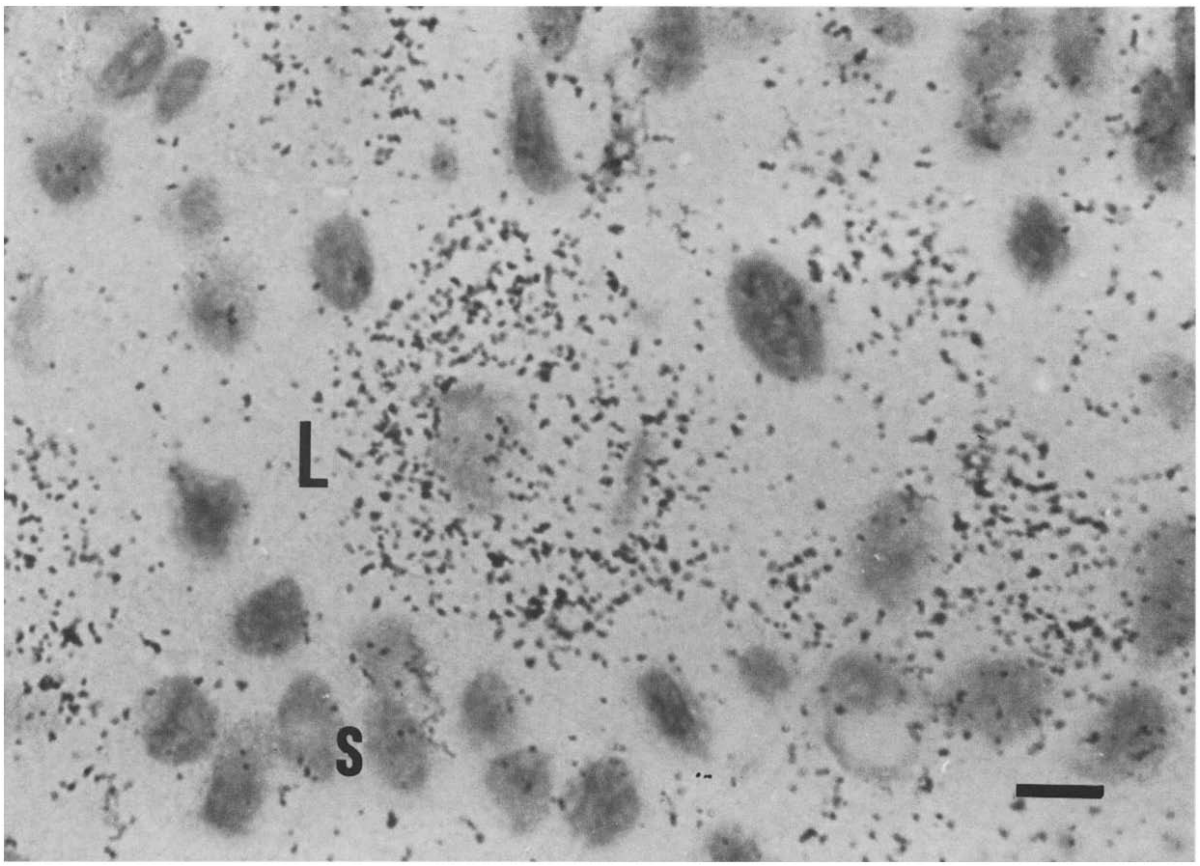

Fig.3. In situ hybridization of a day 3 bovine corpus luteum, exposed for only 2 days. L, large luteal cell; S, small luteal cells. Black bar, 1 μm.

3.2. *In situ hybridization of oxytocin mRNA*

Cryotome sections of bovine corpora lutea from various stages of the non-pregnant cycle as well as from pregnancy were prepared for in situ hybridization with single-stranded cRNA probes specific for oxytocin mRNA as described in section 2. The use of RNA single-stranded probes of high specific activity and subsequent RNase treatment of sections to reduce background significantly increased the sensitivity of this methodology. In macroscopic, dark-field view (fig.2, insets), the sections, which have been hybridized and autoradiographically exposed in parallel, indicate a high level of oxytocin gene expression on day 3 – white areas are the silver grains resulting from the mRNA-positive hybridization signals. The hybridizing cells belong at this young luteal stage to the layers of involuting and proliferating follicle wall. The dark areas are occupied by small epithelial cells, blood vessels and connective tissue. In the later corpora lutea (fig.2), there is clearly much less oxytocin mRNA and the convoluted gross anatomy gradually disappears.

At the microscopic level, here in normal incident illumination with only cell nuclei visualized by hematoxylin staining (figs 2,3), silver grains are localized in the cytoplasm, forming a halo around the larger, more lightly staining nuclei, belonging to the large luteal cells.

Comparing the microscopic distribution of oxytocin mRNA through the cycle shows firstly that this mRNA is confined to the large luteal cells, small cells, connective tissue and blood vessels being negative. Secondly, the quantitative decline in oxytocin mRNA is due to both a decrease in levels within individual large cells and to a reduction in the number of cells giving positive signals. As expected, very few positive cells could be identified in tissue from the luteolytic half of the cycle, and none at all from the corpus luteum of pregnancy (fig.2). A control using a cRNA probe specific for the vasopressin gene product (not shown) was also negative; levels of vasopressin mRNA are present in the bovine corpus luteum in 1000-fold lower amounts than those of the oxytocin-specific transcript ([4]; Ivell and Morley, unpublished), but lie below the detection limits of the in situ hybridization technique.

4. DISCUSSION

The current view on the genesis of the corpus luteum is that, accompanying ovulation, the cells of the follicular wall proliferate and involute with the inner granulosa layer forming the large luteal cells, and the outer theca layer giving rise to the interstitial small cells. As the cycle progresses the number of granulosa-derived large cells, based on expression of specific surface antigens, declines and theca-derived cells enlarge and replace the former cells [12]. In pregnancy the majority of cells are then of thecal origin.

A basal expression of the oxytocin gene including the secretion of the nonapeptide is associated with the granulosa layer of the preovulatory follicle [13,14]. Upon ovulation, there is a massive up-regulation of the oxytocin gene to high expression in the same cells, now part of the corpus luteum. No new cell types have started expressing the gene, agreeing with immunohistochemical observations [15,16]. The up-regulation is maintained only for a few days, and as demonstrated by the considerable drop in the oxytocin mRNA pool, is then cancelled, even though other metabolic processes of the corpus luteum, such as steroidogenesis (fig.1C) are maintained well into the latter half of the cycle.

However, what cannot be explained is the delay of 4–6 days between the peak of the mRNA pool and that of oxytocin peptide. Eucaryotic mRNAs generally have half-lives of only a few hours; oxytocin mRNA appears to be no exception as seen in the rapid drop in mRNA levels after day 6. In the hypothalamo-neurohypophyseal system, in vivo biosynthesis of oxytocin or vasopressin by translation of mRNA, processing and slow axonal transport over a long neuronal distance, has been measured in the rat [7,8] and dog [9] requiring altogether some 2 to 6 h, respectively. Therefore, to account for a delay in the appearance of oxytocin nonapeptide of several days there must be a delay at either the translational or post-translational level.

Electron microscopy reveals dense granule formation already at day 3, some of which are immunoreactive for neurophysin [16], the other component of the oxytocin precursor polyprotein. The number of such granules increases to a maximum at about day 11 [16], corresponding to the

peak of oxytocin observed here. The most likely explanation is therefore that the rapid cell proliferation characterizing luteinization and the growth of the bovine corpus luteum from ~0.1 g on day 1 to ~5 g on day 7 [5] is not accompanied by an equivalent increase in the elements and enzymes of the post-translational processing pathway. This system must 'mature' before the translated precursor can be converted into the physiologically active nonapeptide packaged into secretory granules and ready for release. A similar maturation has been suggested to account for the varying ratios of neurophysin to nonapeptide immunoreactivity in the developing human embryo [17], or in primary cell cultures of rat hypothalamus [18].

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REFERENCES

[1] Ivell, R. (1986) Top. Neuroendocrinol. 6, 1–19.
[2] Flint, A.P.F. and Sheldrick, E.L. (1982) Nature 297, 587–588.
[3] Schams, D., Schallenberger, E., Meyer, H.H.D., Butterman, B., Breitinger, H.J., Enzenhofer, G., Koll, R., Kruip, T.A.M., Walters, D.L. and Karg, H. (1985) in: Oxytocin: Clinical and Laboratory Studies (Amico, J.A. and Robinson, A.G. eds) pp.317–334, Excerpta Medica, Amsterdam, New York.
[4] Ivell, R. and Richter, D. (1984) EMBO J. 3, 2351–2354.
[5] Ivell, R., Brackett, K., Fields, M. and Richter, D. (1985) FEBS Lett. 190, 263–267.
[6] Wathes, D.C. (1983) J. Reprod. Fertil. 71, 315–345.
[7] Brownstein, M.J., Russell, J.T. and Gainer, H. (1980) Science 207, 373–378.
[8] Franco-Bourland, R. and Fernstrom, J. (1981) Endocrinology 109, 1097–1106.
[9] Sachs, H., Fawcett, P., Tabakatake, Y. and Portanova, R. (1969) Recent Prog. Horm. Res. 25, 447–491.
[10] Rentrop, M., Knapp, B., Winter, H. and Schweizer, J. (1986) Histochem. J. 18, 271–276.
[11] Higgins, G.A., Schmale, H., Bloom, F.E., Wilson, M.C. and Milner, R.J. (1986) J. Neurosci., in press.
[12] Alila, H. and Hansel, W. (1984) Biol. Reprod. 31, 1015–1025.
[13] Geenen, V., Legros, J.J., Hazee-Hagelstein, M.T., Louis-Kohn, F., Lecomte-Yerma, M.J., Demoulin, A. and Franchimont, P. (1985) Acta Endocrinol. 110, 263–270.
[14] Jungclas, B. and Luck, M.R. (1986) J. Endocrinol. 109, R1–R4.
[15] Guldenaar, S., Wathes, D.C. and Pickering, B.T. (1984) Cell Tissue Res. 237, 349–352.
[16] Fields, M. and Fields, P. (1986) Endocrinology 116, 1723–1725.
[17] Burford, G.D. and Robinson, I.C.A.F. (1982) J. Endocrinol. 95, 403–408.
[18] Vincent, J.D., Legendre, P., Poulain, D., Arnauld, E. and Theodosis, D. (1985) Front. Horm. Res. 13, 52–68.