Ultrastructural and cytochemical study of the bovine corpus luteum

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Summary. The large luteal cells comprise the bulk of the bovine corpus luteum during the most active secretory period of the oestrous cycle. At least 3 types of granules were present in mid-luteal cells: microperoxisomes, primary lysosomes and secretory granules. Only the last appear to be exocytosed. The concentration of these secretory granules in the luteal cytoplasm correlated with the reported profile of progesterone secretion by these cells throughout the cycle. Changes in the cytoplasmic proportions of the other subcellular organelles were consistent with the mobilization of steroid precursors stored in lipid droplets, synthesis of increased amounts of progesterone and protein, and the packaging of these products into discrete secretory granules.

Introduction

The corpus luteum is a suitable organ for correlating structure with endocrine function since it undergoes a series of defined structural and secretory phases within a short life-span. The histology of the bovine ovary has been described by various authors (McNutt, 1926; Hofliger, 1948; Rajakoski, 1960) and some histochemical reactions have been reported (Moss, Wrenn & Sykes, 1954; Lobel & Levy, 1968) but little morphological information is available at the ultrastructural level (Priedkalns & Weber, 1968; Gemmell & Stacy, 1979). Progesterone is the principal steroidogenic product, together with small amounts of 20 β -hydroxy-4-pregnen-3-one and pregnenolone (Mason, Marsh & Savard, 1962).

Densely staining granules (0.2–0.4 μ m in diameter) have been observed in a range of steroid-secreting cells (Christensen & Gillim, 1969), although their function is presently unknown. Cavazos *et al.* (1969) stated that the electron-dense granules of the pig corpus luteum were lysosomes whilst Belt, Cavazos, Anderson & Kraeling (1970) suggested that the granules stored relaxin. Gemmell, Stacy & Thorburn (1974, 1976) have proposed that in the luteal cells of non-pregnant sheep progesterone is packaged into granules and secreted from the cell via exocytosis of these granules. Recent in-vitro experiments are also consistent with an exocytotic mode of steroid hormone secretion in the sheep (Sawyer, Abel, McClellan, Schmitz & Niswender, 1979). Sub-cellular fractionation of the bovine corpus luteum showed that progesterone could be sedimented by centrifugation at moderate forces of gravity and was associated with the presence of densely staining granules (Quirk, Willcox, Parry & Thorburn, 1979).

We conducted a morphological examination of the luteal cells of the cow in conjunction with the fractionation studies reported by Quirk *et al.* (1979). Our objective was to determine if the bovine luteal cell contained densely staining granules analogous to those in the sheep.

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Cytochemical staining techniques were used to distinguish microperoxisomes and lysosomes from other densely staining bodies. A morphometric analysis of the cytoplasm of luteal cells was undertaken to correlate changes in the proportions of the subcellular organelles throughout the oestrous cycle with known endocrine events.

Materials and Methods

A corpus luteum was obtained within 5 min of death from Friesian cows on known days of the oestrous cycle from the Agricultural Research Council Laboratories at Compton, Berkshire. Representative tissue pieces ($\leq 1 \text{ mm}^3$) were immersion fixed in 3% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 2 h on ice. After overnight washing in buffer, the tissue was post-fixed for 30 min in cacodylate-buffered 1% (w/v) osmium tetroxide, dehydrated through an ethanol series, immersed in propylene oxide and embedded in Araldite. Sections were stained in methanolic uranyl acetate and lead citrate (Reynolds, 1963) and viewed in a Philips 301 electron microscope. For the cytochemical studies, the fresh corpora lutea were sliced into approximately 20- μm sections using a hand tissue-slicer and fixed for 3 h in 3% (v/v) glutaraldehyde buffer, pH 7.4, containing 7% (w/v) sucrose. For the determination of peroxidase activity, tissue sections were incubated in an alkaline diaminobenzidine (DAB) medium (pH 9.0) for 2 h at 37°C (Novikoff & Goldfischer, 1969). For acid phosphatase cytochemistry, 20- μm sections were fixed for 30 min in 1% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer containing 7% (w/v) sucrose and the enzymic activity was detected by the deposition of lead phosphate according to the method of Barka & Anderson described in Essner (1973).

Morphometry

Micrographs of tissue sections were obtained randomly by photographing the bottom two corners of every grid square. The proportion of luteal cells in single corpora lutea obtained on Days 6, 13, 15, 17 and 20 of the oestrous cycle was determined by using a random-point counting technique. This analysis provided the volume fraction of each cell type in the corpus luteum. The area fractions of various subcellular organelles were similarly estimated at higher magnification by the technique of Glagoleff (1933), as described in Williams (1977). In addition, selected sections stained for peroxidase activity were subjected to morphometric analysis.

Results

The large granulosa lutein cells were the most prominent feature of the mid-luteal corpus luteum, which also contained some small luteal cells interspersed with a few fibroblasts and loose connective tissue. As we did not observe morphological differences between large and small luteal cells as previously described (Priedkalns & Weber, 1968), they were regarded as part of the same population. The tissue was highly vascularized so that the large luteal cells (25–40 μm in diameter) were always close to capillaries. Their outer membranes were highly convoluted, providing the cell with a large surface area. The nuclei were similar in appearance to those of the luteal cells of other species, being large, round and approximately 10 μm in diameter. Mitochondria were abundant and dispersed throughout the cytoplasm (Pl. 1, Fig. 1); they were generally large and pleomorphic, and contained tubular cristae, a characteristic feature of other steroid-secreting cells (Christensen & Gillim, 1969) (Pl. 2, Fig. 2).

Agranular endoplasmic reticulum was the most prominent feature of the cytoplasm. It was distributed extensively and was only occasionally arranged in whorls. In this study, fixation of

Fig. 1. A section of corpus luteum obtained from a cow on Day 13 of the oestrous cycle. Luteal cells are adjacent to a capillary (Cp). They contain large nuclei (N) with prominent nucleoli (n); their cytoplasm contains numerous mitochondria (M), abundant agranular endoplasmic reticulum (AER), and patches of electron-dense granules (arrows) which are most abundant near the convoluted cell margins. $\times 3400$.

Fig. 2. A portion of a bovine luteal cell obtained at Day 13 of the oestrous cycle. Mitochondria (M) containing tubular cristae are dispersed throughout the cytoplasm. Golgi complexes (G) and granular endoplasmic reticulum (GER) occur discretely amongst the extensive and vesiculated agranular endoplasmic reticulum (AER). Densely staining granules (arrows) occur widely and some are found close to Golgi membranes. N = nucleus. $\times 10\,900$.

Fig. 3. Densely staining granules (single arrows) at the periphery of a large luteal cell obtained from a cow on Day 13 of the oestrous cycle. Some of the granules are located in the extracellular space (Ex), suggesting that they have undergone secretion (double arrows). $\times 30\,000$.

Fig. 4. Secretion of the granules (single arrows) is accompanied by loss of their surrounding membranes, followed by gradual diminution of their contents (double arrow). $\times 55\,000$.

Figs 5 and 6. Portion of a luteal cell, from a cow on Day 13 of the oestrous cycle, incubated with alkaline diaminobenzidine to demonstrate microperoxisomes. The sections are not counterstained and the dense osmiophilic reaction product can be seen over microperoxisomes (Mp). Figure 5 shows many unstained granules (single arrows) some of which have been secreted from the cell (double arrows). DAB staining is always intracellular. $\times 16\ 600$. In Fig. 6 a large lipid droplet (L) is also present and an unstained secretory granule (arrow). $\times 33\ 750$.

tissue pieces by immersion in glutaraldehyde yielded micrographs in which this reticulum appeared as a collection of swollen membrane vesicles, whereas in the living cell it is thought to consist of a system of branching and interconnected tubules (Enders, 1962; Christensen & Gillim, 1969). Granular endoplasmic reticulum occurred only in small amounts as discrete patches scattered throughout the cytoplasm, which also contained numerous free ribosomes and/or polysomes (Pl. 2, Fig. 2).

Golgi complexes were extensive and numerous in the cytoplasm of the large luteal cells, and consisted of stacks of closely packed cisternae and collections of vesicles. Small, membrane-bound vesicles, which were often electron-dense, were commonly observed in the region of the Golgi complex, suggesting that they had been 'budded off' from this organelle (Pl. 2, Fig. 2). Microtubules and microfilaments were occasionally observed but were not prominent features of the large bovine luteal cell, in contrast to the same cells of women (Crisp, Dessouky, Dessouky & Denys, 1970) and bitches (Crisp, Denys & Channing, 1972).

Electron-dense secretory granules were noticeable in the cytoplasm of the large cells of the mid-cycle bovine corpus luteum (Pl. 2, Fig. 2). They were 0.2–0.4 μm in diameter, compared to 0.2 μm diameter in sheep (Gemmell *et al.*, 1974). A single membrane enclosed the electron-dense contents, which were uniformly opaque at high magnification. The granules were usually spherical, although some pleomorphism was observed, such as a 'dumb bell' configuration. They were distributed in clusters throughout the cytoplasm, especially in the vicinity of the Golgi complexes, and were also often found near the edge of the cell. These electron-dense granules were observed in the extracellular space, which suggested that they had been secreted. Their extrusion from the cell was accompanied by fusion of their membrane with the cell membrane. Once outside the cell, the contents of the granules appeared to disperse: this process was accompanied by a gradual diminution in the granule electron-density (Pl. 3, Figs 3 and 4).

Microperoxisomes were indistinguishable from the secretory granules on morphological grounds. Similarly, primary lysosomes (if present) were not identifiable by morphological examination alone, but secondary lysosomes (containing inclusion bodies) were sometimes seen in the cytoplasm. Microperoxisomes were identified by their peroxidase staining and were distributed discretely throughout the cytoplasm (Pl. 4, Fig. 5). In general, they occurred in those areas of the cytoplasm that contained the greatest number of lipid droplets (Pl. 4, Fig. 6). The diameter of the microperoxisomes ranged from 0.15 to 0.25 μm . The larger osmiophilic granules (0.2–0.4 μm in diameter) did not stain cytochemically for peroxidase activity and were morphologically classified as the putative secretory granules. Deposits of diaminobenzidine were always intracellular.

The putative secretory granules did not stain for acid phosphatase either in the luteal cell cytoplasm or in the extracellular space. These results were confirmed in tissue sections of bovine corpora lutea obtained on Days 6, 12, 15 and 20 of the oestrous cycle.

Lipid droplets were present in some of the cells. Their contents were uniformly opaque and in general their concentration varied inversely to the numbers of electron-dense granules present. In cells containing large numbers of lipid droplets, evidence for exocytosis of electron-dense granules was rare.

Morphometry

Low magnification micrographs were analysed to obtain the proportions of luteal cells (Table 1). One corpus luteum was examined for each stage of the cycle. The luteal cells comprised about 50% of the area fraction of the corpus luteum on Day 6, at which time the secretion rate of progesterone was still rising (Herriman, Harwood, Mallison & Heitzman, 1979). At Day 12, progesterone secretion was maximal and the large luteal cells reached their greatest area fraction of around 70%. At Day 20, when the corpus luteum ceased secreting

significant amounts of progesterone and degenerative processes predominated, the proportion of large luteal cells decreased to represent about 60% of the corpus luteum.

Table 1. The proportions (see 'Materials and Methods') of luteal cells in the bovine corpus luteum throughout the oestrous cycle

		Day of oestrous cycle				
		6	13	15	17	20
Luteal cells	No.	1045	2990	1663	1629	1578
	%	51.2	70.9	71.6	74.2	59.0
Blood vessels + RBC	No.	249	308	173	156	174
	%	12.2	7.3	7.5	7.1	6.5
Extracellular space	No.	606	735	349	322	616
	%	29.7	17.4	15.0	14.7	23.1
Other tissue	No.	142	183	139	89	305
	%	7.0	4.3	6.0	4.1	11.4
No. of random points		2042	4216	2324	2196	2673
No. of fields		17	36	19	20	24

The final magnification was $\times 4130$.

Table 2. The proportions (see 'Materials and Methods') of subcellular components in the cytoplasm of the large cells of the bovine corpus luteum at different stages of the oestrous cycle

		Day of oestrous cycle				
		6	13	15	17	20
Mitochondria	No.	346	570	678	628	669
	%	15.6	25.6	31.0	25.8	34.0
Lipid droplets	No.	204	60	36	121	342
	%	9.2	3.2	1.6	5.0	17.4
Agranular endoplasmic reticulum + groundplasm	No.	1317	1215	937	1033	590
	%	59.4	54.5	42.8	42.4	30.0
Granular endoplasmic reticulum + ribosomes	No.	6	164	167	297	229
	%	0.3	7.4	7.6	12.2	11.7
Densely staining bodies	No.	55	69	64	136	37
	%	2.5	3.1	2.9	5.6	1.9
Golgi vesicles	No.	19	72	111	96	32
	%	0.9	3.2	5.1	3.9	1.6
Golgi cisternae	No.	40	42	56	59	18
	%	1.8	1.9	2.6	2.4	0.9
Lysosomes	No.	2	1	7	30	37
	%	0.09	0.05	0.03	1.2	1.9
Open vesicles	No.	209	29	124	11	7
	%	9.5	1.3	5.7	0.5	0.4
Crystalline bodies	No.	2	2	3	0	0
	%	0.09	0.09	0.14	0	0
Unassigned	No.	12	4	6	23	5
	%	0.5	0.2	0.3	0.9	0.25
No. of random points		2212	2228	2189	2434	1966
		21	22	22	22.5	22

The final magnification was $\times 14\ 030$.

Morphometric analysis of the cytoplasm of large luteal cells on 5 different days of the oestrous cycle showed that the proportion of mitochondria rose throughout the cycle (Table 2). Lipid droplet numbers fell as the rate of secretion of progesterone by the luteal cells increased but rose by Day 20 when progesterone secretion had ceased. The protein-synthesizing compartment, consisting of granular endoplasmic reticulum plus polysomes and/or ribosomes, increased steadily from Day 6 to Day 17 of the cycle. The Golgi apparatus (cisternae plus vesicles) constituted 5–7% of the luteal cell cytoplasm from Days 6 to 17, but by Day 20 its contribution had declined to approximately 1% of the cytoplasm. Until Day 20, agranular endoplasmic reticulum and groundplasm were the most abundant cytoplasmic components, as expected in a steroidogenically active tissue (Christensen & Gillim, 1969).

The population of densely staining bodies included not only the putative progesterone secretory granules but also microperoxisomes and any other electron-dense, membrane-bound body (including primary lysosomes) of 0.2–0.4 μm diameter. The general class of densely staining bodies varied with the stage of the oestrous cycle. The concentration of microperoxisomes doubled between Days 6 and 15 to constitute approximately one-third of the total population of electron-dense granules (Table 3).

Table 3. The cytoplasmic proportions of microperoxisomes in the large luteal cells at different times of the oestrous cycle

	Day of oestrous cycle		
	6	15	17
No. of microperoxisomes	42	76	72
No. of other densely staining bodies	216	156	148
% of microperoxisomes	16.3	32.8	32.7
No. of fields examined	14	13	14

The final magnification was $\times 14\ 030$.

Discussion

The morphological features of the large luteal cells of the cow were comparable to those described for steroid-secreting cells generally and for the luteal cells in particular of other mammals (Fawcett, Long & Jones, 1969; Christensen & Gillim, 1969; Enders, 1973). Although bovine luteal cells contained abundant agranular endoplasmic reticulum, it was only rarely in whorls, and even then the whorls were not as extensive as those observed in the luteal cells of other animals (Bjersing, 1967; Crisp *et al.*, 1970; Crombie, Burton & Ackland, 1971).

Previous cytochemical studies of steroidogenic cells have concentrated on the role of lysosomes in steroidogenesis (see Elfont, Roszka & Dimino, 1977) or luteal cell regression (see Bjersing, Hay, Moor, Short & Deane, 1970; Gemmell *et al.*, 1976) and on the distribution of microperoxisomes among different cell types. Microperoxisomes have been found in the cells of the adrenal cortex (Beard, 1972), the testicular interstitium (Reddy & Svoboda, 1972) and the primate corpus luteum (Gulyas & Yuan, 1975). Gulyas & Yuan (1975) observed three distinct populations of granules in luteal cells of the pregnant rhesus monkey: large granules of irregular outline, medium-sized granules (0.5–1.2 μm diameter), and small granules (0.1–0.45 μm diameter) which they identified as microperoxisomes by cytochemistry. They were unable to suggest a function for the largest granule; they could have been lysosomes or analogous to the relaxin storage granules in the luteal cells of pregnant pigs (Cavazos *et al.*, 1969; Belt, Anderson, Cavazos & Melampy, 1971) and rats (Long, 1978). The medium-sized granules were not microperoxisomes (Gulyas & Yuan, 1975), but were morphologically very similar to the secretory granules found in the luteal cells of sheep (Gemmell *et al.*, 1974) and cows (present

study). The role of microperoxisomes in the metabolism of luteal cells is unknown. The fact that they appear to be derived from the agranular endoplasmic reticulum and that they are found in association with (or at least adjacent to) lipid droplets in the cytoplasm of a number of different cells (Leuenberger & Novikoff, 1973; Novikoff & Novikoff, 1973; Novikoff, Novikoff, Quintana & Davis, 1973) has led to the suggestion that microperoxisomes are involved in steroidogenesis in luteal cells (Gulyas & Yuan, 1975).

The localization of relaxin antibodies over granules in the pregnant sow has been achieved by the immunoperoxidase technique (Kendall, Plopper & Bryant-Greenwood, 1978). The human corpus luteum is a rich source of relaxin during pregnancy also, and its secretion correlates well with that of luteal progesterone at mid-pregnancy and term (Weiss *et al.*, 1977). However, relaxin was undetectable in the corpora lutea of non-pregnant women (O'Byrne *et al.*, 1978) and during the oestrous cycle of sheep maximal circulating concentrations of progesterone and relaxin did not coincide (Chamley, Stelmasiak & Bryant, 1975). Thus, the intragranular sequestration of relaxin may be a phenomenon restricted to pregnancy, especially as Cavazos *et al.* (1969) and Belt *et al.* (1971) did not obtain evidence for the secretion of relaxin granules from the luteal cells of non-pregnant sows.

In sections of bovine luteal cells stained for acid phosphatase activity, membrane-bound granules other than lysosomes were also adjacent to the cisternae of the Golgi apparatus, suggesting that they had been formed by this organelle. These granules were free of product resulting from acid phosphatase activity. Such an observation has also been made in the sheep (McClellan, Abel & Niswender, 1977). These findings, together with the observation that not all of the Golgi cisternae were acid phosphatase-positive, suggest that the organelle has functions additional to the packaging of lysosomes and correlates with the observation that Golgi membranes were prominent in luteal cells of the mid-cycle cow, when lysosomes were not numerous. Elfont *et al.* (1977) have observed acid phosphatase staining around the rim of lipid bodies in gonadotrophin-treated, immature rats and suggested that the lysosomal system is involved in making substrate available for steroid synthesis and/or facilitating steroid secretion. However, the possibility that microperoxisomes are also involved in steroidogenesis (e.g. mobilization of cholesterol from lipid droplets) in the cow cannot be excluded. They comprised at least 4-fold more of the luteal cell cytoplasm than lysosomes during the active luteal phase and were commonly observed adjacent to lipid droplets, whereas the lysosomes present at these times appeared to occur randomly throughout the cytoplasm. Acid phosphatase staining around the rim of lipid droplets was only observed during luteal regression in the cow, by which time secondary lysosomes were common and secretion of progesterone had practically ceased.

It is possible that small numbers of primary lysosomes are required during the luteal phase to degrade unwanted compounds including gonadotrophin or even steroid receptors. Polypeptide hormones such as insulin, prolactin and growth hormone have been localized within cells using immunofluorescent techniques (Kolata, 1978) and specific binding of LH to lysosomes has been demonstrated in ovine luteal cells (Chen *et al.*, 1977). Prostaglandin and gonadotrophin receptors have also been found associated with lysosomal membranes in bovine luteal cells (Mitra & Rao, 1978).

Increased concentrations of mitochondria in luteal cells at mid-cycle correlate with the expected requirement for greater amounts of cholesterol side-chain cleavage activity to accompany increased synthesis of progesterone. Lipid droplets contain cholesterol in the cow (Flint & Armstrong, 1971) and cholesterol esters in other species (Fienberg & Cohen, 1965). The concentration of lipid droplets in bovine luteal cytoplasm varied inversely with the rate of progesterone synthesis throughout the oestrous cycle, in agreement with their projected role as stores of steroid precursors (Deane, Hay, Moor, Rowson & Short, 1966). Groundplasm-agranular endoplasmic reticulum, whilst remaining the most abundant subcellular compartment of the luteal cytoplasm, nevertheless declined during the course of the oestrous cycle as the proportion of other organelles increased. The role of secondary lysosomes in luteal regression is

well known (Deane *et al.*, 1966; Dingle, Hay & Moor, 1968; Bjersing *et al.*, 1970; Gemmell *et al.*, 1976; McClellan *et al.*, 1977; Elfont *et al.*, 1977). The first structural signs of luteolysis in the sheep were seen at Day 12 of the oestrous cycle when a decrease in the secretion of progesterone and concentration of densely staining granules coincided with the emergence within luteal cells of secondary lysosomes and autophagocytic bodies (Gemmell *et al.*, 1976).

Secretory granules were present throughout the luteal phase of the bovine oestrous cycle. Although they comprised only 2–4% of the luteal cell cytoplasm, they are probably packaged and released rapidly so that few mature granules would be in the luteal cytoplasm at any time. The concentration of secretory granules was maximal on Day 17, at the onset of luteolysis. This is compatible with the finding that structural dislocation of the secretory process precedes the cessation of progesterone synthesis in the cow (Hansel, Concannon & Lukawzewska, 1973). The numbers of secretory granules would then increase temporarily in the cytoplasm before being degraded by secondary lysosomes.

The presence of large nucleoli during the active secretory phase, together with patches of well developed granular endoplasmic reticulum and numerous ribosomes and/or polysomes scattered throughout the cytoplasm, suggests that protein synthesis is an important feature of luteal metabolism in the cow. Secretion of protein and steroid occurs in the adrenal and corpus luteum (Rubin, Sheid, McCauley & Laychock, 1974; Laychock & Rubin, 1974; Gemmell, Laychock & Rubin, 1977; Abel, Verhage, McClellan & Niswender, 1977). The bovine corpus luteum contains binding proteins for progesterone (Willcox, Jenkin, Quirk & Thorburn, 1978; Willcox, 1979) which may be involved in intracellular synthesis and/or transport of the hormone. It is likely that the presence of a progesterone-binding protein in the secretory granules of the bovine corpus luteum renders the granules electron-dense.

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