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Co-Localization of Rab3B and Oxytocin to Electron Dense Granules of the Sheep Corpus Luteum During the Estrous Cycle

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ABSTRACT

Oxytocin and its carrier protein, neurophysin, are both associated with luteal secretory granules which migrate from the paranuclear region to the cell membrane where exocytosis takes place. Rab3 proteins are thought to be associated with membrane vesicles or granules undergoing exocytotic fusion with the plasma membrane. The objective of this study was to determine whether Rab3B is co-localized with oxytocin within the same secretory granules of large luteal cells obtained from corpora lutea of 16 Merino cross ewes at day 3, 7, 12 or 15 of the estrous cycle using immunocytochemistry.

The mean granule density (granules/μm^3) was not significantly different ($P > 0.05$) between the days examined. Electron microscopic immunocytochemistry showed that oxytocin and Rab3B were co-localized to the secretory granules on all days evaluated. Rab3B immunostaining was primarily located within secretory granules scattered throughout the cytoplasm. The mean intensity of labelling (number of gold particles) for oxytocin per μm^2 cytoplasmic luteal tissue was significantly decreased on day 15 compared to those observed on days 3, 7 and 12 of estrous cycle. No significant changes were observed in the mean intensity of the Rab3B label at the different times of the cycle.

The present study provides evidence that a member of the subfamily of Rab proteins, Rab3B, is present and co-localized with oxytocin in the same secretory granules of the ovine corpus luteum. These results implicate Rab3B protein directly or indirectly in the hormone secretory pathway of ovarian tissue. Anat Rec 254:214–221, 1999.

Key words: **oxytocin; Rab3; exocytosis; granules; immunohistochemistry**

The corpus luteum of the ewe is composed of two types of steroidogenic cells, small and large luteal cells, and non-steroidogenic cells, fibroblasts, capillary endothelial cells and pericytes (O'Shea et al., 1979; Wiltbank, 1994). Densely staining secretory granules have been identified in the large luteal cells of the non-pregnant ewe (Gemmell et al., 1974; O'Shea et al., 1979) and cow (O'Shea et al., 1990). Similar granules have also been observed in luteal cells of the pig (Belt et al., 1971; Gemmell and Stacy, 1979) and rat (Long, 1973).

Immunocytological studies have indicated that oxytocin and its carrier protein, neurophysin-I, are both found

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associated with luteal secretory granules in the ewe (Watkins, 1983; Theodosis et al., 1986) and cow (Ivell et al., 1985) and oxytocin-neurophysin-specific mRNA has also been localized within the cow large luteal cells (Fehr et al., 1987), the site of oxytocin synthesis (Rodgers et al., 1983).

The electron dense staining granules are formed by the Golgi complex, accumulate at a paranuclear region, and then migrate to the cell membrane where exocytosis takes place (Gemmell and Stacy, 1979).

In cells in which exocytosis has been investigated in detail, exocytosis is carried out by transport organelles that bud from one compartment and fuse with another (Pryer et al., 1992) and is associated with both constitutive and regulated secretion. Constitutive exocytosis is apparently unregulated, secretory substances are not packaged into densely staining secretory granules but are released continuously to the cell surface as a bulk flow process whether or not the cells are stimulated (Kelly, 1985). Regulated exocytosis is associated with neuronal communication and hormone secretion. Proteins assigned to this pathway are stored in the trans-Golgi network, where they are packaged into secretory granules that undergo a process of maturation (Tooze and Stinchcombe, 1992). The mature secretory granules are stored in the cytoplasm and fuse with the plasma membrane in response to a trigger event (Burgoyne and Morgan, 1993) activated by a rise in free Ca^{+2} (Pryer et al., 1992).

A previous study by Rice et al. (1986) has shown that in homogenates ovine luteal cells, oxytocin immunoreactivity is associated with the particular fraction containing the granules. The large luteal cells of the secretory granules will deplete in response to $PGF_{2\alpha}$ (Braun et al., 1988) and induce an ovarian release of oxytocin (Flint and Sheldrick, 1982). Hirst et al. (1986) demonstrated that calcium is also involved in oxytocin release from ovine luteal cell slices as hormone output is reduced in calcium-free medium and increased by calcium ionophore. These results imply that oxytocin release from the cell is by a regulated rather than a constitutive pathway.

More than 30 GTP-binding proteins related to Ras have been identified from mammalian cDNA libraries which are termed Rab proteins. The low molecular weight GTP-binding proteins of the Rab family act as central regulators of vesicular traffic (Goud et al., 1988). Distinct Rab proteins are associated with different organelles involved in exocytotic (Goud et al., 1990) and endocytotic pathways (Chavrier et al., 1990) as well as synaptic vesicles (Fischer von Mollard et al., 1990), suggesting that they function in the selective targeting and binding of carrier vesicles to their receptor sites.

Studies on synaptic vesicles and chromaffin granules have demonstrated that the Rab3 proteins are associated with membrane vesicles or granules undergoing exocytotic fusion with the plasma membrane (Fischer von Mollard et al., 1990; Oberhauser et al., 1992). The Rab3 proteins consist of four members, termed Rab3A, Rab3B, Rab3C and Rab3D (Baldini et al., 1992; Fischer von Mollard et al., 1994), and are expressed exclusively in cells that have a high activity of regulated exocytosis (Mizoguchi et al., 1989; Baldini et al., 1992). Rab3B protein, which is mainly cytosolic, is present in the synaptic vesicles of brain (Moya et al., 1992), platelets, endothelial cells, pituitary and epithelial cells (Karniguian et al., 1993; Lledo et al., 1993; Weber et al., 1994). Rab3A protein shares 80% amino acid identity with Rab3B protein, is specific for synaptic vesicles in neural tissue and is absent from platelets and endothelial cells. The expression of Rab3B in rat anterior pituitary cells was abolished when lactotroph cells were microinjected with specific antisense RNA and resulted in inhibition of exocytosis (Lledo et al., 1993).

The present study was undertaken to investigate the co-localization of Rab3B and oxytocin within secretory granules of large luteal cells of tissue collected at different times during the ovine estrous cycle.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Experimentation Ethics Committees of Victoria University of Technology and Monash University, Department of Physiology.

Sixteen 2-year-old Border Leicester Merino cross ewes, average weight 40 kg, which showed regular cyclic activity were used in this study. The estrous cycles of the ewes were synchronized by the insertion of an intra-vaginal controlled internal drug release (CIDR) device impregnated with 300 mg progesterone (EAZI-breed CIDR, InterAg, Hamilton, New Zealand) for 14 days followed by an intra-muscular injection of Folligon Serum Gonadotrophin (400 IU; Intervet Pty., NSW Australia) at the time of CIDR removal. Ewes were kept in the pasture with vasectomized rams fitted with a harness and marking crayon with access to water and were fed lucerne chaff, oats and ewe and lamb pellets ad libitum. Ewes were checked twice daily for behavioral estrus. The day that the ewe displayed estrous behavior (first marking by the ram) was designated as day 0 of the estrous cycle.

Corpora lutea were collected from each of 16 ewes at laparotomy on days 3, 7, 12 and 15 of the estrous cycle (n = 4 per selected day). Day 15 of the cycle represents just prior to the commencement of functional luteolysis as measured by progesterone concentrations in peripheral blood in previous study using same breed of sheep (Al-Matubsi et al., 1998).

Tissue Collection

Anaesthesia was induced and maintained by gradual injection of Letha barb (euthanasia injection, Virbac) via the jugular catheter. The reproductive tract was then located through a ventral midline incision and a catheter (24 G; Optiva, Critikon, Florida, USA) was inserted into the ovarian artery of the ovary bearing the corpus luteum or into the ovarian vein (back perfusion). The position of the catheter was confirmed by injection of a small quantity of food dye (C.I. 42090). Rapid coloring of the ovary indicated that the catheter was appropriately placed. Before commencing the perfusion, the ovarian artery or vein was ligated at a point proximal to the anastomosis with the catheterized branch. Krebs Ringer bicarbonate (Rodgers and O'Shea, 1982) was first perfused at 4 ml/min to remove blood from the ovary over a period of approximately 5 min via a hypodermic syringe using gentle manual pressure. The tissue was then fixed primarily by infusion of 0.067 M cacodylate buffer containing 1% paraformaldehyde (Merck, Darmstadt, Germany), 1.5% glutaraldehyde (grade I, 25% aqueous solution; Sigma, St. Louis, MO), and 0.05% calcium chloride (Analytical; Ajax Chemicals, Sydney, Australia) pH 7.2 followed by 50 ml of

0.067 M cacodylate buffer containing 4% paraformaldehyde, 5% glutaraldehyde pH 7.2. The ewes were killed at the end of the perfusion by an overdose of Letha barb. The perfused ovary was removed. The corpus luteum was decapsulated and cut into fine pieces (3–5 mm^3) using a razor blade and placed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 24 hr. The glutaraldehyde was removed and the tissue washed and placed in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C until processed for electron microscope.

Processing for Electron Microscopic Morphology

Two cubes of tissue from each corpus luteum were randomly selected, dissected into 1–3 mm^3 cubes and postfixed in 1% osmium tetroxide (Probing and Structure, Central Queensland, Australia) in sodium cacodylate buffer, rewashed in buffer, dehydrated by passing through increasing concentrations of ethanol followed by 100% ethanol-treated with copper sulfate. The tissue blocks were immersed in propylene oxide (BDH Chemicals, Victoria, Australia), then overnight with 2:1, followed by 1:2 (v/v), propylene oxide:epoxy resin (Probing and Structure). The tissue blocks were placed in pure resin for 8 hr, replaced with fresh resin overnight, and finally embedded in pure resin using flat embedding mould (14 × 5 × 6 mm; Probing and Structure) at 60°C for 48 hr.

Processing for Electron Microscopic Immunocytochemistry

Cubes of corpus luteum (two per corpus luteum) of approximately 1 mm^3 were randomly selected and used for immunocytochemistry. The tissue was dehydrated, then immersed overnight in 1:1 (v/v) 100% ethanol-treated with copper sulfate:LR White resin (acrylic resin, hard grade; Probing and Structure) followed by pure LR White resin. The tissue blocks were then embedded in pure LR White resin using embedding beam capsules (Probing and Structure) and kept at 60°C for 48 hr.

Transmission Electron Microscopy

Tissues embedded in epoxy resin were used for morphological study. The blocks were trimmed to localize areas which contained large luteal cells as visualized under light microscopy. Silver to silver-gold interference color sections (0.05–0.08 μm thick) were then cut on a Leica ultramicrotome. Three to four sections were mounted on the silver side of 200 mesh copper grids (maxtaform HR 24 Cu/Rh; Probing and Structure), stained with uranyl acetate and lead citrate and subsequently examined in a Phillips 100 transmission electron microscope (TEM). For the immunohistochemisty study, tissues embedded in LR White resin were used. The 200 nickel grids (Probing and Structure) were first coated with 2% of collodion in amyl acetate (Electron Microscopy Sciences, NSW Australia). The sections were cut as above and mounted on the coated side of nickel grids which were then blotted, stained with uranyl acetate and subsequently examined by TEM.

Immunohistochemistry

Before commencing the specific immunoco-localization of oxytocin and Rab3B protein, several experiments were undertaken to determine the optimum dilutions of the primary and secondary antibodies as well as the most appropriate incubation period for both compounds. All incubations were carried out at room temperature unless otherwise stated. The solution used to dilute the antisera was phosphate buffered saline (PBS, pH 7.4) supplemented with 1% goat serum or ovalbumin (same species as secondary antibody). The specificity of the oxytocin antisera has been determined by Serotec (Oxford, England). The oxytocin antisera alone and the oxytocin antisera with the peptide, pre-incubated with an excess of peptide for at least 2 hr at room temperature, were incubated on individual sections of rat brain and visualized with diaminobenzidine. No specific signal was observed on the tissue sections treated with the oxytocin antisera incubated with peptide compared to the sections treated with the oxytocin antisera alone. Rab3B antisera is not cross-reactive with Rab3A or any other characterized protein using immunohistochemistry techniques as has been determined by Santa Cruz Biotechnology (California, USA).

The grids were floated facing down on a drop of dH_2O for 5 min. The sections were then etched by floating on a drop of saturated sodium metaperiodate (Analytical Univar Reagent, Melbourne, Australia) for 10 min (Bendayan and Zollinger, 1983) and washed vigorously with dH_2O. Following blotting, the tissue sections facing down were incubated five times for 10 min each (5 × 10 min) with 0.02 M glycine (Sigma) and blotted side-on on filter paper. Nonspecific binding sites were blocked by floating the grids, tissue side down on drops of phosphate buffer saline (PBS) supplemented with 10% goat serum and 0.1% Triton-X 100 (BDH Chemicals) for 20 min. After the excess serum was blotted, the sections were subsequently exposed to drops of primary rabbit anti-oxytocin serum (Serotec) diluted 1:50 in buffered 1% goat serum for 1 hr at room temperature and then overnight at 4°C in a humidified container. The antibody solution was rinsed 6 × 5 min each in drops of buffered 1% ovalbumin (Albumin, chicken egg; Sigma), blotted as above after the last rinse and followed by drops of goat anti-rabbit IgG-colloidal gold (5 nm diameter, AuroProbe EM GAR G 5, Amersham Life Science, NSW Australia) diluted 1:10 in buffered 1% goat serum for 90 min. After 6 × 5 min washes in buffered 1% ovalbumin followed by four washes of 1 min in PBS only, the grids were floated on drops of free protein-A solution (1 mg/ml PBS, Sigma) for 90 min, blotted and washed 6 × 5 min each in buffered 1% ovalbumin. The same procedure was repeated to localize Rab3B on the same face of the grids. The grids were incubated with Rab3B antiserum as the second primary rabbit polyclonal antibody (Santa Cruz Biotechnology) which was diluted 1:100 in buffered 1% ovalbumin. The secondary antibody was protein-A gold particles (10 nm diameter, Sigma) diluted 1:40 in buffered 1% ovalbumin. Following washing with buffered 1% ovalbumin and PBS only, the grids were washed in a series of four small beakers of dH_2O and counter stained in uranyl acetate for 4 min. The stained grids were then washed immediately in 70% alcohol followed by four washes with dH_2O, blotted on a piece of filter paper and placed in a dry labelled petri dish. During the immunohistochemical procedures, negative control sections were processed in parallel. The grids were incubated in primary antibodies in the absence of secondary antibodies and in secondary antibodies in the absence of primary antibodies. These antibodies were substituted by antibody diluent only.

TABLE 1. Characteristics of luteal cell secretory granules at four stages of estrous cycle in the ewe

	Stage of estrous cycle (days)			
	3	7	12	15
No. of granules/μm^2 (N_A)	0.47 ± 0.14	0.55 ± 0.09	0.35 ± 0.05	0.49 ± 0.06
No. of granules/μm^3 (N_V)	1.68 ± 0.5	1.96 ± 0.32	1.25 ± 0.18	1.75 ± 0.21
% of granules labelled for both oxytocin and Rab3B	61.5	79.7	72.7	42.4
% of granules labelled for oxytocin only	5.64	8.37	7.27	4.55
% of granules labelled for Rab3B only	6.66	7.97	8.18	6.66
No. of gold particles labelled for oxytocin per granule[a]	19.63 ± 2.25*	10.06 ± 0.95**	13.61 ± 1.82**	4.62 ± 0.82
No. of gold particles labelled for oxytocin per μm^2[b]	9.23 ± 2.72	5.54 ± 0.87	4.66 ± 0.62	2.26 ± 0.26*
No. of gold particles labelled for Rab3B per granule[a]	5.2 ± 0.49	4.58 ± 0.36	4.66 ± 0.58	4.15 ± 0.52
No. of gold particles labelled for Rab3B per μm^2[b]	2.44 ± 0.72	2.52 ± 0.39	1.60 ± 0.21	2.04 ± 0.24

Values are mean ± SEM.
[a]Calculated from granules labelled for both oxytocin and Rab3B.
[b]Calculated from mean number of gold particle labelled for oxytocin or Rab3B multiplied by N_A.
*$P < 0.05$ compared with all other days.
**$P < 0.001$ compared with day 15.

Morphometric Measurements of the Volume Fraction Occupied by Secretory Granules

The method used for the measurement of volume density has been reported previously (Weibel, 1969; Gemmell et al., 1983). In brief, the profile cytoplasmic area of all electron micrographs was calculated using the formula $A_T = n \times d^2$ (coherent test system) where A_T, n and d^2 represent cytoplasmic (test) area, number of square points making up the test and length of square lines respectively. However, the profile of cytoplasm may not always fill the entire micrograph so if a fraction of nucleus was present on the micrograph, the test area of that fraction was calculated and subtracted from the cytoplasmic area. The number of granules in the particular micrograph was redefined with respect to the recalculated cytoplasmic area. The number of secretory granules present in a cross-section of a luteal cell was obtained from the electron micrographs. Granule number per unit volume of luteal cytoplasm (N_V) was calculated using the formula $N_V = N_A/D$ (Weibel, 1969), where N_A is the number of granules per μm^2 and D is the mean diameter of the granules and was calculated from the relation $D = d4/\pi$ (Weibel, 1969) where D = 0.28 μm. The mean diameter of granule profile (d) was 0.22 ± 0.06 (± SD) as estimated by measuring the largest and shortest axis of 206 granules from 28 electron micrographs, magnification 21,000×. The granules counted only if the distribution of the profile diameters were between 0.15 and 0.3 μm. This represented 80% of total granules population. The other granules which represent lower (10%) and higher (10%) than the range were omitted from this calculation. Individual characteristic of secretory granules, N_A, N_V, number of gold particles labelled for oxytocin and Rab3B were analyzed using Student's unpaired *t*-test. Data are reported as means ± SEM.

RESULTS

An abundance of small secretory granules of 0.22 ± .06 μm (mean ± SD) diameter with, frequently, a two part core structure of light and dark electron-dense material was observed near the nuclear periphery of the large luteal cells. The mean granule density (N_V) was not significantly ($P > 0.05$) different between the days examined (Table 1) nor were there ultrastructural differences in the large luteal cells of the corpora lutea excised at the different times of the cycle (Fig. 1). On day 15 of the estrous cycle, the corpora lutea still contained apparently healthy large luteal cells as indicated by the presence of secretory granules and the absence of swollen mitochondria with dense inclusions.

Co-localization of oxytocin and Rab3B in the ewe's corpus luteum secretory granules, using IgG (5 nm) and protein-A (10 nm) gold particles respectively for immunohistochemical labelling, was evident on all days evaluated. Rab3B immunostaining was primarily located within secretory granules scattered throughout the cytoplasm. None of the other organelles in the large luteal cells (mitochondria, Golgi body, nucleus and rough endoplasmic reticulum) were labelled above background levels. Furthermore, no oxytocin or Rab3B immunostaining was observed in any other structures of the corpus luteum such as the endothelial cells, connective tissue cells and blood cells.

In negative control sections, background (non-specific) staining associated with secretory granules was minimal compared to positive-staining secretory granules. This indicated the specific binding of the oxytocin and Rab3B antisera.

In any large luteal cell examined of days 3, 7 and 12 of estrous cycle, 60–80% of the secretory granules were labelled by both oxytocin and Rab3B antisera protein compared to only 40% on day 15 (Fig. 2).

The mean intensity of labelling (number of gold particles) for oxytocin was significantly ($P < 0.05$) higher on day 3 compared to those obtained from day 7, 12 and 15 of estrous cycle. This intensity of oxytocin labelling was similar on days 7 and 12 of the cycle. There was a significant decline in intensity of staining on day 15 compared with days 7 and 12 of the estrous cycle. The mean intensity of labelling for oxytocin per μm^2 cytoplasmic luteal tissue was significantly decreased on day 15 compared to that on days 3, 7 and 12 of the estrous cycle. No significant changes were observed in the mean intensity of the Rab3B label of luteal secretory granules of corpus luteum on days 3, 7, 12 and 15 of the estrous cycle (Table 1).

During the estrous cycle, granules moved from the nuclear periphery towards the plasma membrane and on day 15 post-estrus most of the granules were found to be concentrated along the plasma membrane. Several granules without any labelling were seen around the nuclear

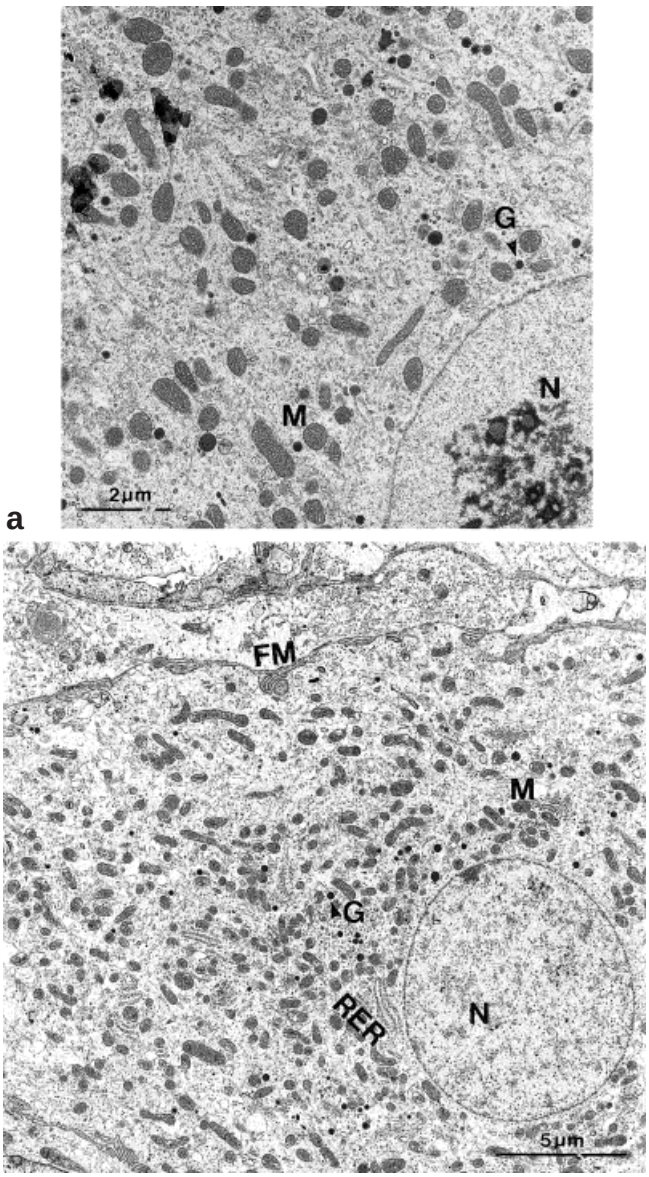

Fig. 1. Representative electron micrographs of large luteal cells from corpora lutea at different stages of the estrous cycle. **a:** Day 7. **b:** Day 12. N, nucleus; M, mitochondria; FM, folded membrane. Note the presence of secretory granules within cytoplasm (G) and rough endoplasmic reticulum (RER). Magnification in a, ×5.4 K; in b, ×2.95 K.

periphery region and near the plasma membrane at day 15 of the estrous cycle (Fig. 2d).

DISCUSSION

The ultrastructure of large luteal cells observed during the ovine estrous cycle in this study confirmed the previous morphological description of these cells (Gemmell et al., 1974; Alila and Dowd, 1991). Our observations showing that the luteal secretory granules were concentrated around nuclear periphery region of the large luteal cells and contained, frequently, a two part core structure (Fig. 2b,c) with an average size of 0.22 µm in diameter are similar to the results reported previously by Gemmell et al. (1974) and Paavola and Christensen (1981). Thus these granules can readily be distinguished from 0.1–0.15 µm neurosecretory granules, which have a core of a homogeneous density (Morris and Cannata, 1973). As indicated in previous studies (Gemmell et al., 1974; Sawyer et al., 1979) and in the present study, these secretory granules appear to migrate to, and fuse with, the plasma membrane at the time of exocytosis. The number and nature of proteins located in secretory granules is still uncertain. Previous studies have shown that oxytocin (Wathes et al., 1983), neurophysin (Wathes et al., 1983; Theodosis et al., 1986), relaxin (Fields and Fields, 1985) and tissue inhibitor of metalloproteinases-1 (TIMP-1) (McIntush et al., 1996) are localized to secretory granules of luteal cells. The present study provides evidence that a member of the subfamily of Rab proteins is also present and co-localized with oxytocin in the same secretory granules of the luteal cells of the corpus luteum. Although the antiserum we used in this study was purportedly specific for Rab3B, the possibility that it cross reacts with other members of this family cannot be discounted as pre-absorption of Rab3B antisera with other members of the Rab3 family peptides has not been undertaken in this study.

The findings of this study have shown that the mean number of secretory granules per μm^2 (Table 1) of the cytoplasmic luteal tissue did not vary significantly ($P > 0.05$) throughout the period studied. However, there was a significant decrease in the intensity of labelling for oxytocin on day 15 compared with days 3, 7 and 12 post-estrus.

The highest concentrations of oxytocin-neurophysin mRNA were measured within the corpus luteum on day 3 of the ovine cycle (Jones and Flint, 1988). A similar pattern has been observed on days 3 to 6 of the bovine cycle (Ivell et al., 1985; Fehr et al., 1987). Maximum concentrations of luteal oxytocin were observed during the mid-luteal phase of the cycle in these species, approximately 3–5 days after maximum increase in oxytocin mRNA (Fehr et al., 1987; Jones and Flint, 1988). The findings of this study give further support to this concept, since the percentage of secretory granules labelled for both oxytocin and Rab3B as well as for oxytocin alone was maximum on day 7 post-estrus and declined thereafter. Furthermore, a previous study by Lamsa et al. (1989) has shown that there was a decrease in the amount of total oxytocin released in response to $PGF_{2\alpha}$ stimulus as the luteal phase progressed. Theodosis et al. (1986) found that corpora lutea with a high oxytocin content had a higher intensity of oxytocin gold labelling over their secretory granules than those shown to have low oxytocin content. These findings are consistent with the results of this study showing that the number of gold particles labelled for oxytocin declined significantly with advancing age of the corpus luteum. Oxytocin-neurophysin-(I) complex has been detected in the early corpus luteum in addition to unbound oxytocin. This complex became less abundant as the corpus luteum matured (Shukovski et al., 1991). This observation may explain the higher intensity of labelling for oxytocin detected in this study on day 3 compared to day 7 of estrous cycle.

Rab proteins are thought to play an important role in the regulation of vesicular traffic (Goud et al., 1988). The physiological function of low molecular weight GTP-binding proteins is thought to depend on the interconver-

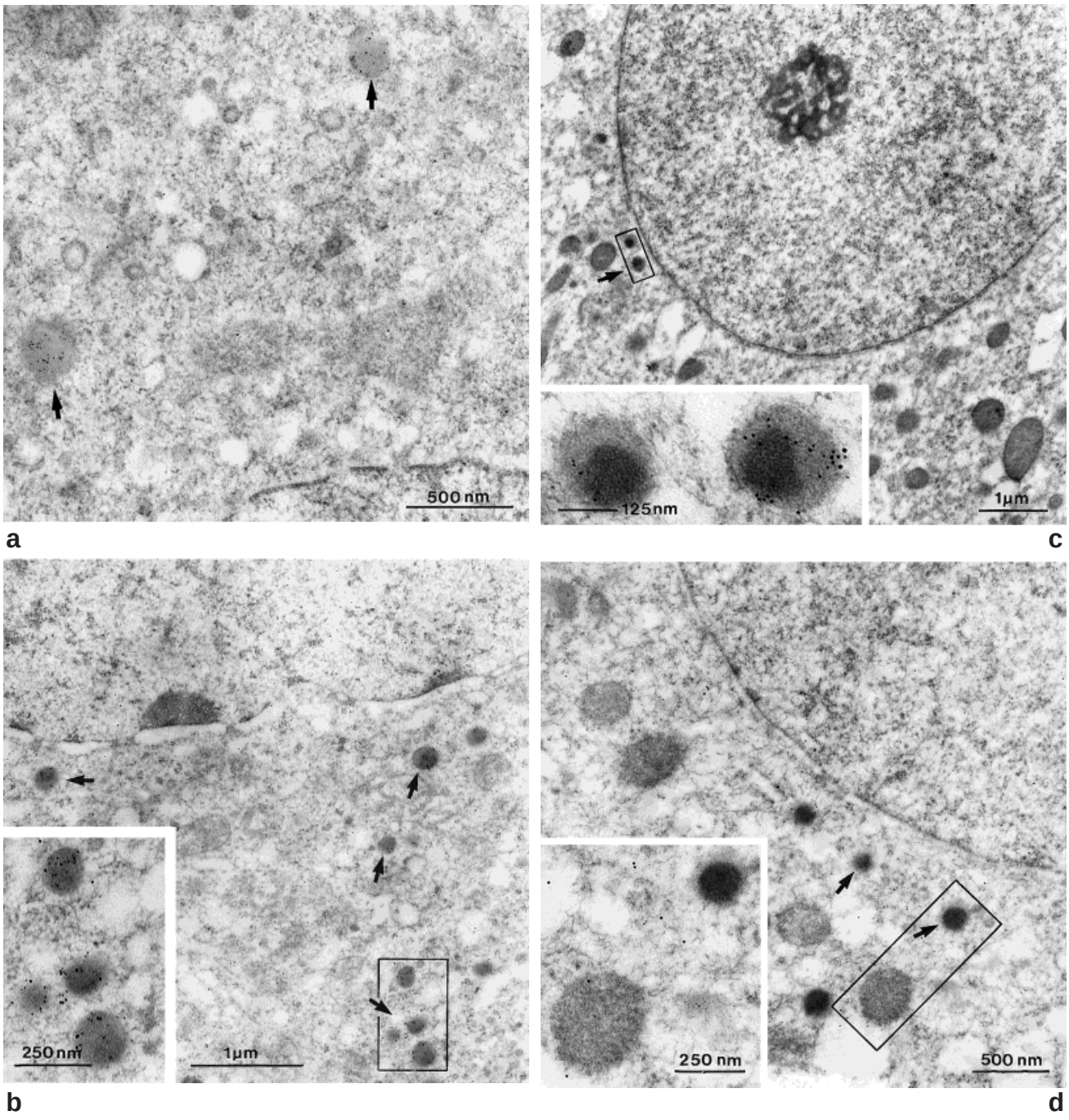

Fig. 2. Electron micrographs illustrating the portion of luteal cells from the corporal utea collected at different stages of ewe's estrous cycle (a–d). By double immunohistochemical labelling, ultrastructural co-localization of oxytocin and Rab3B in ewe's corpus luteum using IgG and protein-A gold particles respectively was evident. Colloidal gold particles, indicative of positive immunostaining for oxytocin (5 nm) and Rab3B (10 nm), were restricted to secretory granules (arrows) dispersed throughout cytoplasm of large luteal cells. **a:** Corpus luteum collected at day 3 (×28.5 K). **b:** Corpus luteum collected at day 7 (×15.5 K); note frequent granules with a two part core structure. The inset shows the boxed area on the large micrograph at a higher magnification (×31 K). **c:** Corpus luteum collected at day 12 (×8.9 K). The inset shows the boxed areas on the large micrographs at a higher magnification (×62.3 K). **d:** Corpus luteum collected at day 15 (×21 K). The inset shows the boxed area on the large micrograph at a higher magnification (×35 K). Note several granules without any labelling near nuclear periphery region.

sion between a cytosolic "inactive" (GDP-bound) form and a membrane-associated "active" (GTP-bound) form (Novick and Brennwald, 1993; Zerial and Stenmark, 1993) that is controlled by regulatory proteins (Macara, 1994). Several of these regulatory proteins for Rab3A have been identified and characterized. These include GTPase-activating pro-

tein (GAP), guanine nucleotide releasing factor (GRF) (Macara, 1994) and GDP dissociation inhibitor (Matsui et al., 1990; Macara, 1994). According to Fischer von Mollard et al. (1991, 1994) using neural tissue from the brain of animals, Rab3A is removed from the transport vesicle-fusion complex upon completion of neurotransmitter exocytosis and following hydrolysis of GTP, presumably by interaction with one the regulatory proteins. Thereafter, the protein is recirculated in the cytoplasm where it may participate in subsequent exocytotic processes. If Rab3B is controlled in the same way as Rab3A, results of the present study support this concept since the percentage of labelled granules for Rab3B was similar on days 3 and 15 of the cycle and the number of gold particles labelled for Rab3B over luteal secretory granules remained relatively constant throughout the period of study, suggesting that, despite continuous exocytosis, the availability of Rab3B remains more or less constant.

This study provides the first evidence that Rab3B is present in ovarian tissue and that it is co-localized with oxytocin to the same luteal staining granule of the corpus luteum during the luteal phase of the ovine estrous cycle. Rab3B protein is thus implicated directly or indirectly in the hormone secretory pathway of the corpus luteum. The precise role of Rab3B protein in ovarian function and whether ovine secretory granules contain other Rab3 isoforms, other Rab proteins or other small GTP-binding proteins have yet to be established.

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