Zygote **7** (August), pp 261–269. © 1999 Cambridge University Press Printed in the United Kingdom

Ultrastructural changes during nuclear maturation in *Bufo arenarum* oocytes

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Date submitted: 5.12.98. Date accepted: 9.3.99

Summary

During progesterone-induced nuclear maturation the oocytes of *Bufo arenarum* undergo a series of nuclear and cytoplasmic changes. The breakdown of heterocellular communications between the follicular cell projections and the oocyte microvilli, and the consequent enlargement of the perivitelline space, were observed at the animal pole. The more evident cytoplasmic feature during nuclear maturation comprised the gathering of glycogen granules in clusters, some phagocytosed by empty vesicles. With respect to the location of these vesicles, some were observed in close proximity to the oolemma and others were freely suspended in the perivitelline space, extruded from the oocyte. Other visible events were the disruption of the annulate lamellae, the formation of an elaborate cortical endoplasmic reticulum and the rearrangement of the cortical granules in a monolayer immediately beneath the oolemma together with aggregates of endoplasmic reticulum cisternae. Our results show that during nuclear maturation the nuclear oocyte changes include a flattening of the spherical oocyte nucleus, its migration towards the surface of the animal pole, the disappearance of the nucleoli and the dissolution of the nuclear envelope.

Keywords: Amphibian, Nuclear maturation, Oocyte, Ultrastructure

Introduction

Throughout the intraovarian growth phase, amphibian oocytes remain arrested at the prophase of the first meiotic division (Masui, 1985; Matten *et al.*, 1996). During the later stages of follicle development the preovulatory fully grown oocytes are meiotically competent, a fact that implies the ability of the gamete to progress in the cell cycle (Downs, 1997).

Meiosis resumption or nuclear maturation *in vivo* is physiologically induced by progesterone (Gerhart, 1980) secreted by the follicular cells under gonadotropin stimulation (Nagahama, 1987). Progesterone acts on the oocyte surface (Sadler & Maller, 1982; Liu & Patiño, 1993), both *in vivo* and *in vitro* (Maller, 1985; Jalabert *et al.*, 1991), triggering meiotic resumption and its progression to the second metaphase.

Oocyte maturation is a complex process involving structural (Imoh & Miyazaki, 1984; Wasserman *et al.*, 1986; Ramos, personal observation), biochemical and functional changes (Wasserman *et al.*, 1988; Lessman & Kessel, 1992) at the nuclear and cytoplasmic levels. This process culminates in ovulation, thus providing a gamete competent for fertilisation and development.

In *Bufo arenarum*, as in many amphibian species, the first visible event in nuclear maturation is the migration of the nucleus or germinal vesicle (GV) towards the animal pole (Brachet *et al.*, 1970; Wasserman *et al.*, 1986). Following nuclear migration, several processes occur in the oocyte including nuclear envelope breakdown, condensation of chromosomes, meiotic spindle formation and the extrusion of the first polar body (Brachet *et al.*, 1970; Wasserman *et al.*, 1986).

In spite of the significance of these biological aspects, information concerning the ultrastructural changes that take place in *Bufo arenarum* oocytes during the maturation process is quite limited (Mariano de Bozzini, 1987). The purpose of the present study was to describe the temporal sequence of the ultrastructural modifications that occur during progesterone-induced

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https://doi.org/10.1017/S0967199499000659 Published online by Cambridge University Press

nuclear maturation in the oocytes of the anuran amphibian *Bufo arenarum*, with a view to arriving at a better understanding of this physiological process.

Materials and methods

Animals

Sexually mature *Bufo arenarum* female toads (n = 8) were freshly collected in the neighbourhood of San Miguel de Tucumán, Argentina, during the breeding period and used within 24 h after capture.

Nuclear maturation induction

Fully grown follicle oocytes (1.5–1.7 mm in diameter) with one layer of follicle cells (hereafter referred to as oocytes or follicles) were dissected from ovarian tissue with watchmaker's forceps under a stereo dissecting microscope and placed in amphibian Ringer's solution. They were then pooled at random in lots of about 100 oocytes each in Petri dishes containing 30 ml of Ringer's solution supplemented with penicillin G sodium (30 mg/l) and streptomycin sulphate (50 mg/l) as incubation medium.

Nuclear maturation was induced by incubating lots of oocytes in the presence of 1 μg/ml progesterone (Sigma Chemical, St Louis, MO) dissolved in ethanol at a ratio of 1 mg/ml. Incubations were performed at 26 °C. Then, the oocytes were removed for fixation at different intervals ranging from 1 to 24 h after treatment. Control oocytes were incubated without the hormone under the same conditions.

Ultrastructural studies

Both treated and control oocytes, selected at random, were fixed in glutaraldehyde buffered in 0.1 M phosphate buffer pH 7.4 for 3 h, rinsed in the same buffer and postfixed in 1% osmium tetroxide overnight. Samples were dehydrated in a graded ethanol series, exchanged through acetone and embedded in Spurr resin (Pelco, Redding, CA). Silver-grey sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 transmission electron microscope.

Results

The ultrastructural events that occurred during the process of nuclear maturation in *Bufo arenarum* oocytes were analysed at the animal pole where the most striking morphological changes take place.

Control oocytes were surrounded by a single layer of follicular cells showing cell process endings that penetrated the vitelline envelope and established a close contact with the oolemma through gap junctions. There was no perivitelline space. The oocyte surface was characterised by the presence of numerous microvilli arranged perpendicularly (Fig. 1).

The most prominent ooplasmic structures at the cortical level were pigment granules distributed in the cortex, and electron-lucent, spherical, homogeneous cortical granules located in the cortical and subcortical cytoplasm. The latter were arranged either singly or in small clusters between some membrane-bound vesicles (Fig. 1). The peripheral cytoplasm also contained numerous dictyosomes of the Golgi complex and glycogen granules as well as yolk platelets and lipid droplets. In the subcortical cytoplasm, another remarkable feature was the presence of scattered stacks of annulate lamellae composed of multiple parallel layers of double membrane sheet (Fig. 2). Each lamella in the stack contained a highly organised array of pores. Sites of continuity between the annulate lamellae and cisternal elements of the endoplasmic reticulum could be observed. The euchromatic nucleus, located in the proximity of the equatorial ooplasm, exhibited an envelope with numerous pores and showed deep invaginations at the basal side (Fig. 3), thus producing an irregular profile. At this side nucleoli of different sizes and moderate electron density were present. Even though the mitochondria were distributed throughout the ooplasm, in the perinuclear cytoplasm, close to the nuclear invaginations, an important mitochondrial mass made up of numerous small rounded mitochondria associated with β glycogen particles and ribosomes was observed (Fig. 4).

During the first 2 h following progesterone treatment, the microvilli and their relationship with the follicular cells were still present, although some displayed signs of retraction. At this time there were no changes in the different structures of the cortical ooplasm, but at the basal side of the germinal vesicle indentations became more evident.

About 3 h after progesterone treatment, the beginning of nuclear migration towards the surface of the animal pole was observed. In the cortical region of the oocyte, a few cisternae of smooth endoplasmic reticulum were distributed in the cytoplasmic matrix between lipid droplets, yolk platelets and pigment granules. The cortical granules changed from a cortical position to a more superficial one, close to the plasma membrane. At this time, the peripheral glycogen granules tended to gather in clusters, some appearing in close contact with empty vesicles (Fig. 5) that tended to phagocytose them (Fig. 6). Glycogen-filled vesicles were also observed both in the proximity of the oocyte surface (Fig. 7) and freely suspended in the perivitelline space between the microvilli (Fig. 8). The

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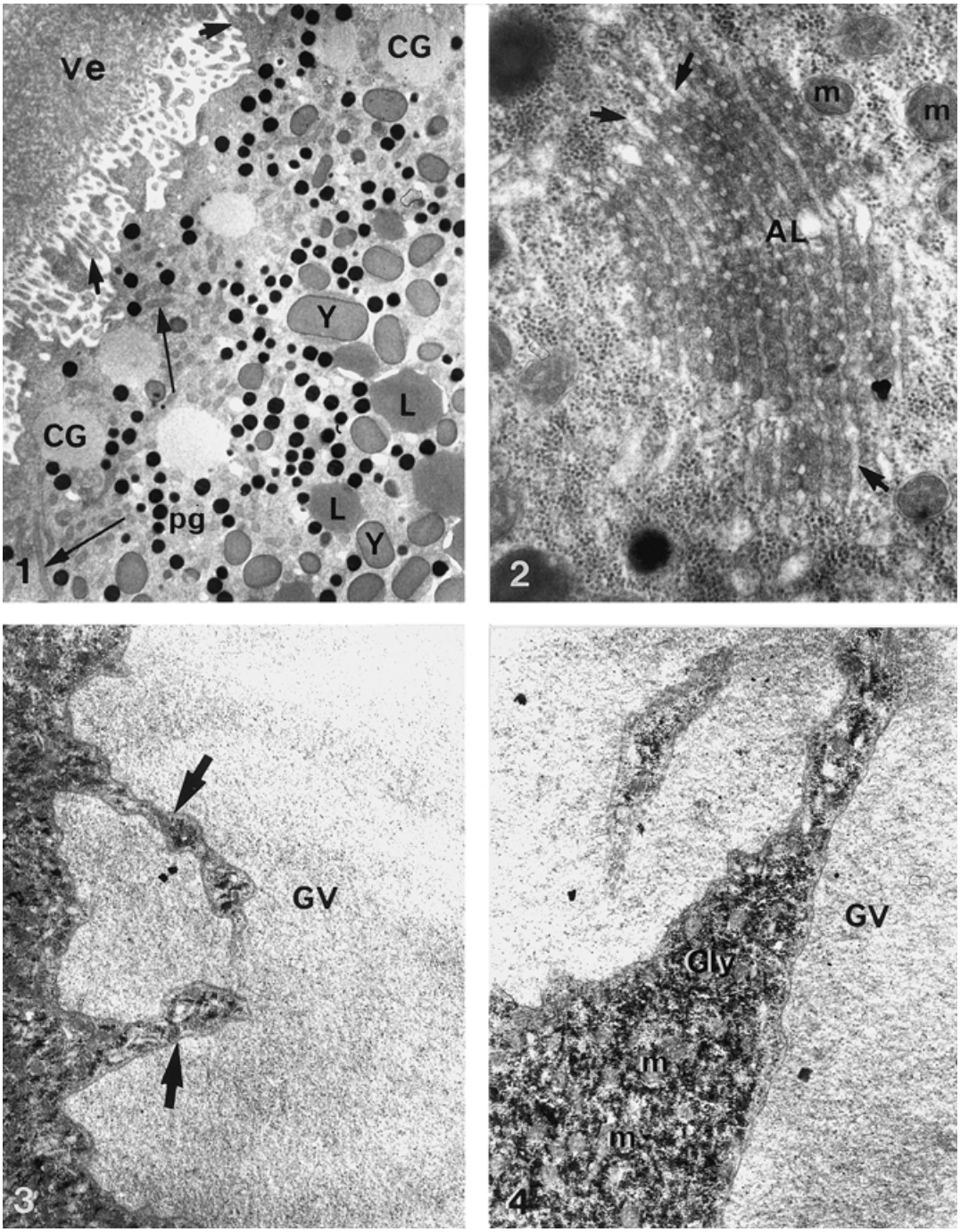


Figure 1 *Bufo arenarum* control oocyte. The oocyte surface, at the animal pole, exhibits numerous and prominent microvilli (arrows). ve, vitelline envelope; CG, cortical granules; L, lipid droplets; Y, yolk platelets; pg, pigment granules; thin arrows, mitochondria. ×4600. **Figure 2** *Bufo arenarum* control oocyte. Stacks of annulate lamellae (AL) are observed in the subcortical cytoplasm. Arrows indicate sites of continuity between the annulate lamellae and the endoplasmic reticulum. m, mitochondria. ×32300. **Figure 3** *Bufo arenarum* control oocyte. Basal side of the germinal vesicle (GV) showing deep invaginations (arrows). ×6560. **Figure 4** *Bufo arenarum* control oocyte. Basal side of the germinal vesicle (GV). Numerous β glycogen particles (Gly) associated with small mitochondria (m) are present in the perinuclear cytoplasm. ×9800.

release of these vesicles would involve exocytosis and/or an apocrine process (Fig. 9). Our observations also demonstrated the association of some clusters of glycogen granules with concentric multilamellar membrane arrays (Fig. 10). During this period we still observed stacks of annulate lamellae with no modifications.

About 4–5 h after progesterone treatment the

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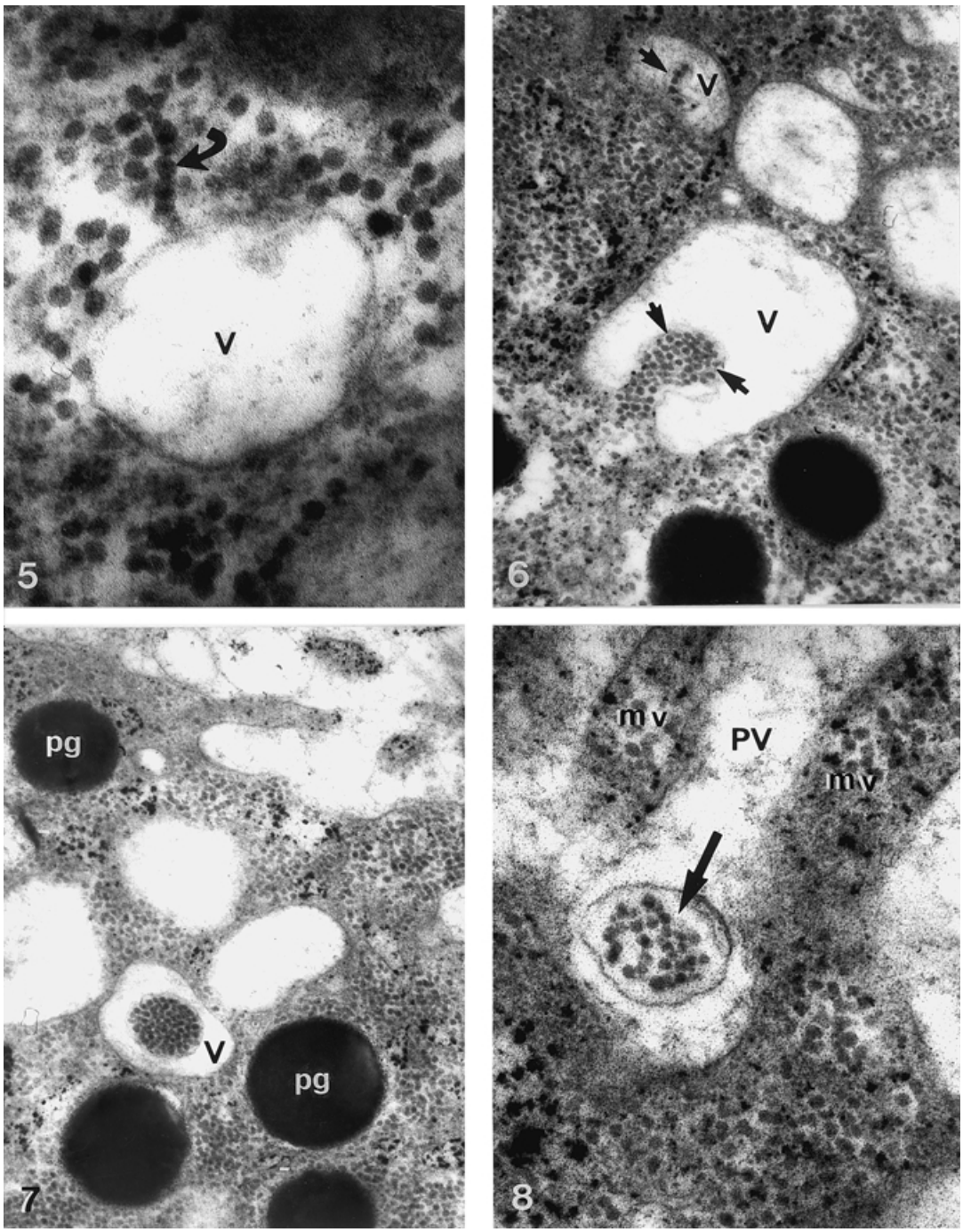


Figure 5 *Bufo arenarum* oocyte after 3 h of progesterone treatment. Arrow indicates a file of glycogen granules close to an empty vesicle (V). ×131 000. **Figure 6** *Bufo arenarum* oocyte after 3 h of progesterone treatment. Masses of glycogen granules (arrows) are being phagocytosed by empty vesicles (V). ×45 500. **Figure 7** *Bufo arenarum* oocyte after 3 h of progesterone treatment. A cluster of glycogen granules surrounded by a membrane is completely included in a vesicle (V) which is near the oocyte surface. pg, pigment granules. ×38 400. **Figure 8** *Bufo arenarum* oocyte after 3 h of progesterone treatment. A glycogen-filled vesicle (arrow) is freely suspended in the perivitelline space (PV). mv, microvilli. ×77 800.

cortical ooplasm did not show any relevant changes, but in the subcortical cytoplasm the vesiculation of annulate lamellae occurred (Fig. 11), resulting in an increased number of vesicles and cisternae distributed in the regions previously occupied by the stacks (Fig. 12). These vesicles seemed to migrate and accumulate in the oocyte cortex, showing a high degree of confluence. The nucleus located in a peripheral position evidenced several gradual modifications that included the onset of nuclear envelope breakdown at the basal side, the

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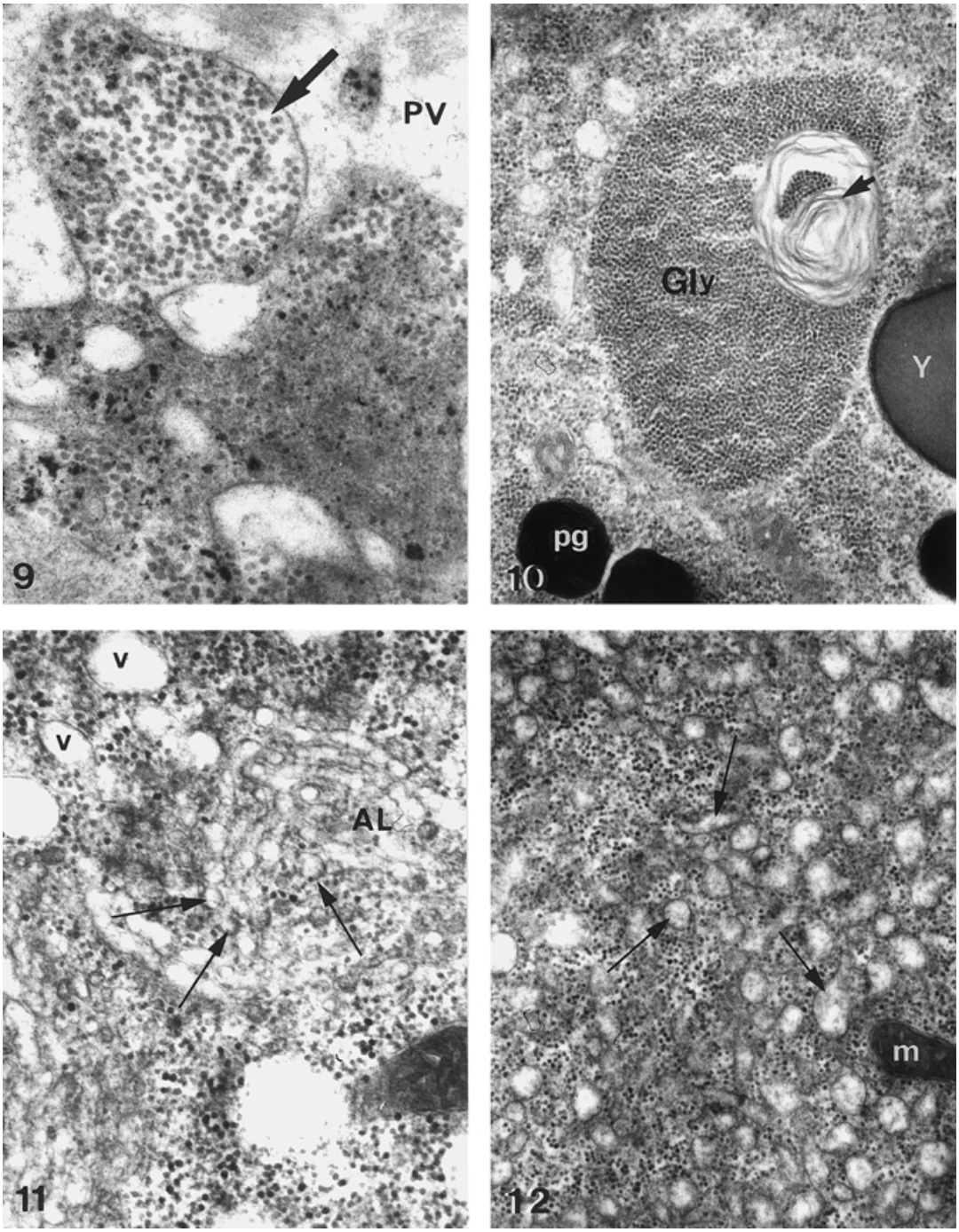


Figure 9 *Bufo arenarum* oocyte after 3 h of progesterone treatment. An important glycogen mass (arrow) is being released by an apocrine process. PV, perivitelline space. ×54 000. **Figure 10** *Bufo arenarum* oocyte after 3 h of progesterone treatment. A close association between a glycogen mass (Gly) and multilamellar membrane arrays (arrow) is clearly observed. pg, pigment granules; Y, yolk platelet. ×29 000. **Figure 11** *Bufo arenarum* oocyte after 4–5 h of progesterone treatment. Note the disruption of the annulate lamellae (AL) in numerous vesicles (V) exhibiting pore-like structures (arrows). ×51 500. **Figure 12** *Bufo arenarum* oocyte after 4–5 h of progesterone treatment. Arrows indicate vesicles and cisternae located in the cytoplasm previously occupied by the stacks of annulate lamellae. m, mitochondria. ×31 000.

rearrangement of the nuclear material and the disappearance of the nucleoli.

At the end of the nuclear migration (6–7 h after treatment) a general smoothing of the oocyte surface with noticeably shorter microvilli was observed (Fig. 13). The perivitelline space became clearly evident at the animal pole. The cortical granules were then located very close to the plasma membrane. Another feature

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was the presence of multivesicular bodies (Fig. 14) distributed randomly in the subcortical region. As regards the nucleus, the nuclear envelope exhibited a complete dissolution when it reached the oocyte surface. The zone previously occupied by the nucleus was now invaded by mitochondria, ribosomes, glycogen granules and vesicles.

In the mature oocyte (8–9 h after progesterone treatment) and after the first polar body extrusion, almost all the cortical granules were organised forming a monolayer (Fig. 15) located immediately below and/or in contact with the plasma membrane. Extended throughout the cortex interconnected tubules and cisternae of the endoplasmic reticulum formed a well-developed network. The cortical endoplasmic reticulum was observed next to the plasma membrane and around the adjacent cortical granules (Fig. 16). In the perivitelline space numerous small vesicles and multivesicular bodies were observed.

Discussion

Bufo arenarum are seasonal breeders; during the spawning period their ovaries contain the maximum number of fully grown oocytes capable of undergoing nuclear maturation when exposed to progesterone.

Previous studies have shown that progesterone-treated oocytes undergo germinal vesicle breakdown (100%) after an 8–9 h treatment during the breeding period of the species (Ramos de Romero *et al.*, 1998).

The present results demonstrate that this process produces extensive ultrastructural modifications at cytoplasmic and nuclear levels. Prior to hormone treatment, a close association was observed between cytoplasmic projections of the follicular cells and microvilli of the surface of the oocyte, via gap junctions (Villecco *et al.*, 1996). These heterocellular communications are believed to be responsible for the transfer from the follicular cells to the oocyte of metabolites and nutrients that play an essential role during oocyte growth. Moreover, follicular cells are involved in the regulation of the maturation process by means of the provision through the gap junctions of an oocyte maturation inhibitor that arrests meiosis (Vilain *et al.*, 1980), and also a gonadotropin-induced maturation-inducing substance (Patiño & Purkiss, 1993). Both seem to require heterocellular gap junctional coupling for their transfer to the oocyte (Patiño & Purkiss, 1993).

During progesterone-induced maturation, the oocytes showed microvilli retraction at the animal pole as a consequence of the disruption of the gap junctions, thus triggering meiosis resumption. Similar results were reported for *Rana pipiens* (Lessman & Kessel, 1992), Japanese quail (Yoshimura *et al.*, 1993) and bovine oocytes (Zhang *et al.*, 1995). These processes were accompanied by the progressive formation of a perivitelline space between the maturing oocyte and the surrounding follicular cells, with the concomitant loss of intercellular contact between them.

Progesterone treatment allowed us to observe cortical granule translocation from the subcortical and cortical cytoplasm to a more superficial position when nuclear migration to the cell surface occurred. This event has also been described for other species such as *Xenopus laevis* (Campanella *et al.*, 1984), sea urchin (Berg & Wessel, 1997) and pig (Kim *et al.*, 1996; Wang *et al.*, 1997). It is generally believed that the formation of a more-or-less continuous cortical granule monolayer beneath the plasma membrane is an uninterrupted process until ovulation (Wang *et al.*, 1997).

These changes in cortical granule distribution are essential for the oocyte activation process, which involves the fusion of the cortical granule membrane with the plasma membrane previous to the release of the cortical granule contents into the perivitelline space. The products released modify the chemical, structural, physicochemical and biological properties of the vitelline envelope; such modifications are considered essential for the prevention of polyspermy (Gómez *et al.*, 1984; Cabada *et al.*, 1987).

The subcortical ooplasm of fully grown immature oocytes exhibits, as in other amphibian species (Campanella *et al.*, 1984; Larabell & Chandler, 1988), parallel stacks of annulate lamellae. During progesterone-induced nuclear maturation, disruption of the annulate lamellae took place, as did the formation of numerous vesicles that migrate to the cortex. After the migration of these vesicles, a network of endoplasmic reticulum developed in the oocyte cortex. Similar results were reported for *Xenopus laevis* and *Rana pipiens* (Kessel & Subtelny, 1981). It has been postulated that annulate lamellae would act as a storage site for membranes that will later develop into the cortical endoplasmic reticulum network seen in the cortex of mature oocytes (Larabell & Chandler, 1988). This endoplasmic reticulum network would represent possible sites of calcium sequestration as reported for *Xenopus laevis* by Andreuccetti *et al.* (1984) and Campanella *et al.* (1984). Furthermore, the use of osmium pyroantimonate fixation allowed us to observe the ultrastructural localization of calcium deposits both in the annulate lamellae of maturing oocytes and in the cortical endoplasmic reticulum of *Bufo arenarum* mature oocytes (unpublished results). Our observations showed a close proximity between endoplasmic reticulum cisternae and cortical granules. This morphological relation is a very important event given that the primordial role of the cortical endoplasmic reticulum is calcium release, which is followed by the exocytosis of the cortical granule contents after sperm penetration (Terasaki & Sardet, 1991).

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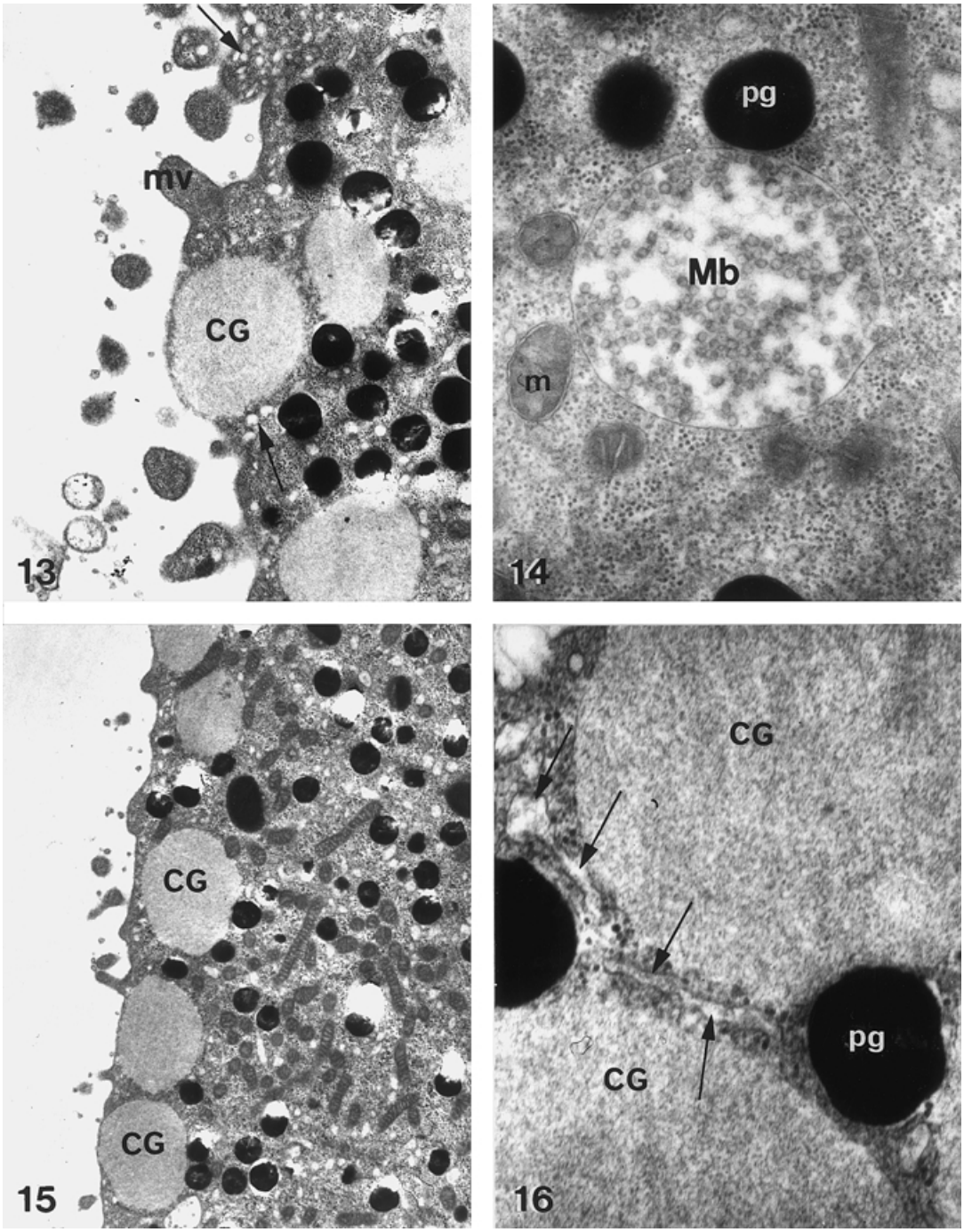


Figure 13 *Bufo arenarum* oocyte after 6–7 h of progesterone treatment. The microvilli (mv) are noticeably shorter, giving a general smoothing of the oocyte surface. The cortical granules (CG), located close to the plasma membrane, and a well developed endoplasmic reticulum (arrows) are observed. ×13 600. **Figure 14** *Bufo arenarum* oocyte after 6–7 h of progesterone treatment. Mb, multivesicular body in the subcortical cytoplasm; pg, pigment granules; m, mitochondria. ×28 300. **Figure 15** *Bufo arenarum* oocyte after 8–9 h of progesterone treatment. Cortical granules (CG), forming a monolayer, are located immediately below the plasma membrane. ×8000. **Figure 16** *Bufo arenarum* oocyte after 8–9 h of progesterone treatment. Arrows indicate cisternae of the endoplasmic reticulum surrrounding the cortical granules (CG). pg, pigment granules. ×49 000.

In fully grown immature oocytes we observed that glycogen particles were randomly dispersed in the peripheral and also in the perinuclear cytoplasm. At the time of nuclear migration, the glycogen granules seemed to gather in clusters, some of which were endocytosed in vesicles. Later, some of these glycogen-containing vesicles were found close to the plasma membrane and others in the perivitelline space. These

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observations are in agreement with those reported for oocytes of the urodeles *Pleurodeles waltlii* and *Ambystoma mexicanun* but not for *Xenopus* oocytes (Taghy-Sadak & Vilain, 1985). The biological significance of the release of such vesicles into the perivitelline space is unknown. To the best of our knowledge, this phenomenon is reported here for the first time in an anuran oocyte during the maturation process.

Glycogen incorporation into cytoplasmic vesicles and its association with multilamellar bodies suggests a possible role in the storage of membranes and energy-containing compounds probably required during embryo development.

Investigations are in progress for determining the physiological meaning of these events, bearing in mind that the morphological changes that occur during progesterone-induced nuclear maturation would probably be an important prerequisite for the oocyte to achieve the competence to undergo fertilisation and embryonic development at a normal rate.

Acknowledgements

This work was supported by a grant from CIUNT, Consejo de Investigaciones de la Universidad Nacional de Tucumán, Argentina.

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