Cinemicrographic analysis of cytoplasmic and nuclear events associated with germinal vesicle breakdown in rat oocytes exposed to LH in vitro

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Summary. Cinemicrography, with Nomarski differential interference optics, was used to study the motion of cytoplasmic organelles and events leading to germinal vesicle breakdown (GVB) in rat oocytes perfused with a defined medium with or without LH. Initially, cytoplasmic organelles, 0-3-1.5 μm diam., appeared to move randomly and were uniformly distributed. A gradually increasing vigour of organellar movement, saltatory translocation of organelles and discontinuous convergent flow of groups of organelles around the nucleus were observed. Ultimately, most of the organelles, which possessed several properties indicative of lysosomes, accumulated around the nucleus. In the presence of 5-6 μg LH/ml perfusate, GVB was usually complete by 80 min, whereas in the absence of hormone, or in the presence of LH + antiserum to LH, GVB took up to 130 min. A sudden slight decrease in nuclear size, undulating activity in the nuclear envelope, and the appearance of intranuclear filaments indicated imminent GVB. The appearance of spherical bodies at the nucleolar surface, and the subsequent mobilization and collapse of the nucleolus in the nucleoplasm, were advanced events in the nuclear response. It is suggested that LH promotes GVB through activation of lysosomes.

Introduction

The nucleus of the mammalian oocyte remains arrested at the diplotene stage of meiosis until a gonadotrophic stimulus, or some other mechanism, causes the cytoplasmic-nuclear activation that induces either further meiotic maturation or degeneration of the oocyte (Schuetz, 1969, 1974). The primary site of action and the nature of the stimulus that triggers resumption of meiosis in an oocyte within a Graafian follicle are difficult to determine because of the complex interrelationship between the oocyte and its surrounding corona and cumulus cells (Odor, 1960; Franchi, 1960). Cytoplasmic processes of the corona cells penetrate the zona pellucida to reach the surface of the oocyte where tight intercellular junctions are formed between the plasma membranes, often within deep infoldings of the egg surface (Zamboni, 1970). The significance of these close contacts between the oocyte and the processes of the corona cells has not yet been established. Neither is it known whether a relationship exists between the stimulus for oocyte maturation and the retraction of the cell processes during meiotic maturation (Sotelo & Porter, 1959; Odor, 1960; Zamboni & Mastroianni, 1966).

Meiotic maturation has been studied in oocytes retained within explanted ovarian follicles to keep to a minimum the disturbance of the physical relationships between the egg and its investments (Tsafiri, Lindner, Zor & Lamprecht, 1972; Lindner et al., 1974). However, even when there is little disruption of intrafollicular cell layers during dissection, the follicle-enclosed oocyte is less suitable than the liberated oocyte for meiotic maturation studies for several reasons, e.g. the inability to make frequent or continuous observations of the oocyte, the possibility that oxygenation of the oocyte is not adequate, and the variable permeability of the follicular wall, particularly to steroids (Schuetz, 1974). Oocytes released from Graafian follicles into a substrate-enriched tissue-culture
medium resume meiosis in the absence of gonadotrophins and steroids, whether the oocytes retain or are devoid of their investment of follicular cells (Cross, 1973; Zeilmaker & Verhamme, 1974). Such oocytes can be observed continuously for the onset of 'spontaneous' maturation or the effects of hormones introduced into the culture medium.

The present report describes experiments in which cinemicrography was used to study the interaction between the cytoplasm and nucleus of rat oocytes perfused with defined medium in the presence and absence of LH. The aim of these studies was to identify changes, if any, in the movement and distribution of subcellular organelles before and during germinal vesicle breakdown.

**Materials and Methods**

Inbred Sprague–Dawley rats weighing 180–200 g were used after establishing that they were undergoing regular oestrous cycles. Ovaries were excised on the morning of the 1st day of dioestrous and on the morning of pro-oestrus from selected rats lightly anaesthetized with subcutaneously administered Nembutal (5 mg/100 g body weight). Graafian follicles, about 1 mm in diameter, were punctured with a fine scalpel blade to release the oocytes into a medium of the following constituents (mM): NaCl, 94.6; KCl, 4.78; MgSO₄, 1.19; calcium lactate, 1.27; KH₂PO₄, 1.19; NaHCO₃, 25.07; sodium pyruvate, 0.5; sodium lactate, 21.58. This solution was gassed with 5% CO₂ in air at 37°C before use; antibiotics and surface-active substances such as silicone grease were excluded in the preparation of culture vessels and the perfusion apparatus.

Each released oocyte was immediately taken up in about 20 µl medium and placed between two parallel supporting threads of fine glass (60–75 µm in diam.) on a microscope slide. A small coverslip was placed over the droplet and gently lowered until it just touched the oocyte; petroleum jelly was used to anchor the coverslip. The slide containing the oocyte formed the floor of a perfusion chamber which was placed on a heated microscope stage maintained at 37°C. Filming was begun as soon as an appropriate area of the oocyte was located in the camera’s viewfinder. After filming for 30–60 sec, the chamber was perfused with medium containing ovine LH (NIH-S18), at concentrations ranging from 0.3 to 6.0 µg/ml, or the hormone plus an antiserum to LH (raised in rabbits), or with the medium alone. The motion of organelles within the oocyte was filmed at 4 frames/sec on Ektachrome No. 7252 Commercial film, employing Nomarski interference optics, a × 40 water-immersion objective, and a × 12.5 ocular with an Arriflex camera mounted above it.

For 12 oocytes, filming was continuous for the first 16 min; thereafter 60-sec sequences were taken every 5 min. Two oocytes were filmed continuously for up to 140 min.

**Results**

A summary of the experiments is given in Table 1. The motion of cytoplasmic organelles in the oocytes appeared to be random and the vigour of movement varied in different oocytes independently of the stage of the oestrous cycle. In oocytes with sluggish cytoplasmic activity the vigour of organellar movement increased noticeably after culture for 15–20 min, particularly in those perfused with LH. Analysis of the film showed that in some oocytes, especially those perfused with an adequate concentration of LH (see below), the random movement of cytoplasmic organelles was associated with their bulk translocation from the peripheral cytoplasm to a perinuclear region. This feature could not be satisfactorily demonstrated in a series of photographic prints, except as a relatively higher concentration in the later compared with the earlier sequences of frames. Circumferential flow of groups of organelles around the nucleus, first in one, then in the reverse direction, accompanied by a brisk rocking motion of the nucleus, was observed regularly in oocytes perfused with adequate concentrations of LH as well as control medium, but it could not be convincingly represented in photographic prints. The nucleus remained in about the same position in the cytoplasm throughout all experiments. After about 30 min of perfusion, most of the cytoplasmic organelles generally accumulated around the nucleus (Pl. 1, Fig. 2); a thin zone of granules remained close to the oolemma and the remainder formed bands of actively moving organelles connecting the two main regions (Pl. 2, Fig. 13).
Irregularities of the nuclear envelope were initially present in some oocytes obtained from pro-oestrous rats, but most oocytes contained a perfectly spherical germinal vesicle. However, areas of localized nuclear envelope activity, seen as transitory invaginations (Pl. 1, Fig. 1) up to 6 µm in depth, or occasionally as apparent evaginations, were often evident in oocytes recovered at dioestrus and pro-oestrous stages of the cycle. All germinal vesicles contained a brightly refractile nucleolus which was either ring-shaped, due to the presence of a large vacuole-like structure in its substance (Pl. 1, Fig. 3), or spherical with one or more small vacuolar structures at its surface (Pl. 1, Fig. 4). Frequently the nucleolus was surrounded by fine granular and filamentous material (Pl. 1, Fig. 3) which appeared to anchor it to a segment of the nuclear membrane. This perinucleolar material sometimes contained vibrating fibrils, with attached granules. Some nucleoli had at their surface one or more ring-shaped structures (Pl. 1, Fig. 4), 2–3 µm in diameter, which enlarged and became diffuse before germinal vesicle breakdown. The nucleoplasm contained various numbers of mobile spherical structures, 0.5–5 µm in diameter, which moved at speeds of 5–10 µm/sec.

A complete cinemicrographic record of germinal vesicle breakdown was obtained in all 5 oocytes exposed to 5–6 µg LH/ml, in 2 oocytes preincubated for 10 min in antiserum to LH and subsequently perfused with 5–6 µg LH/ml and in 2 out of 5 oocytes perfused with medium not containing LH. In the presence of an adequate concentration of LH without its antiserum, undulating activity of the nuclear envelope (Pl. 1, Fig. 7) was evident between 35 and 60 min after the start of perfusion and GVB was completed by 80 min. In oocytes perfused with medium without LH, undulating activity of the nuclear envelope was observed only after 85 min and its dissolution occurred no earlier than 105 min. A similarly long interval was required for germinal vesicle breakdown when a low, presumably ‘inadequate’, concentration of LH (0.3 µg/ml) was used, or when oocytes were first preincubated in antiserum to LH and then perfused with effective concentrations of LH (5–6 µg/ml; approximately $2 \times 10^{-7}$M) in the presence or absence of antiserum in the medium.

A sudden decrease in size of the germinal vesicle (Pl. 1, compare Figs 5 and 6), not always perceptible, increasing undulating activity of the nuclear envelope (Plate 2), and often the appearance of vibrating intranuclear fibrils (Pl. 1, Figs 8 and 9; Pl. 2, Fig. 14) with attached spherical bodies, were the earliest visible changes that signalled imminent germinal vesicle breakdown. Disappearance of the nucleolar vacuole (Pl. 1, Figs 8 and 9), generally in less than 4 sec, and condensation of nucleoplasmic material at the inner surface of the nuclear envelope were also observed. As the undulating activity of the nuclear envelope became more pronounced, invaginations of the nuclear membrane began to reach the nucleolus (Pl. 2, Figs 12 and 15). When some of these pseudopod-like processes
retracted, small globules appeared to be left behind at the surface of the nucleolus (Pl. 2, Fig. 13). In addition, larger spherical bodies, 2–4 µm in diameter, which originated at the nucleo-cytoplasmic junction, appeared to fuse with the surface of the nucleolus (Pl. 2, Fig. 15). The perinucleolar material, which had previously seemed to tether the nucleolus to the inner nuclear membrane, disappeared and the nucleolus became mobile within the diminishing nucleoplasm. Just before, or about this time, the nuclear envelope became less distinct. Additional clumps of nucleoplasmic material appeared to fuse with the surface of the free floating nucleolus. The latter soon collapsed to form an irregular mass. Portions became separated from the bulk of this aggregate and gained access to the cytoplasm as a result of final dissolution of the nuclear envelope (Pl. 2, Figs 16 and 17), and groups of fibrils, possibly destined to form the first meiotic spindle, became visible in the residual nucleoplasm and in the adjacent cytoplasm.

Discussion

The present results demonstrate that at concentrations of approximately 2 × 10^{-7} m, LH promotes the onset and accelerates the progress of germinal vesicle breakdown in rat oocytes explanted into a defined medium. The 45–80 min required for dissolution of the nuclear envelope in oocytes exposed to this concentration of LH were similar to the time required for germinal vesicle breakdown in intrafollicular oocytes (approx. 90 min) in rats injected intraperitoneally with pituitary gonado-trophin (Vermeiden & Zeilmaker, 1974). In the present investigations, longer times (105–130 min) were required for nuclear membrane breakdown in the absence of hormone. Similar intervals (75–130 min) have been reported for germinal vesicle breakdown in rat oocytes denuded of follicular cells and cultured in the absence of hormone in droplets of medium under oil (Zeilmaker & Verhamme, 1974). Although the present results suggest that nuclear envelope disintegration of LH-stimulated oocytes is accelerated, as judged by the generally shorter times from the onset of nuclear undulating activity to the disappearance of the membrane, an extensive study would be required to define with more precision the rates of disintegration of the nuclear membrane in the two groups of oocytes. LH has been reported to increase the rate of maturation of hamster oocytes in vitro (Gwatkin & Anderson, 1976). The sudden onset of the appreciable decrease in size of the germinal vesicle,
which occurred before nuclear disintegration, may correspond to the shrinkage of the germinal vesicle before breakdown in frog oocytes in response to progesterone (Schuetz, 1969). It would be of interest to evaluate whether the collapse of the system of nuclear envelope pores, such as occurs in mouse oocytes at the onset of germinal vesicle breakdown (Szollosi, Calarco & Donahue, 1972), is related to the abrupt decrease in nuclear size.

The present experiments were primarily concerned with the qualitative nature of the cytoplasmic and nuclear events culminating in germinal vesicle disintegration. Because the resolution of the objective was 0.27 µm, the visible elements included a population of the larger lysosomes, multivesicular lysosomes, mitochondria, lipid granules and vesicles of various types (Sotelo & Porter, 1959; Odor, 1960). The ultrastructure and disposition of organelles at the time of germinal vesicle breakdown has been described in sections of mouse oocytes (Calarco, Donahue & Szollosi, 1972). In the present study the specific nature of the mobile cytoplasmic organelles which became associated with the nuclear envelope before dissolution could not be determined by the criteria available during the filming process. However, additional observations carried out in related experiments with u.v.-fluorescence microscopy after intravital staining with acridine orange (Szego & Seeler, 1973) provided strong circumstantial evidence that the perinuclear organelles were lysosomal (unpublished observations). The organelles we saw corresponded in size (Novikoff, Essner & Quintana, 1964) and mobility (Freed & Lebowitz, 1970) with lysosomes and our finding of the altered cohesiveness of intranuclear elements suggests that lysosomal activities (see Szego, 1974, 1975) may underlie the process of germinal vesicle breakdown and associated phenomena.

Peptide and steroid hormone action in a number of tissues is accompanied by the release of cathepsin B1 from lysosomes (Szego, 1975; Pietras, Seeler & Szego, 1975; Pietras & Szego, 1975). Corresponding experiments with LH may demonstrate that the 'proteolytic factor' from the cytoplasm believed to be responsible for germinal vesicle breakdown (Wasserman & Masui, 1976; Kishimoto & Kanatani, 1976) and some aspects of progressive follicular maturation may have a lysosomal origin.

This study was aided by a Lalor Foundation Fellowship to A.L. and by Grants HD 04354 and FR-7009 (USPHS) and PCM 76-01826 (NSF) to C.M.S. We are grateful to Dr Theodore L. Jahn for the photomicrographic equipment; Dr C. H. Li for the antiserum against ovine LH (ICSH) and corresponding control sera; Mrs Margaret Patullo for the photographic prints; and Ms Barbara J. Seeler and Ms Karen A. Thompson for technical and secretarial assistance.

EXPLANATION OF PLATE 2

Germlinal vesicle breakdown in a pro-oestrous oocyte which had been preincubated for 10 min in antiserum to LH, and then perfused with a medium containing 5-6 µg LH/ml. × 770.

Fig. 10. After 90 min a distinct and regular nuclear envelope (NE) was still present.

Fig. 11. By 110 min undulating activity of the nuclear envelope had started and a deep pseudopod-like invagination (pp) was forming.

Fig. 12. The deep invagination had extended to the nucleolus (n); the nuclear envelope was out of focus in this region but was visible in other parts.

Fig. 13. The nuclear invagination (pp) has retracted. Perinuclear accumulation of cytoplasmic organelles was evident and irregular bands of organelles (bg) extended towards the sub-oolemmal granules (sg).

Fig. 14. A nucleoplasmic fibril (f) had appeared between the apex of the retracting invagination (arrow) and the surface of the nucleolus. Several globular structures (g) are present over the nucleolar surface which was approached by the invagination.

Fig. 15. By 115 min a new invagination (arrow) had formed in the nucleus and had established contact with the mobilized nucleolus. A nucleoplasmic mass (m) appeared to attach to the nucleolus. The nuclear envelope had become indistinct.

Fig. 16. By 120 min the nuclear envelope was no longer visible, the nucleolus had collapsed and nucleoplasmic masses at its surface gained access to the cytoplasm (arrows).

Fig. 17. At about the same time groups of fibrils (fs) appeared in the shrinking nucleolus and an adjacent area of cytoplasm.
References

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Received 23 August 1976