Effects of some cytoskeleton inhibitors on ooplasmic segregation in the *Nereis virens* egg

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ABSTRACT The ooplasmic segregation in the *Nereis virens* egg starts after completion of cortical reaction. During ooplasmic segregation the radial symmetrical pattern of the oocyte transforms into polarized stratified structure with different kinds of cytoplasm regularly distributed along the animal-vegetal axis. The beginning of oocyte polarization is associated with the generation of the mitotic spindle and is sensitive to microtubule inhibitors. By analyzing the effect of colchicine, taxol, nocodazole and cytochalasin B, we show that the mechanisms of ooplasmic segregation have an integrated complex nature and include interactions of microtubules and microfilaments. The inhibition of any of these elements delays or disturbs the ooplasmic segregation and simultaneous disruption of microtubules and microfilaments functions leads to total cessation of the process.

KEY WORDS: *polychaete, ooplasmic segregation, cytoskeletal inhibitors, colchicine, cytochalasin B*

Introduction

Ooplasmic segregation is a process of redistribution of different types of cytoplasm within the oocyte which occurs following fertilization during the period of meiotic divisions and preparation for the first cleavage (Costello, 1948; Jeffery and Bates, 1989). Ooplasmic segregation seems to play an important role in polarization and axis formation of the embryo (Houliston *et al.*, 1993), and in determination of the cleavage type. At the same time, ooplasmic segregation is often an instrument of cytoplasmic localization of morphogenetically active substances (Davidson, 1986), which assures a strict spatial redistribution of factors for cell specification. Ooplasmic segregation has been demonstrated in many taxa and there is a great diversity in its manifestation (Conklin, 1905; Zalokar, 1974; Shimizu, 1982; Jeffery, 1984; Dorresteijn and Fischer, 1988; Hill and Strome, 1988; Sardet *et al.*, 1989; Houliston *et al.*, 1993; Longo et al., 1993). Even among animals belonging to a single phylum (e.g. Annelida) there are very different patterns of ooplasmic segregation.

In the leech *Theromyzon rude*, formation of the perinuclear cytoplasmic domain and polar accumulation of animal and vegetal teloplasm are observed just before cleavage of the egg. During development, the cytoplasm of these domains is distributed into five pairs of teloblasts which produce ectoderm and mesoderm (Fernández *et al.*, 1987). It has been shown that colchicine, a potent inhibitor of microtubules, disrupts the separation of polar cytoplasmic domains and blocks the movement of male pronucleus toward animal-vegetal axis. At the same time, cytochalasin B, and inhibitor of actin filament polymerization disturbs the normal localization of perinuclear cytoplasm. It was therefore concluded that leech ooplasmic segregation requires integrated activities of both microtubules and actin filaments (Fernández *et al.*, 1994).

It has been suggested that in the oligochaete *Tubifex* the process of ooplasmic segregation starts after meiosis and includes two steps (Shimizu, 1982). The first step consists of centrifugal movement of endoplasm which forms a subcortical layer devoid of lipid droplets and yolk granules at the egg surface. The second step is the localization of the subcortical ooplasm along the surface toward the poles, which results in separation of animal and vegetal pole plasm (Shimizu, 1982). It has been recently reported that *Tubifex* egg contains two configurations of the actin cytoskeleton: an endoplasmic network and a cortical shell (Shimizu, 1996). These cytoskeletal formations are characterized by different sensitivities to the botulinum C3 exoenzyme. Microinjection of drug disrupts cortical actin filaments but not actin cytoskeleton of endoplasm (Shimizu, 1996). In C3-injected eggs of *Tubifex*, only the first step of ooplasmic segregation remains; whence it follows that actin filaments of the endoplasm network may produce driving forces for the centrifugal movement of the endoplasm, whereas cortical actin filaments are capable of driving the poleward movement of subcortical ooplasm (Shimizu, 1996).

The ooplasmic segregation in the polychaete, *Platynereis dumerilii*, is different (Dorresteijn and Kluge, 1990; Kluge, 1991). The authors distinguish two phases of ooplasmic segregation which start just after fertilization. Dorresteijn and Kluge showed that the fully developed oocyte of *Platynereis dumerilii* prior to fertilization is ellipsoidal. The nucleus of the oocyte lies in a central domain of clear cytoplasm. This domain is surrounded by a layer

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of yolk components (i.e., yolk granules and lipid droplets). The cortical layer with numerous cortical granules is formed at the periphery of the oocyte. After fertilization and cortical reaction, the oocyte changes its form and becomes spherical. The clear cytoplasm now extends from the center to the periphery of the egg (at the future animal pole). The area of yolk-free cytoplasm located near egg surface is about 35 μm in diameter and does not contain any inclusions. This is the region where the spindle of the first meiotic division comes in contact with the egg cortex. During meiotic divisions, this area does not grow, but 20 min after meiosis yolk granules of the peripheral layer begin to move towards the vegetal pole. At the same time, clear cytoplasm moves from the central region to the periphery and occupies the animal part of the egg. Ooplasmic segregation finishes shortly before the onset of the first cleavage (Dorresteijn and Kluge, 1990). Accumulation of the clear cytoplasm was also described in animal region of *P. massiliensis* egg (Schneider *et al.*, 1992).

According to Dorresteijn and Kluge, the first phase of ooplasmic segregation can be inhibited by nocodazole (5 μg/ml) and taxol (5 μg/ml), but is insensitive to cytochalasin B (10 μg/ml). The authors suggest that microtubules might play a decisive role during this phase. The second phase of ooplasmic segregation is not so sensitive to microtubule inhibitors which delay the process of segregation rather than block it. On the contrary, cytochalasin B inhibits this step of segregation, and consequently actin filaments are thought to be a driving force during the second phase of the process (Dorresteijn and Kluge, 1990; Kluge, 1991).

We have studied the ooplasmic segregation in eggs of *Nereis virens* using microtubule inhibitors (colchicine, nocodazole, taxol) and a microfilament inhibitor (cytochalasin B) to disrupt the cytoskeletal structures. *Nereis virens* has some advantages over other species because it develops relatively slowly at low temperature (10-11°C) so that the period of ooplasmic segregation, i.e. the period from fertilization to the first cleavage, lasts about six hours. The results of our experiments show that ooplasmic segregation in *Nereis virens* has at least two phases directed successively by microtubules and actin filaments. The inhibition of one of these components delays or disturbs the ooplasmic segregation, but simultaneous disruption of microtubule and microfilament functions leads to total block of the process, suggesting that some steps of ooplasmic segregation may be of integrated complex nature that includes interactions between microtubules and actin filaments.

Results

Cortical reaction and ooplasmic segregation

Immediately after shedding, the unfertilized oocyte appears spherical with a diameter up to 200 μm (Fig. 1). The oocyte exhibits radial symmetry and does not display any signs of polarization. It is surrounded by the vitelline envelope, and there is a narrow perivitelline space between the envelope and egg surface. The egg periphery is occupied by a vast cortical zone with numerous cortical alveoli. Inward from the cortex, the zone contains lipid droplets and yolk granules of variable size. Larger inclusions in the zone are concentrated near the central sphere of the oocyte which contains yolk-free cytoplasm free of lipid droplets and rich in both mitochondria and ribosomes. In the very center of the oocyte is a large nucleus with a diameter of about 60 μm.

The cortical reaction starts 30 min after fertilization. During the period of cortical reaction cortical alveoli are discharged into the perivitelline space. The vitelline envelope elevates due to a swelling of the material released and the space between the egg surface and envelope (the fertilization envelope) increases up to 3 μm. The fertilization envelope becomes stretched so much that its thickness decreases from 225±8 nm to 197±5 nm. The cortical reaction lasts about 1 h. During the reaction, yolk- and lipid-rich cytoplasm moves centrifugally to form the superficial layer (superficial sphere) of the oocyte.

At 1 h post-insemination, apart from the changes directly connected with cortical granule exocytosis, some other structural reorganizations of the oocyte were observed. By this time nuclear envelope has broken down so that an extensive domain of clear myxoplasm (i.e., a mixture of the yolk-free cytoplasm and the nucleoplasm) is formed in the center of the oocyte. The appearance of myxoplasm is a prelude to the segregation of the ooplasm. Soon the domain loses its spherical form and becomes ellipsoidal. The animal pole is marked by the point where the clear cytoplasm reaches the surface of the oocyte. The yolk granules and lipid droplets move slightly laterally from this point. These first signs of oocyte polarization are clearly connected with the first meiotic spindle formation, the long axis of which is located perpendicular to the egg surface. During these translocations both the peripheral yolk-rich layer and the deeper one with lipid inclusions develop a cup-like structure with a narrowed opening, filled with clear cytoplasm where the meiotic spindle is positioned. The first polar body is formed at 3 h and the second one at 4.5 h postinsemination (Fig. 2).

Following completion of the meiotic divisions the second phase of ooplasm segregation sets in. During this period, the area of clear cytoplasm in the oocyte cortex becomes larger as if the brim of the yolk cap were moving towards the vegetal pole. This bipolar segregation of the egg cytoplasm marks the beginning of the polarized stratified structure of the oocyte, replacing the previous concentric pattern of radial symmetry. After segregation, the zygote nucleus lies in the animal hemisphere together with clear cytoplasm rich in ribosomes and mitochondria. The cytoplasm with lipid droplets and yolk is concentrated in the equatorial and subequatorial regions. The cytoplasm with small yolk granules is located at the periphery of the vegetal hemisphere. A partial confluence of lipid droplets is observed during the concentration of inclusion in the vegetal hemisphere. The first cleavage is initiated at 6 h post-insemination.

Effect of colchicine

Two main variants of experiment with colchicine were performed. In one (A) the incubation with colchicine was started at 15 min post-insemination, in the other (B) the treatment began 70 min post-insemination. Cortical reaction was not affected by colchicine in the variant (A). But the first step of the oocyte polarization was depressed to a large degree during the first 4.5 h post-insemination. The clear cytoplasm domain retained its central location in the oocyte. However, at 6.5 h post-insemination the cytoplasm was observed in the animal hemisphere. In the variant (B) where samples were fixed every hour, it was ascertained that the clear cytoplasm kept its central location during the first 4 h of development. The nuclear envelope broke down and the chromosomes became centralized in the clear cytoplasm domain.

Starting at 4 h post-insemination, the central cytoplasmic domain often gave rise to protrusion(s) extending toward the egg surface (Fig. 4). The chromosomes together with specific sur-

rounding material usually appeared in these protrusions, though sometimes chromosomes were still observed in the central domain, in spite of the presence of well developed protrusions.

At 8 h of post-insemination, when cleavage is proceeding in control eggs, the clear cytoplasm together with nuclear material was observed in the animal hemisphere of the oocyte (type B experiment).

Effects of nocodazole and taxol

Incubation with nocodazole beginning at 25 min post-insemination did not stop cortical reaction but completely inhibited any movement of clear cytoplasm for several hours: the earliest sign of ooplasmic segregation took place 3.5-4 h post-insemination. Cytoplasmic protrusions connecting the central cytoplasm domain with oocyte periphery were observed as early as 4 h post-insemination in the experiment where incubation with nocodazole started after cortical reaction. The number of oocytes with such protrusions increased during the experiment. A stratification of cytoplasm occurred 5-6 h after continuous incubation with nocodazole (i.e. 6-7 h post-insemination). Thus, nocodazole can markedly hinder the ooplasmic segregation but not fully inhibit it.

Similar results were obtained when a mixture of nocodazole and taxol was used. In this case, the first signs of egg polarization were noticed at 4 h post-insemination.

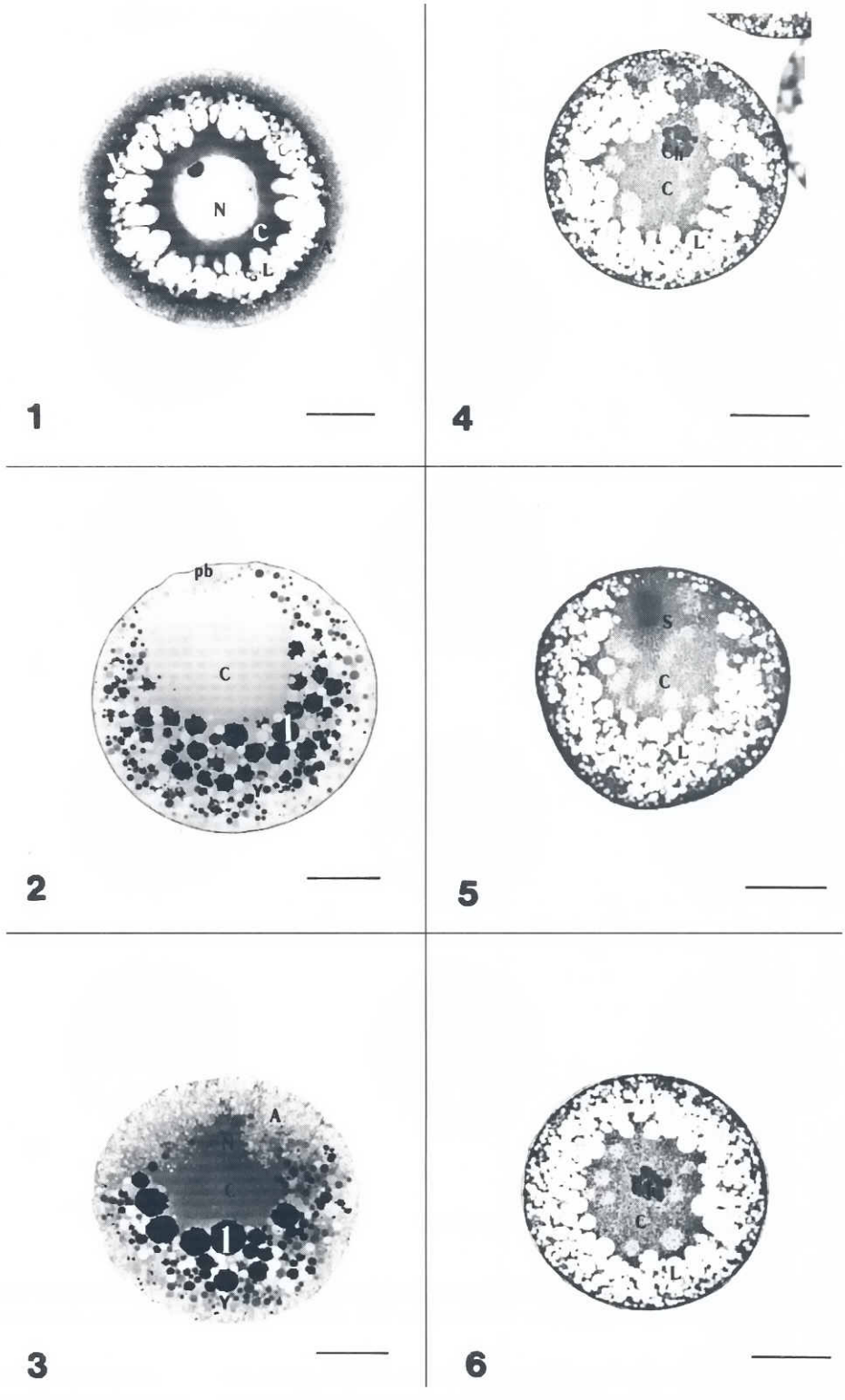

Fig 1. A full-grown oocyte of *Nereis virens* immediately after shedding. *Material was fixed in 2% gluteraldehyde and post-fixed in 1% OsO_4. The oocyte has not any signs of polarization. A, cortical zone with alveoli; C, yolk-free cytoplasm; L, lipid droplets zone; N, nucleus; Y, yolk granules.*

Fig 2. An egg after the second meiotic division (4.5 h post-insemination). *Material was fixed in 2% gluteraldehyde and postfixed in 1% OsO_4. C, zone of clear cytoplasm; l, lipid droplets; pb, polar bodies; Y, yolk granules.*

Fig 3. Effect of cytochalasin B on ooplasmic segregation (6 h post-insemination). *The incubation with cytochalasine B has been started 15 min after fertilization. Material was fixed in 2% gluteraldehyde and post-fixed in 1% OsO_4. Delayed ooplasmic segregation. A, cytoplasm with cortical alveoli; C, clear cytoplasm; l, lipid droplets zone; N, nucleus; Y, yolk granules.*

Fig 4. Effect of colchicine on ooplasmic segregation (5 h post-insemination) *Normal polarization is depressed. There is a central cytoplasmic domain with protrusion. Material was fixed in Zenker fluid. C, clear cytoplasm; L, lipid droplets; ch, chromosomes surrounded by homogeneous material.*

Fig 5. Effect of cytochalasin B on ooplasmic segregation (6 h post-insemination). *The incubation with cytochalasine B has been started 70 min after fertilization. Material was fixed in Zenker fluid. C, clear cytoplasm; L, lipid droplets zone; S, spindle and chromosome material*

Fig 6. Combined effect of colchicine and cytochalasin B (6 h post-insemination). *Fully blocked ooplasmic segregation. Material was fixed in Zenker fluid. C, clear cytoplasm; L, lipid droplets; ch, chromosomes surrounded by homogeneous material.*

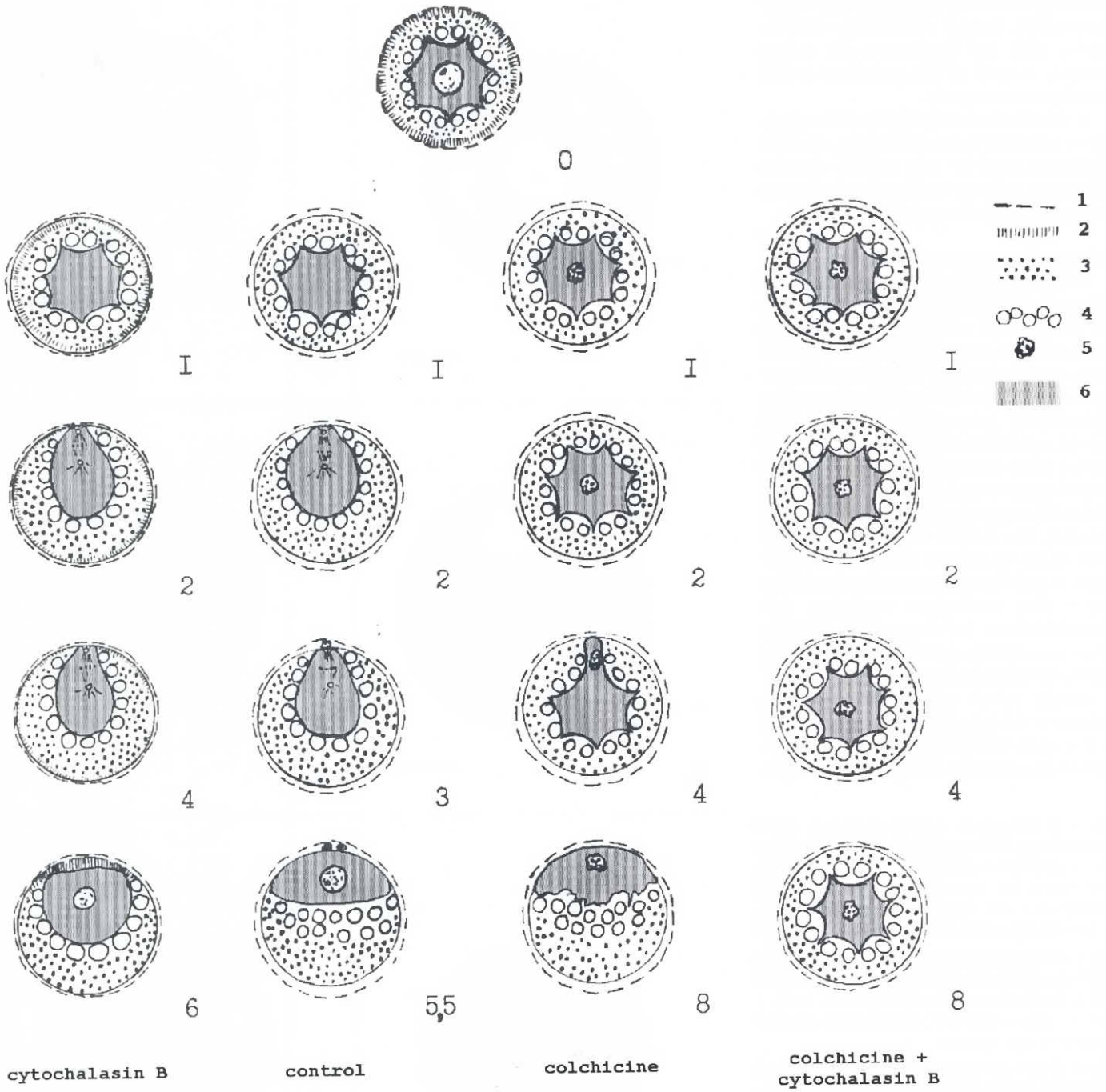

Fig 7. The scheme of effect of cytoskeleton inhibitors on ooplasmic segregation in *Nereis virens*. *1, vitelline envelope; 2, cortical alveoli; 3, yolk granules; 4, lipid droplets; 5, nucleus; 6, clear cytoplasm; ciphers to the right of pictures designate hours of development .*

The protoplasm protrusions (often with chromosomes) connected to the central cytoplasm, i.e., domain with the egg surface. At seven hour postinsemination stratification of the oocyte was observed.

Effect of cytochalasin B

Cytochalasin B inhibited the cortical reaction even at 0.2 μg/ml. In this case, cortical alveoli did not shed their material into the perivitelline space.

In oocytes treated with cytochalasin B at 1 h post-insemination, the nuclear envelope broke down, myxoplasm was located in the center of the oocyte and astral rays were clearly observed. The structure of the oocyte is concentric: the central domain of clear cytoplasm is surrounded by the sphere rich in lipid droplets which in turn is enclosed in the sphere rich in yolk and, finally the external sphere (with cortical alveoli) surrounding all these components. Three hour post-insemination, in the case when the experiment

began at 10 min post-insemination, or after 2 h when the experiment began at 70 min post-insemination (when cortical reaction was completed), the central cytoplasm domain stretched control along the spindle of the first meiotic division towards the periphery of the oocyte. This configuration persisted for a long time and it was observed sometimes 7 h after continuous incubation with the drug (Fig. 5). If the treatment began immediately after fertilization, interphase nucleus was observed at 6 h post-insemination (Fig. 3). However stratification of the egg was abnormal. The sphere of clear cytoplasm was eccentrically displaced and surrounded by cup-like layers of cytoplasm including the layers with lipid droplets and cortical alveoli. Some alveoli had been destroyed *in situ*. Later, this material was concentrated at the animal pole over the nucleus. It should be emphasized that the normal animal cap of clear cytoplasm did not appear after cytochalasin B treatment.

Effect of combined colchicine and cytochalasin B treatment. Incubation of oocytes in the mixture of colchicine and cytochalasin B began 70 min post-insemination. During the first 8 h of incubation, oocytes did not change the initial structure typical for oocytes after cortical reaction: the nuclear material (i.e., chromosomes surrounded with specific homogeneous plasma) was located at the very central point of the oocyte (Fig. 6). Further towards the periphery, the spheres of cytoplasm that were observed in the normal oocyte before ooplasmic segregation, particularly, the sphere with lipid-rich cytoplasm and sphere with yolk granules were detected. At the end of 8 h of incubation, ooplasmic segregation was completely inhibited.

Discussion

The preparation for karyogamy and subsequent cleavage divisions occur in the *Nereis virens* oocyte. This preparation is accompanied by structural reorganization of the oocyte, in particular by cortical reaction and ooplasmic segregation.

During cortical reaction the content of the cortical alveoli is released from the oocyte, and a thick jelly coat is formed from material released. It has already been established that cortical reaction in nereids is sensitive to cytochalasins (Dondua and Sidorova, 1986; Kluge et al., 1995). In this publication we have shown that the cortical reaction can be blocked by cytochalasin B even at concentration as low as 0.2 μg/ml. After completion of the cortical reaction, the cortical alveoli becomes occupied by yolk-rich cytoplasm in the external sphere of oocyte.

During ooplasmic segregation the radial-symmetric structure of *Nereis virens* oocyte is transformed into a polarized stratified one where different types of cytoplasm become aligned along the animal-vegetal axis. The sequence of the events occuring in *Nereis virens* oocytes is generally consistent with the descriptions for *Platynereis dumerilii* (Dorresteijn and Fischer, 1988; Dorresteijn and Kluge, 1990; Kluge, 1991) and can be subdivided into two phases.

The first phase involves the polarization of the central cytoplasmic domain (from spherical to an ellipsoidal shape). During this process, the clear cytoplasm of the central domain "perforates" the external strata rich in lipid and yolk inclusions and reaches the surface marking the animal pole where the first polar body soon appears.

The second phase of ooplasmic segregation starts after the second polar body separation: all clear cytoplasm is translocated into animal hemisphere whereas yolk-rich cytoplasm and cytoplasm with lipid vacuoles is shifted in the opposite direction.

The first step of the ooplasmic segregation is sensitive to colchicine, nocodazole and taxol, suggesting that microtubules are of primary importance for the initial stages of *Nereis virens* oocyte polarization. This may be a common property of nereids (Dorresteijn and Kluge, 1990). The fact that the external appearance of a central cytoplasmic domain depends on the availability of functional microtubules underlines the conditionality of "Hertwig's rule", which postulates that mitotic spindles can align themselves along the largest axis of the clear cytoplasmic domain. In the case of *N. virens* oocytes, active function of spindle microtubules is responsible for the formation of the cytoplasm domain, so that an inhibition of spindle assembly by colchicine or nocodazole prevents the polarization of cytoplasm. Although the results obtained underline unequivocally the leading role of microtubules in the promotion of ooplasmic segregation in *N. virens* oocytes, they also indicate the complexity of the mechanisms of segregation.

Indeed, when microtubule assembly is completely blocked, the cytoplasm of central domain can reach the oocyte surface via protrusions which often contain chromosomes. The formation of these protrusions when microtubule assembly is inhibited, seems to be a consequence of the centrifugal movement of cortical yolk-rich cytoplasm along the surface of oocyte from some particular point of the cortex. It may be hypothesized that actin microfilaments represent a driving force of this process because, when microtubules and microfilaments were simultaneously inhibited with a mixture of colchicine and cytochalasin B, the central cytoplasmic domain of the oocyte remained intact for at least 8 h, i.e. during the period of cleavage progressing in control embryos. This delayed and slow movement (as compared with control eggs) of the external cytoplasm toward the vegetal pole may be interpreted as the element of the ooplasmic segregation which plays a leading role during the second phase of the process and which is mediated by actin microfilaments.

In summary, ooplasmic segregation in *Nereis virens* implies existence of interactions between microtubules and microfilaments. Inhibition of either of these cytoskeletal elements will hinder or disturb the ooplasmic segregation but simultaneous disruption of microtubules and microfilaments will result in total cessation of this process (Fig.7). Integral functions of microtubules and microfilaments during ooplasmic segregation has already been shown in the leech (Fernández *et al.*, 1994).

Further studies on structural organization of oocyte cytoskeleton will be required to provide a better insight into the mechanisms of ooplasmic segregation. It would be interesting to examine reorganization of the cytoskeleton; after fertilization, during meiotic divisions and during subsequent preparation to the cleavage. Nereid oocytes may be of particular interest in this regard since their type of ooplasmic segregation is characterized by some inherent "primitive" traits. This "simplicity" can be perceived, for example, in the comparatively late animal-vegetal axis formation or in the obvious conjunction between the mechanisms of initiation of ooplasmic segregation and oocyte nucleus translocation to the cell periphery where meiotic divisions occur.

During the process of evolution these mechanisms could serve as a basis for the development of more complicated types of ooplasmic segregation and as an approach for precise localization of specific cytoplasmic factors that determine cell lineages in the embryo.

Materials and Methods

Experiments on *Nereis virens* oocytes and embryos were carried out at the Marine Biological Station of the St. Petersburg State University, on the White Sea. Adult animals were collected during spawning, from the second half of June until the beginning of July. The males and females were kept separately. Oocytes obtained were washed twice during 1.5 h with Millipore-filtered sea water. Artificial fertilization and cultivation of the embryos was carried out in a thermoregulated room at 10.5-11.5°C (Dondua, 1975). Ten minutes after the addition of sperm diluted with sea water, the eggs were washed three times with Millipore-filtered sea water and cultivated in 4 l glass vessels with constant stirring.

To study effect of cytoskeleton inhibitors on the ooplasmic segregation, the fertilized eggs were placed in various drug solutions in pasteurized (80°C for 20 min) Millipore-filtered (Synpor 8, VUFS, Chemapol, Prague) sea water. Experimental variants differing in inhibitor concentrations and in time of the treatment were carried out. The variants, where the start of the treatment was 60 -70 min after fertilization, were characterized by completion of the cortical reaction and by the first polar body separation accordingly. The following concentrations of inhibitors were used: colchicine (Ferrak Berlin)-25 μg/ml, 100 μg/ml and 200 μg/ml; cytochalasin B (Sigma)-0.2 μg/ml, 5 μg/ml, and 10 μg/ml; nocodazole (Sigma)-5 μg/ml; taxol (Calbiochem)-5 μg/ml. The inhibitors were also used in combinations, namely, the mixture of nocodazole and taxol with concentrations 5 μg/ml for each component and the mixture of colchicine ($5x10^{-4}$M) and cytochalasin B (5 μg/ml).

The embryos were fixed in Zenker fluid for 8 h at 0.5 h intervals. The fixed material was stained with Mayer and Hansen hematoxylin.

For transmission electron microscopy, the material was fixed with 2% glutaraldehyde solution in PBS for 2 h and post-fixed with 1% OsO4 in PBS for 2 h. After dehydration, the samples were embedded in Araldit. Blocks were cut with a LKB- Nova ultratome, and after staining with uranil acetate and lead citrate, viewed using a Tesla BS 500 transmission electron microscope.

For serial semithin sections, Zenker or glutaraldehyde and OsO_4 fixed material was stained with gallocyanin (Fluka) at pH 0.85, dehydrated in ascending alcohol concentrations and embedded in Araldit. Sections were cut using a LKB-Nova ultratome and post-stained with methylene blue.

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