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Attribution of Cortical Granules to Formation of Fertilization Envelopes and Polyspermy Block in *Urechis unicinctus*

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Abstract: Cortical reaction and polyspermy block are well defined in most marine invertebrates. In *Urechis* species, the function of cortical granules (CGs) is not yet known, and there is controversy on whether the cortical reaction occurs, or the fertilization envelope (FE) is attributed to CG releases or functions to prevent polyspermy. This study was carried out to determine the cortical reactions and functions of the FE in *Urechis unicinctus*. Artificial insemination of the eggs revealed that CG release occurred to give rise to perivitelline space (PS) into the final FE. Both PS and final FE effectively blocked polyspermy. The final FE was accomplished within 10 min after sperm-egg initial binding. No massive release of CGs occurred within the early phase of 5 min after the initial binding, initially and the PS seemed to play a role to prevent polyspermy. The CG massively released its content into the PS in late phase of FE formation, and differentiated PS into five intermediate layers. The layers opened into each other by anastomosis, so that the final FE consisted of two layers, the inner layer (15 μm in thickness) and the outer layer (1 μm in thickness). The outer layer derived from vitelline layer and the inner layer consisted of PS and CG secretions. Immunofluorescence and confocal laser microscopy revealed that the spermatozoon took up residence in the egg cortex during FE formation and successive meioses of the fertilized egg. These results suggest that both PS and final FE of *U. unicinctus* were equivalent to the early and late block, respectively, of other marine animals.

Key words: Cortical granules, fertilization envelope, polyspermy, *Urechis unicinctus*

In many animals, fertilization envelope (FE) prevents polyspermy and provides extracellular space in which the fertilized eggs undergo cleavage and development (Berg and Wessel, 1997). The FE is attributed to oocyte specific secretory granules, cortical granules (CGs) (Matese et al., 1997). It is known especially in echinoderm, where the CGs draw spatially closer and fuse to oolemma and its contents are expelled to be secreted into perivitelline space (PS). The cortical reactions are mediated by free Ca^{++} and several membrane proteins (Conner et al., 1997; Stricker, 1999).

Although the CGs appear in eggs of *Urechis* species (spoon worm, innkeeper), whether the FE is attributed to CGs secretions, or the FE plays a role to block polyspermy is not clear. Gould-Somero (1975) observed in *U. caupo* that FE was elevated without a statistical decrease in the number of CGs, which were intact until gastrular stage. There are pro (Gould-Somero, 1975) and contra (Paul, 1975) views on whether FE is effective in preventing polyspermy. It seemed likely that a part of the unsettled situations repeats also in other animals. In echinoderms, molluscs and some annelids, the cortical reactions occur, and FE prevents polyspermy (Vacquier, 1975). The CG has not yet been reported in *C. elegans* and *D. melanogaster* (Vacquier, 1981). In *Mytilus edulis*, when the eggs are exposed to sea water, CGs are discharged prior to fertilization and the FE failed to block polyspermy (Humphreys, 1967).

In this study, in *U. unicinctus* and another *Urechis* species, the PS was elevated from the oolemma after initial sperm-egg binding, cortical reactions occurred and the CGs released its contents massively into the PS. Immunofluorescence staining followed by confocal laser microscopy showed that both PS and FE were effective barriers to polyspermy. This work reports that cortical reactions and functions of the FE in *U. unicinctus* are largely similar with those of already known echinoderms, molluscs and some annelids, but deviated from the previous reports in *U. caupo*. There are indications that the mature egg was fertilized at the stage of 2n karyotype, because the fertilized spermatozoon

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had patient residence in the egg cortex during the time periods of two successive meioses of the egg.

MATERIALS AND METHODS

Sample preparation for electron microscopy

In the season, mature sperm and eggs can be prepared from two pairs of testes and ovaries of both sexes, respectively. The mature generative cells can also be prepared from coelomic fluid of this tide worm. The freshly prepared mature sperm showed no activities, but the sperm showed strong activities upon contacting with natural sea water (NSW, 22‰, pH 8.1-8.2, 15-17°C). But the strong activities, supposedly required to fertilization, were exhibited for about 1 min, decreased rapidly thereafter, while the sperm survived for 3 min.

The mature eggs (>130 μm in diameter) have cytoplasmic indentation and nuclear invagination, but the eggs restored round shapes in sea water of lower salinity (<18‰). The round shape can also be recovered when the eggs are fertilized in NSW of normal salinity (≒ 22‰). The eggs were washed several times in 0.45 μm millipore filtered NSW. Artificial insemination was performed by intermixing the sperm and eggs in ratio of 400:1 (v/v) for 2-3 min.

The fertilized eggs were used for EM and immunofluorescence preparations. For electron microscopy for early events of the fertilization, such as initial sperm-egg binding and elevation of fertilization cone, the materials were prefixed in 0.1 M phosphate buffered 4% formaldehyde containing 0.5% glutaraldehyde for 1 h at 4°C and fixed in 0.1 M phosphate buffered 2.5% glutaraldehyde for 2 h. The materials were then washed in 0.2 M phosphate buffer and postfixed in 1% OsO_4 for 2 h. After washing in DW, the materials were dehydrated in a series of graded concentrations of ethanol and propylene oxide, and embedded in araldite mixture. The blocks were incubated for overnight at 40°C, 60°C and 65°C, and then sectioned with OM-2 Reichert microtome. The sections were contrasted in saturated aqua solution of uranyl acetate and lead citrate and examined on electron microscope JEOL 1010B.

Immunofluorescent preparations and confocal laser scanning microscopy (CLSM)

To prepare CLSM materials, the fertilized eggs were fixed in cold methanol (-20°C), and then washed three times in phosphate buffered saline solution (PBS; pH 6.8). To observe meioses, polar body formation and the fertilized spermatozoon taking residence in the egg cortex, the fertilized eggs were treated in 2% Triton X-100 for 12 h to enhance reaction efficiency to anti-tubulin, and then washed three times in the same PBS. The primary antibody (Ab), anti-mouse β-tubulin were diluted in PBS containing 0.1% Triton X-100 in ratio 1:200, and the materials were incubated in the Ab solution for 1 h at 37°C. Microtubules and egg cytoplasm were stained with FITC-conjugated goat anti-mouse IgG for 1 h at 37°C. The male and female pronuclei were stained with propidium iodide diluted in alcohol, embedded in glycer gel (Dako), and observed on CLSM (MRC-1024, Bio-Rad) using Kr-Ar dual laser.

RESULTS

Shortly after sperm-egg initial binding in *U. unicinctus,* the fertilized eggs showed several fertilization events, such as elevation of vitelline layer to give rise to perivitelline space (PS), retractions of microvilli, cortical reactions, germinal vesicle break down (GVBD) and formation of fertilization envelope (FE).

The final FE was established at about 10 min after the initial binding sperm-egg. During the early phase (5 min), PS reached its full thickness (15 μm) without massive release of the CGs. The CGs appeared in the egg cortex and increased during the late phase of about 4-6 min after the initial binding. The cortical reactions occurred in the late phase of FE formation at which the CGs massively released its contents into the PS. The fertilized eggs underwent two successive meioses producing three polar bodies, while the fertilized spermatozoon took residence in the egg cortex.

Formation of perivitelline space

Unfertilized eggs were characterized by prominent germinal vesicle (GV, 30 μm in diameter) and well developed nucleolus (3 μm in diameter). The GV was slightly eccentric, so that animal and vegetal poles can be distinguished despite of weak polarity of the eggs (Fig. 1A-2). Cytoplasmic indentation of the unfertilized egg seemed to depend on concentrations of NSW salinity; round shapes have been observed in salinity below 22‰. The fertilized eggs also recovered its round shape. Microvilli extrude over vitelline layer (VL) of unfertilized eggs (Fig. 1A-1). There was evidence that intial sperm-egg binding occurred between acrosomal processes and tip of microvilli extruded over the VL. The initial binding brought the VL to be elevated to form fertilization cone (Fig. 1B). The elevation of the VL spread over the whole oolemma to establish perivitelline space (PS) until it reached 15 μm in thickness. Distance between polysperm outside of the VL and oolemma increased in direct proportion to the thickness of elevating PS (14-16 μm) (Fig. 3B). The PS seemed to be filled with amorphous substances on LM, and some retracted forms of microvilli and membrane debris were observed on EM (Fig. 1D). GVBD was observed during PS formation (Fig. 3A).

It seemed that cortical reactions in *U. unicinctus* began in

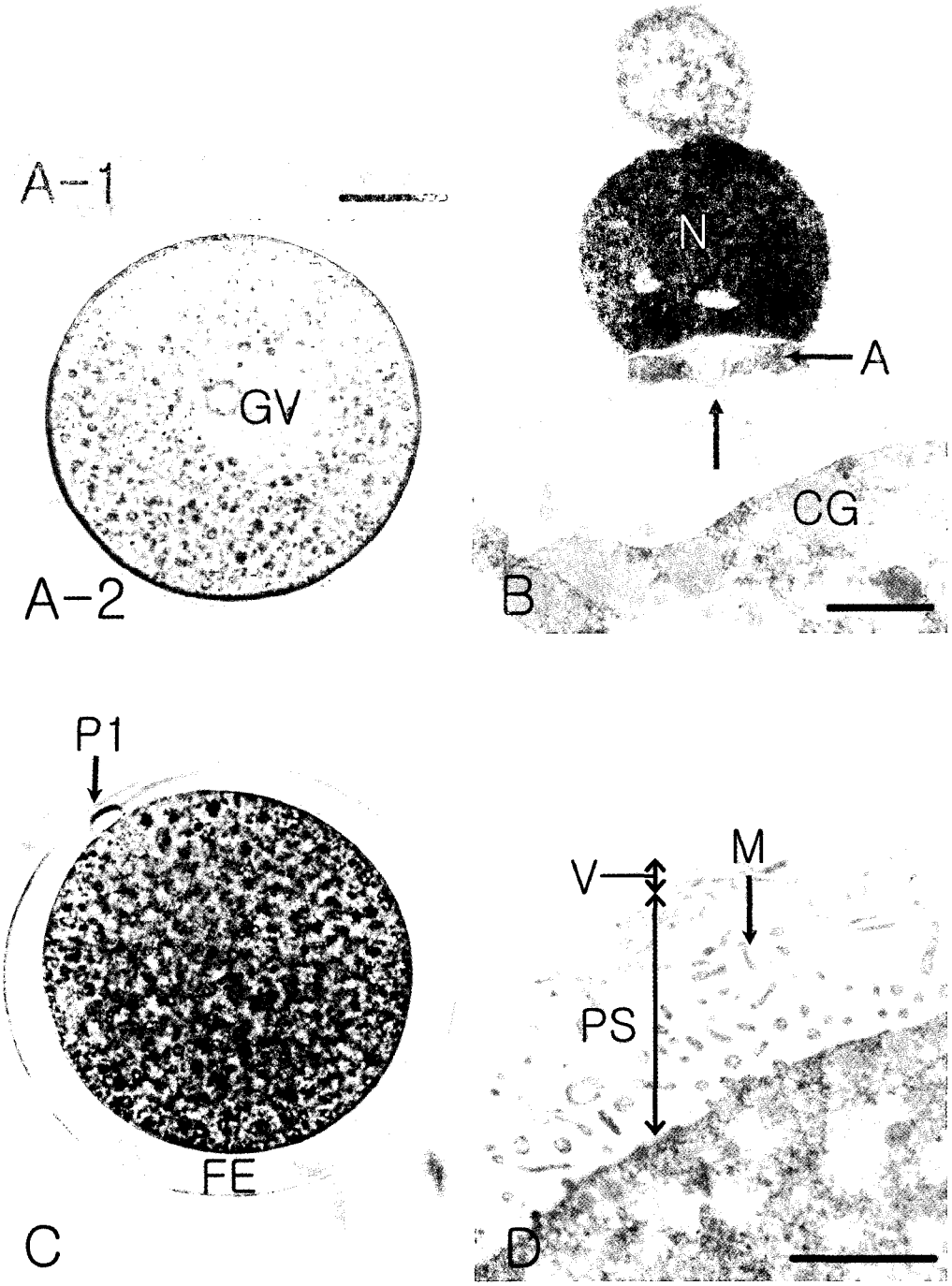

Fig. 1. A-1, Numerous microvilli of unfertilized egg. A-2, Germinal vesicle (GV) is prominent in an unfertilized egg of *U. unicinctus*. B, At fertilization, the acrosomal process (A) was inserted into elevating vitelline layer (arrow) of the egg. CG; cortical granule, N; nucleus of sperm. C, The GV has been broken down after fertilization and the perivitelline space elevated without discharges of cortical granules in the early phase of fertilization envelope formation. P1; first polar body. D, The perivitelline space (PS) in early phase of FE formation. Cortical granules have not yet appeared in egg cortex, but the PS reached its full thickness of 14-16 μm. M; cross sectioned microvillus, PS; perivitelline space, V; vitelline membrane. *Scale bars* = 1 μm (A-1, A-2, B) and 2 μm (C, D).

the late phase of FE formation. After 4-6 min of the initial binding, CGs (1 μm in diameter) began to appear in the egg cortex. Some CGs aligned along and approached the oolemma, while the others associated closely with the oolemma, and another opened its membrane outwardly into the PS, like exocytotic vesicles of somatic secretory cells

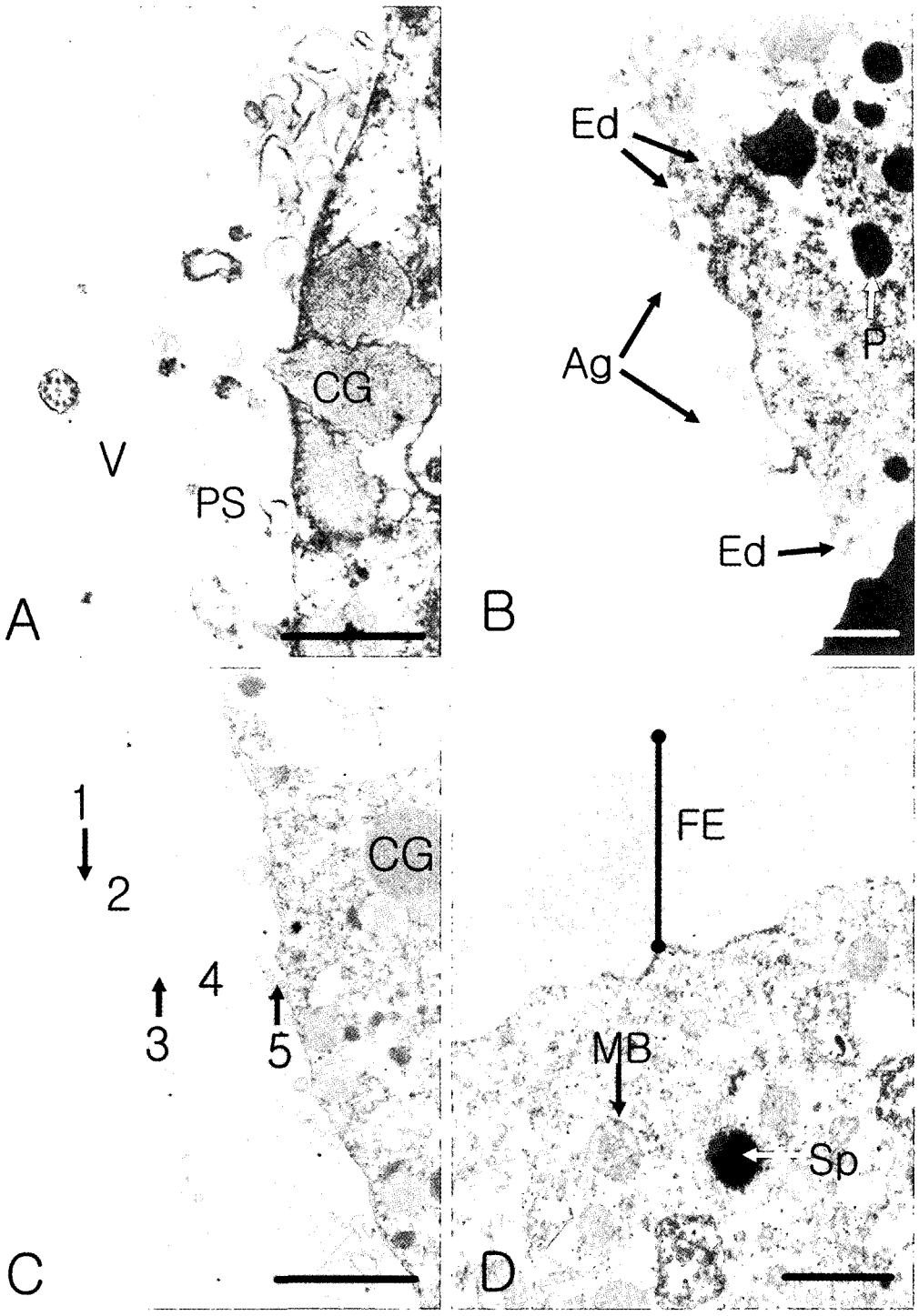

Fig. 2. A, During the late phase of fertilization envelope formation, the CGs and endocytotic vesicles have appeared and the CGs are aligned in the egg cortex, fused with the oolemma while the vitelline layer (V) diffused inwardly into the perivitelline space (PS). B, The aggregates (Ag) in perivitelline space arose from the CG contents secreted into the PS. Ed; endocytotic vesicle, P; pigment granule. C, The aggregates were opened into each other by anastomosis, so that the FE differentiated into 5 intermediate layers which were numbered from the vitelline layer inwardly. D, Continuous diffusion of the aggregates furnished a monolayer beneath the vitelline layer. The final fertilization envelope (FE) in *U. unicinctus* consisted with 2 layers, the inner layer arose from the cortical granules secretions and the outer layer derived from the vitelline layer. The fertilized spermatozoon (Sp) resided in inner cortex. MB; multivesicular body. Scale bar=2 μm.

(Fig. 2A). Since the number of secretory CGs were increased during the late phase, it could be inferred that massive secretions of the CGs occurred in the late phase of FE formation.

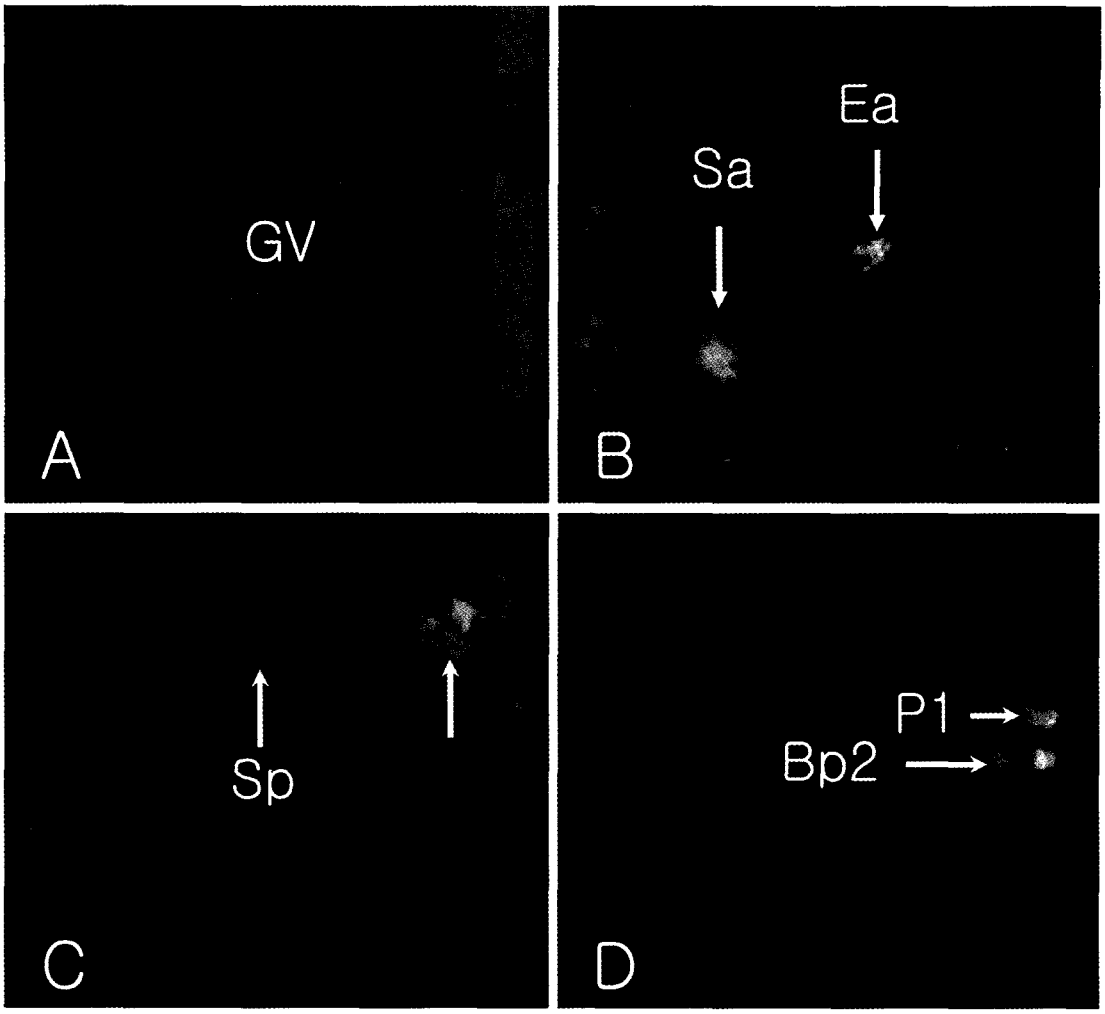

Fig. 3. A, An immunofluorescence microscopy taken immediately after fertilization revealed that the germinal vesicle (GV) has not yet been broken down while the spermatozoon penetrated into egg cortex. B, Asters of sperm (Sa) and egg (Ea) were activated while polyspermy were blocked effectively by the fertilization envelope. C, A process extruding the 1st polar body. Sp; fertilized spermatozoon, Arrow; Meiotic chromosomes of the egg. D, The fertilized egg performed the 1st and 2nd meiosis. P1; first polar body, Bp2; second polar body. <x400>

Formation of the intermediate and final fertilization envelope

The oolemma undulated deeply to create exocytotic pits, when much more numbers of CGs were opened wide into the PS. The contents of the secretions were visible on EM as fine particles in the exocytotic pits. Small vacuole-like vesicles of various sizes appeared beneath the oolemma, when secretory activity of the CGs attained higher level. The vesicles were observed as endocytotic vesicles which appeared to be a counterpart of the oocyte specific exocytosis in some echinoderm. In the beginning, the CG secretions aggregated into globular masses (2-3 μm in diameter) in the PS (Fig. 2B). Morphologically, the fine particles of marginal globular masses showed diffused contour. The marginal fine particles dispersed toward middle portion of the PS, while the VL diffused inwardly separating itself into two layers by optical densities. So, the PS was differentiated into five intermediate layers (intermediate FE, iFE) at about 8 min of the initial sperm-egg binding (Fig. 2C).

The five iFEs opened each other by anastomosis into two final FE at near the end of FE formation. The iFEs were numbered inwardly for convenient description in this work: The outermost layer was the layer 1 and the innermost layer was the layer 5. Outer layers 1 and 2 derived then from the VL of the unfertilized egg and the layers 3-4 came from the PS filled with CG secretions. The layers 1-4 were combined to become a homogeneous monolayer (15 μm in thickness), and the inner layer of the final FE. The layer 1 of the iFE became the outer layer of the final FE. There was optical evidence that the inner layer was hardened at 10 min after the initial sperm-egg binding.

Other fertilization events were observed during FE formation. As GVBD and two successive meioses proceeded

(Fig. 3C, D), polar bodies extruded into the PS (Fig. 1C). Immunofluorescence microscopy revealed that the fertilized spermatozoon took residence in the egg cortex until the egg accomplished the first and second meioses (Fig. 2D).

DISCUSSION

In *U. unicinctus* (spoon worm, innkeeper), beginning at the fertilization cone, the vitelline layer (glycocalyx: Bennett, 1963; vitelline envelope and activation calys; Anderson, 1968) elevated to make the perivitelline space (PS) thicker shortly after intial sperm-egg binding and the CGs released its contents into the PS to form final FE. Both PS and final FE played roles in preventing polysperm and providing space, in which the embryo was cleaved and processed for further development. These fertilization events in *U. unicinctus* deviated from what is known in *U. caupo*, another *Urechis* species. In *U. caupo*, the FE failed to prevent polyspermy and the cortical granules (CGs) were not statistically decreased in number during FE formation, persisting until gastrular stage as a cytoplasmic protrusion (Gould-Somero, 1975). There is also controversy whether the FE effectively to prevent polyspermy. The FE was functional in preventing polyspermy without participation of CG secretions (Gould-Somero and Hollander, 1975), but Paul (1975) and Gould-Somero (1975) described that it was unable to block polyspermy. Gould-Somero and Hollander (1975) suggested that the mechanism of polyspermy block in *Urechis* species appears to be fundamentally different from other animals. It was not known whether the FE prevents polyspermy in *Urechis* species.

It has been already known that the prevention of polyspermy is accomplished by restricting the number of sperm approaching the egg, either by narrow micropyle, or by development of extracellular coat, such as fertilization envelope and zona pellucida (Wolf, 1981), or by restricting the number of sperm nuclei that combine with the egg nucleus when numerous sperm enter the egg (Jaffe and Gould, 1985). *Urechis unicinctus* seem to belong to large group of developing extracellular FE to prevent polyspermy. However, in detail, there are various cases where the CGs provide the FE and block polyspermy. The CG releases its contents into the PS to establish the FE in echinoderm (sea urchin: Vacquier, 1975; Berg and Wessel, 1997), star fish (Sardet and Chang, 1987), mollusc (Speksnijder et al., 1986), ascidian (*Phallusia mammillata*; Sardet et al., 1994), annelid (*Tubifex hattai*; Shimizu, 1995), amphibian (*Xenopus*; Larabell, 1993; Chang et al., 1999; Nuccitelli and Ferguson, 1994) and mammals (mouse; Wasserman, 1977, 2001). Cortical reactions occurred in those animals to furnish the FE. But in *Mytilus edulis,* the CGs are released prior to insemination, there is no visible cortical reaction at the insemination (Pasteels, 1958; Humphreys, 1962). In *Spisula,* CGs are not released in response to insemination, but polyspermy is rapidly prevented (Rebhun, 1962). In *Sabellaria,* the FE elevation is triggered before insemination by exposure to sea water (Pasteels, 1965). In mammals, CG secretion occurred without significant elevation of the zona pellucida (Szollos, 1967). Very little is known about CGs in *Caenorhabditis elegans* and *Drosophila melanogaste.* It is likely that polyspermy block with regard to the substantial contributions of the CGs is yet unknown or different from species to species. As described above, this discrepancy also repeated in *Urechis* species.

In *U. unicinctus,* the situations of FE formation and cortical reactions as well as polyspermy block were different from *U. caupo,* but similar with those of other marine animals, as the above mentioned echinoderms, molluscs and annelids. In the early phase of FE formation, the vitelline layer was elevated from the oolemma to create perivitelline space (PS, 14-15 μm in thickness). Numerous microvilli retracted inside the PS. In the late phase, the CGs appeared and displaced towards the oolemma. Membrane fusion between the CGs and oolemma brought the CG materials to be massively released into the PS. During CG exocytosis, the membrane components may merge into the oolemma and a part of the mosaic oolemma can be retrieved by uptake into coated vesiclesin sea urchin species (Eddy and Shapiro, 1976; Fisher and Rebhun, 1983). It is possible that in *U. unicinctus*, the vacuole-like endocytotic vesicles of various sizes were closely associated in function with the coated vesicles of the echinoderm. The CG secretions gathered firstly into globular masses in the PS. Because of continuous diffusion of the globular masses, the PS differentiated into five intermediate layers which opened later to each other into the homogeneous monolayer, the final FE. Thus, the elevation of the PS and cortical reactions in *U. unicinctus* may be equivalent to what is already known and the slow block to polyspermy of the other marine animals, such as sea urchin (Rothschild, 1952).

Both final FE and PS were effective to prevent polyspermy in *U. unicinctus.* It was suggested that the PS might play a role in initial prevention of polyspermy and the cortical reaction provide a permanent block, as reported in echinoderm (Foerder and Shapiro, 1977). Different from the case with *U. caupo*, the PS seemed an effective barrier to polyspermy. The distance between oolemma and numerous sperm outside vitelline layer increased in direct proportion to the thickness of PS (15 μm). Shortly after initial sperm-egg binding and as the PS elevated, microvilli on the oolemma retracted rapidly inside the elevating PS. Morphological evidence in *U. unicinctus* indicated that initial sperm-egg binding occurred at the tip of the microvilli. On the other hand, the binding increased intracellular pH by 0.25-0.3 unit (alkalinization), and its

activities (Gould and Stephano, 1993). Since the egg activation and retraction of the microvilli alter the sperm receptors on the oolemma, if may also be important for establishment of permanent polyspermy block in polychaete (Sato and Osanai, 1986), sea urchin and mammalian egg (Barros and Yanagimachi, 1971; Carroll and Epel, 1975). The possibility has been thought that the retractions of microvilli in *U. unicinctus* may also provide a initial block to polyspermy being reasserted later by the CG secretions.

Another reason to believe that PS was an effective barrier for polyspermy is based on correlation between persistence of sperm activity and duration of PS elevation. *Urechis unicinctus* sperm were strongly active when in contact with NSW (22‰, pH 8.1-8.2, 15-17°C) and survived for 3 min. During this period, the PS was elevated to about half of its full thickness, most of its microvilli were retracted to inside, but the massive CG release was not yet observed. This suggested that the sperm in NSW is fertilized within 1 min of *U. unicinctus*, while the sperm persisted in its strong activities. This observation corresponded to observation in *U. caupo*, where polyspermy is established in most cases within 1 min (Paul, 1975) and to echinoderm, where the early block is evoked within 2 sec after sperm-egg initial binding (Hinkley et al., 1986; Sardet et al., 2002). Take a together, it seemed apparent in *U. unicinctus* that the PS played a role to initially prevent polyspermy, equivalent to the early block of echinoderm and the cortical reaction provided a permanent block to polyspermy which is equivalent to slow block of other marine invertebrates.

In the course of studying blocking polyspermy, immunostaining followed by CLSM revealed GVBD, primary and secondary meioses, behavior of meiotic apparatus and polar body formation and activation of spermatozoon monaster in the fertilized egg. The fertilized spermatozoon stayed in the egg cortex while the egg proceeded with two successive meioses. When the second polar body associated closely with the oolemma, monaster of the spermatozoon began to displace the female pronucleus. Fusion of the two pronuclei has also been observed in the course of this work (not published).

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