FUNCTIONS OF THE EGG ENVELOPE OF MYTILUS EDULIS DURING FERTILIZATION

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FUNCTIONS OF THE EGG ENVELOPE OF *MYTILUS EDULIS* DURING FERTILIZATION[1)]

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To examine the roles of the external envelope and the plasma mambrane of bivalve oocytes in the reception and exclusion of sperm, which are essential events for monospermic fertilization, envelope-free (naked) oocytes of *Mytilus edulis* were prepared. Sperm underwent the acrosome reaction on the naked egg surface and penetrated into the oocyte. Electron microscopy showed that a protrusion from the egg cytoplasm developed along the acrosomal process and engulfed the fertilizing sperm head. The manner of sperm entry was almost the same as that into intact oocytes possessing an egg envelope. The data indicate that formation of the cytoplasmic protrusion (penetration cone) is essential for incorporation of sperm into the egg cytoplasm whether the egg envelope exists on the egg surface or not. Polyspermic fertilization occurred when sperm were added to naked oocytes at metaphase I. The ratio of polyspermic fertilization corresponded to the sperm concentration in egg suspensions. The occurrence of polyspermy with naked metaphase I oocytes suggests that the egg envelope may have a role in preventing the entry of extra sperm. Intact metaphase II oocytes were still fertilizable and naked metaphase II oocytes could be fertilized by acrosome-reacted sperm. Re-fertilization experiments revealed that the oocytes were re-fertilized at metaphase II if acrosome-reacted sperm were added after removal of the envelope; intact metaphase II oocytes could not be re-fertilized. These results suggest that the ability to induce an acrosome reaction in the sperm had changed, and that a complete polyspermy blocking mechanism did not develop in the egg plasma membrane of *Mytilus* oocytes even after fertilization.

Introduction

Most molluscan oocytes have a thick envelope on the plasma membrane (Humphreys, 1962; Longo and Anderson, 1969a, b; Popham, 1975; Hylander and Summers, 1977; Kyozuka and Osanai,1985). During fertilization, spermatozoa first interact with this layer and only the fertilizing spermatozoon penetrates it. Adhering to this layer, sperm undergo the acrosome reaction and protrude the acrosomal process into the egg envelope to reach the egg plasma membrane (Hylander and Summers, 1977; Kyozuka and Osanai, 1985). There are two questions concerning the first sperm-egg interaction after induction of the sperm acrosome reaction on the egg envelope. Why does only one spermatozoon enter the egg, and how does it penetrate the egg envelope?

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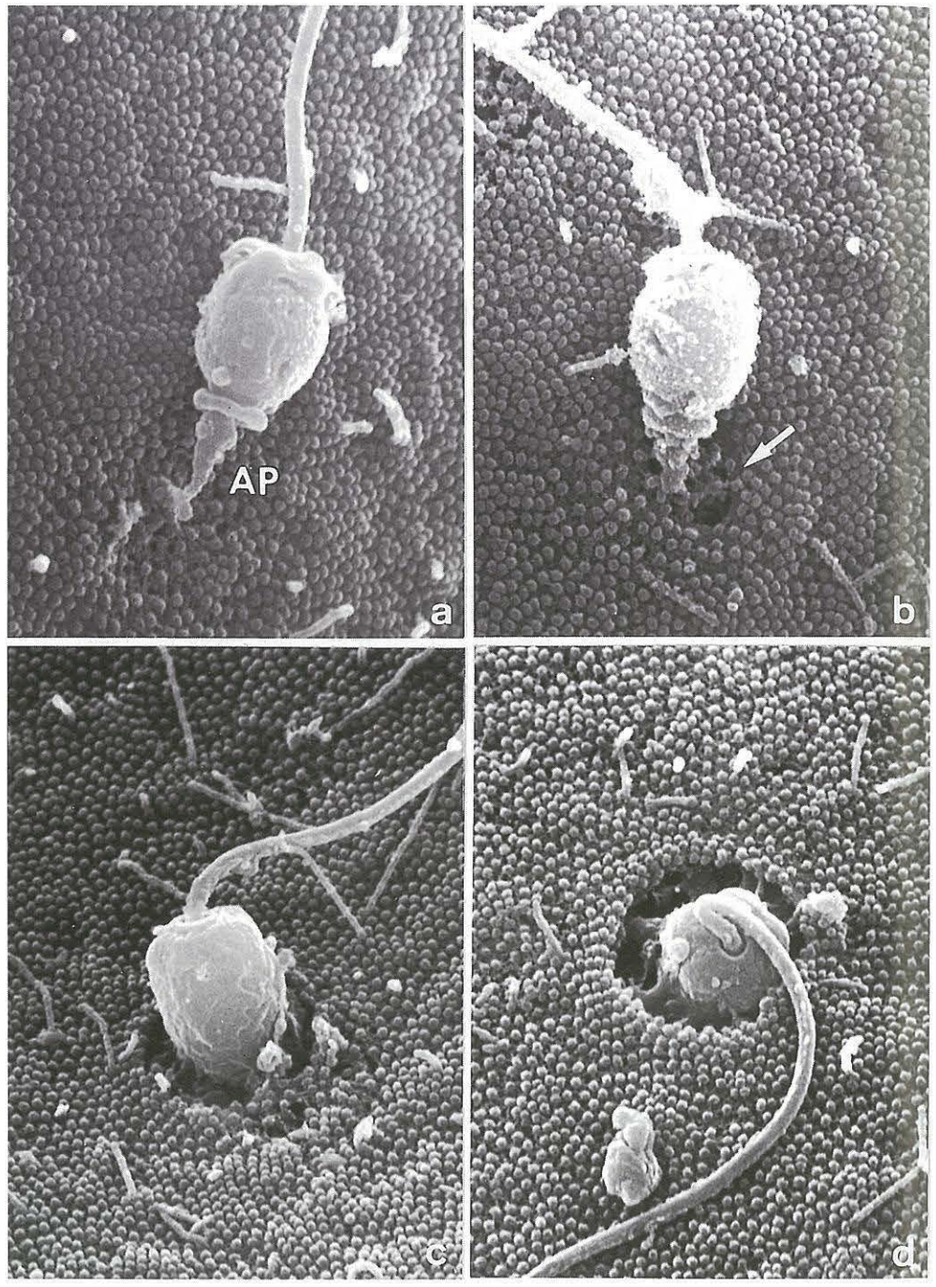


Egg membrane lysin has been detected in the sperm of several species (Tyler, 1939; Haino and Kagawa, 1966; Heller and Raftery, 1973; Ogawa and Haino-Fukushima, 1984; Haino-Fukushima and Usui, 1986) including *Mytilus* (Hauschka, 1963). Egg membrane lysin makes a hole for the fertilizing spermatozoon to penetrate through the egg envelope. In abalone, the lysin makes the egg envelope swell and loosens it, which eases its penetration by fertilizing sperm (Usui and Haino-Fukushima, 1991). However, the function of lysin *in vivo* during fertilization of *Mytilus* is unknown. Alternatively, in some species the penetration cone mediates sperm incorporation into the egg cytoplasm through the egg envelope (Hylander and Summers, 1977; Kyozuka and Osanai, 1985).

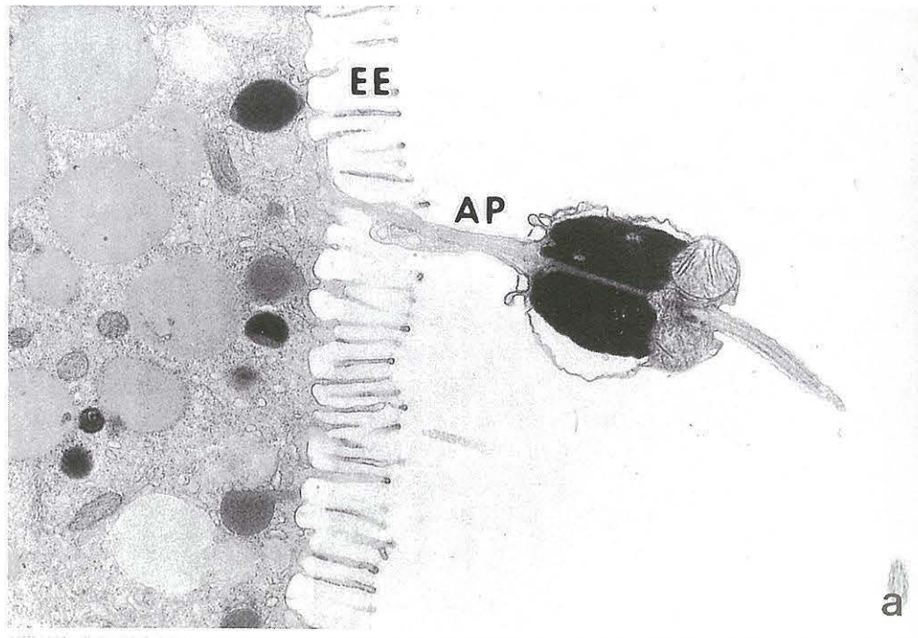
After penetration of a fertilizing spermatozoon, excess sperm can not fuse with the egg surface. Some characteristic change to block the penetration of extra sperm might occur in the egg envelope and/or some other polyspermy blocking mechanism might operate in the egg plasma membrane. Therefore, we romoved the egg envelope from metaphase I oocytes and compared the fertilization process in intact and denuded oocytes to examine the role of the egg envelope in sperm penetration and that of the egg plasma membrane in polyspermy blocking mechanisms.

Materials and Methods

Materials: *Mytilus edulis* specimens were collected from a culture system of the ascidian, *Halocynthia roretzi*, near Asamushi Marine Biological Station in March, 1994. Gemetes were obtained by high-temperature stimulation. Animals kept in running seawater (8°C) were transferrend to seawater at 32°C for 30 min and then returned to cold seawater. Gametes spawned during the 2 h after stimulation were used for these experiments.

Preparation of naked metaphase I and intact or naked metaphase II oocytes: Spawned oocytes remained at metaphase I and were washed twice in Ca^{2+}, Mg^{2+}-free seawater (CaMgFSW) and incubated in 1% actinase (Kaken-Kagaku Co., Japan) in CaMgFSW containing 4 mM ethyleneglycol-bis-(α-amino-ethylether) N, N'-tetra-acetic acid (EGTA, pH 7.8). The egg envelope disappeared during this treatment. After 10 min, the oocytes were washed in seawater several times and used as naked

Fig. 1. Scanning electron microscopy of sperm entry at a metaphase I oocyte of *Mytilus edulis* (×20,000).
(a) 30 s after addition of sperm. The sperm has undergone the acrosome reaction and the tip of acrosomal process (AP) adheres to the egg surface. (b) 30 s after addition of sperm. Microvilli around the sperm acrosomal process are disturbed (arrow). (c) 1 min after addition of sperm. The sperm head penetrates through the egg envelope. (d) 1.5 min after addition of sperm. The sperm head is incorporated into the egg cytoplasm, but the sperm mitochondria and tail still remain on the surface. Regular microvillar patterns on the egg envelope remain unchanged except in the region of spem entry.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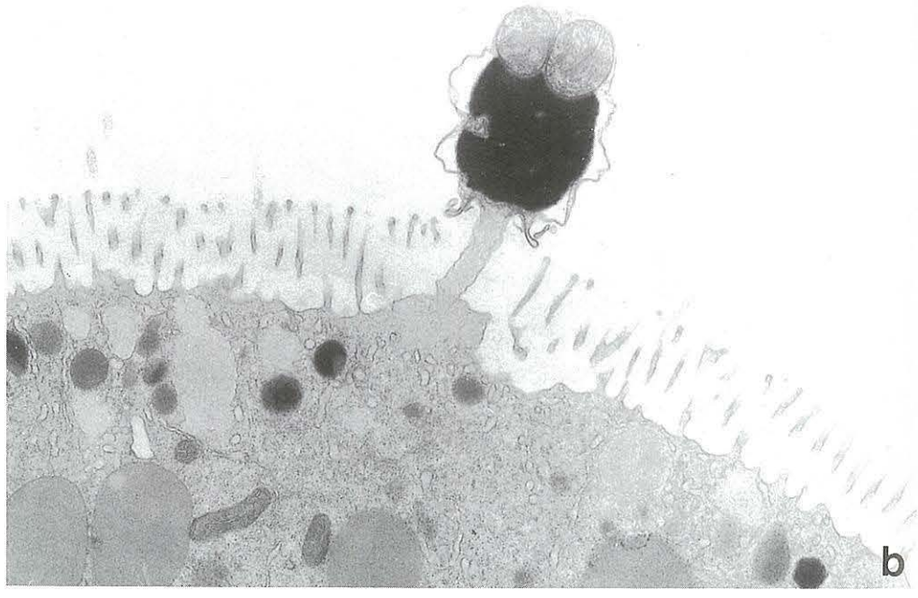
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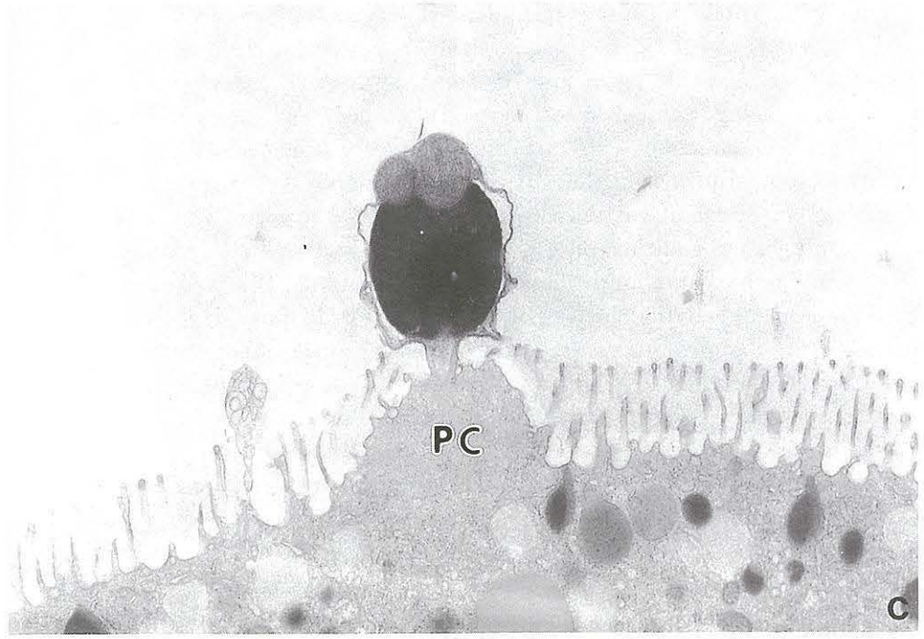
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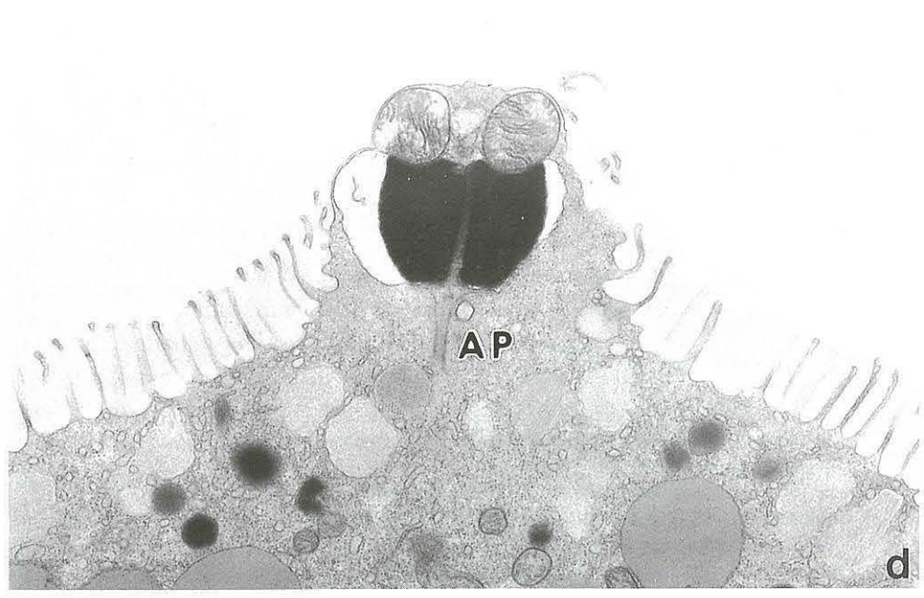
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oocytes at metaphase I. Naked metaphase II oocytes were obtained by treating the spawned oocytes with 1% actinase in CaMgFSW without chelator for 10 min. A first polar body was protruded and the oocytes proceeded to metaphase stage II in 10 min after being returned to seawater. Intact metaphase I oocytes were treated with 2 μM calcium ionophore A23187 in seawater for 10 min and then transferred to pure seawater. Intact metaphase II oocytes developed within 30 min.

Fertilization : Sperm at various dilutions were added to intact and naked oocytes. Ten minutes after addition of the sperm, the oocytes were fixed overnight in CARNOY's fixative containing ethanol and acetic acid (3 : 1) and then stained with 4', 6-diamino-2-phenylindole (DAPI, 1 μg/ml) for 15 min. Incorporated sperm pronuclei in the cytoplasm were counted under an epifluorescence microscope (OPTIPHOT with EFD2, Nikon) to determine whether polyspermic or monospermic fertilization had occurred. In some experiments, sperm were added to an egg suspension in excess Ca^{2+} seawater (CaSW ; One part 1/3 M $CaCl_2$ mixed with 9 parts seawater). In CaSW, sperm underwent the acrosome reaction efficiently (TAMAKI and OSANAI, 1985).

Electron microscopy : For transmission electron microscopy, oocytes were fixed by the triple fixation method described by FOCARELLI *et al.* (1991) : the fixatives employed and the times of fixation were 10 min in 1% glutaraldehyde and 0.05% osmium tetroxide in 0.2 M sodium cacodylate containing 0.1 M sodium chloride and 0.35 M saccharose, 45 min in 4% glutaraldehyde in the same cacodylate buffer, and 1 h in 1% osmium tetroxide in 0.2 M cacodylate buffer containing 0.3 M sodium chloride. After brief dehydration in an ethanol series, they were embedded in SPURR's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Hitachi H-500).

For scanning electron microscopy, oocytes were prefixed in 5% formaldehyde in seawater for 10 min and fixed in 4% glutaraldehyde in seawater overnight. After postfixation with 1% osmium tetroxide in seawater for 1 h and dehydration, they were critical-point dried, coated with gold and examined with a Hitachi S-2250N scanning electron microscope.

Fig. 2. Transmission electron microscopy of sperm entry at a metaphase I oocyte of *Mytilus edulis* ($\times$12,000).
(a) 30 s after addition of sperm. The sperm has undergone the acrosome reaction and the tip of the acrosomal process (AP) has fused with the egg plasma membrane. EE : egg envelope. (b) 30 s after addition of sperm. Beneath the fertilizing sperm, a protrusion from the egg cytoplasm has developed around the acrosomal process. (c) 1 min after addition of sperm. The penetration cone (PC) that has developed in the egg envelope during sperm entry remains on the outside of the egg envelope. (d) 1.5 min after addition of sperm. The sperm head has penetrated the egg envelope through the penetration cone.

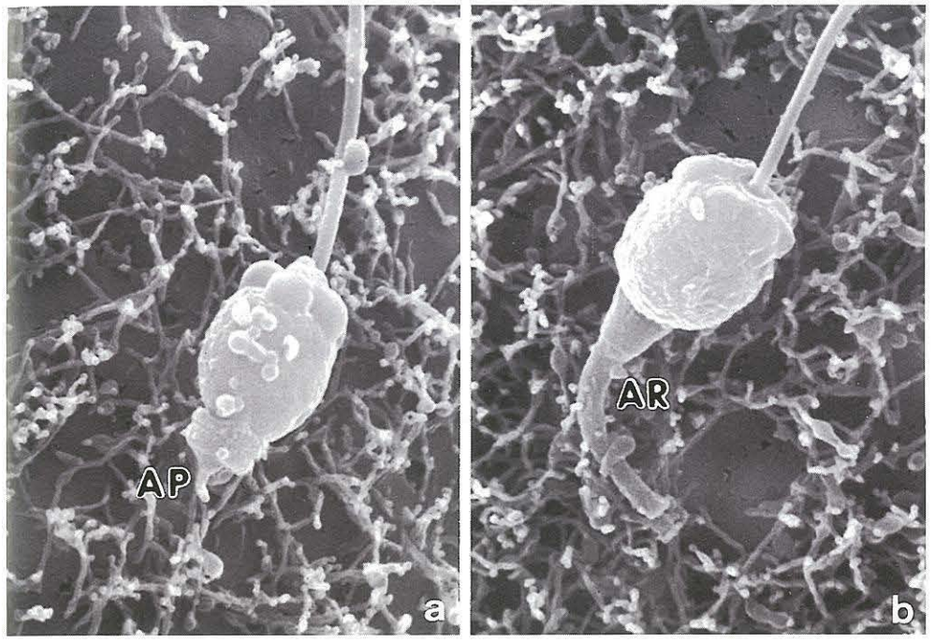


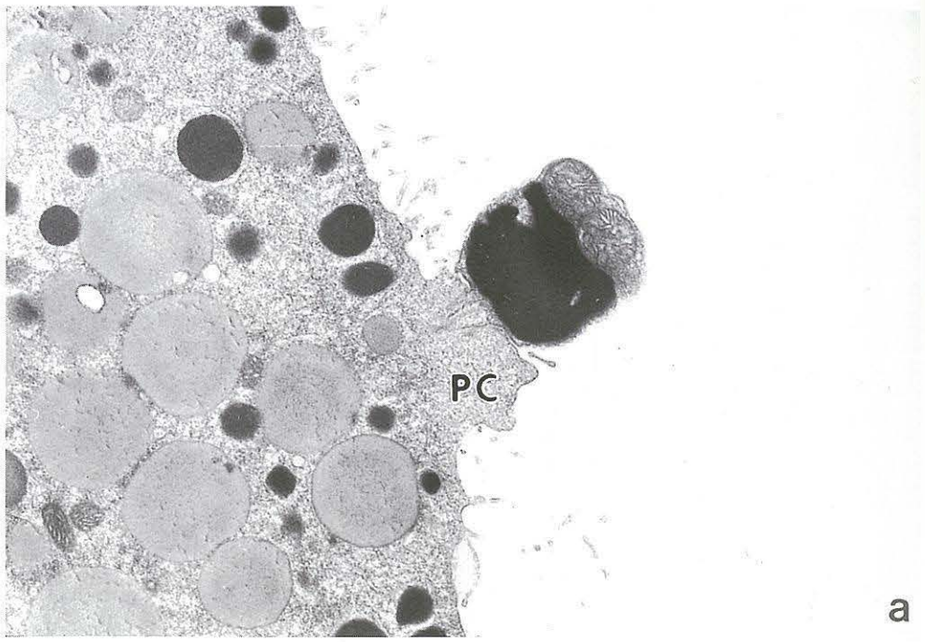
Fig. 3. Scanning electron microscopy of sperm entry at a metaphase I naked oocyte of *Mytilus edulis* ($\times$20,000).
(a) 30 s after addition of sperm. Most of the sperm has undergone the acrosome reaction and the acrosomal process (AP) protrudes into the egg surface. Microvillar patterns are disturbed. (b) 30 s after addition of sperm. A sperm remains unchanged on the naked egg surface with its intact acrosome (AR).

RESULTS

1. *Sperm entry in Mytilus edulis*

Sperm entry into intact and naked metaphase I oocytes was examined with the aid of transmission and scanning electron microscopy.

The diameter of the unfertilized oocyte is 60 μm, and the egg envelope, 0.7 μm thick, tightly surrounds the egg plasma membrane. On the surface of unfertilized oocytes, microvilli were seen to pass through the egg envelope and form regularly-spaced arrays of protrusions (Fig. 1-a, b, c, d). Sperm underwent the acrosome reaction on the egg envelope and protruded the acrosomal process (Fig. 1-a, b), around which a small hole was observed in the egg envelope (Fig. 1-b). Transmission electron microscopy showed that the tip of the acrosomal process penetrated into the egg envelope and fused with the egg plasma membrane (Fig. 2-a). A protrusion of the egg cytoplasm then developed along the acrosomal process to form the penetration cone (Fig. 2-b, c). Sperm penetrated the egg envelope through this



penetration cone (Fig. 2-d). No morphological change, such as elongation of microvilli and/or irregular microvillar patterns on the egg envelope or elevation of the egg envelope, was observed (Fig. 1-c, d, Fig. 2-d). Sperm entry into the cytoplasm through the egg envelope was completed within 2 min after the addition of sperm.

Neither the egg envelope nor the frangments of it were observed on the surface of naked oocytes after actinase treatment. Microvilli elongated and the microvillar patterns were disordered (Fig. 3-a). Most of the sperm observed on the naked egg surface underwent the acrosome reaction (Fig. 3-a), but some remained unchanged (Fig. 3-b). Acrosome-reacted sperm entered the cytoplasm. Transmission electron microscopy showed the acrosomal process to be at the front of the engulfed sperm head (Fig. 4-a). A thick cytoplasmic protrusion then developed around the acrosomal process and engulfed the sperm head (Fig. 4-b). Penetration of the sperm head into the cytoplasm was completed within 2 min after addition of sperm.

2. Addition of different concentrations of sperm to intact and naked oocytes

The role of the egg envelope in sperm entry was examined by adding different concentrations of sperm to intact and naked oocytes (Fig. 5). At a final sperm concentration of 1.25×10^{8} spermatozoa/ml, about 95% of the oocytes were successfully fertilized, whether intact or naked. Polyspermic fertilization occurred in more than 80% of naked oocytes and fewer than 15% of intact oocytes. The number of polyspermic oocytes decreased as the sperm concentration was reduced. About 30 sperm entered naked oocytes when the added sperm concentration was 5×10^{8} spermatozoa/ml, and 3 to 5 sperm pronuclei were observed when only 1.25×10^{7} spermatozoa/ml were added. Further dilution of the sperm concentration, the fertilization ratio in both intact and denuded oocytes decreased. When the final concentration added was 1.25×10^{6} spermatozoa/ml, the fertilization ratio of intact oocytes was about 75% and that of naked oocytes was about 20%.

These data show that removal of the egg envelope allowed polyspermic fertilization to occur in naked oocytes, though it reduced the egg fertility.

3. Fertilization of intact and naked oocytes at metaphase II

The egg plasma membrane of *Mytilus edulis* seemed not to block polyspermy completely. To confirm this, reinsemination of naked oocytes at metaphase II was

Fig. 4. Transmission electron microscopy of sperm entry at a metaphase I naked oocyte of *Mytilus edulis* ($\times$12,000).
(a) 1 min after addition of sperm. The sperm has undergone the acrosome reaction and a protrusion from the egg cytoplasm has developed along the acrosomal process. The protrusion has formed the penetration cone (PC). (b) 1.5 min after addition of sperm. The sperm head has been engulfed by the naked egg surface. The sperm acrosomal process (AP) is still evident at the tip of the sperm head.

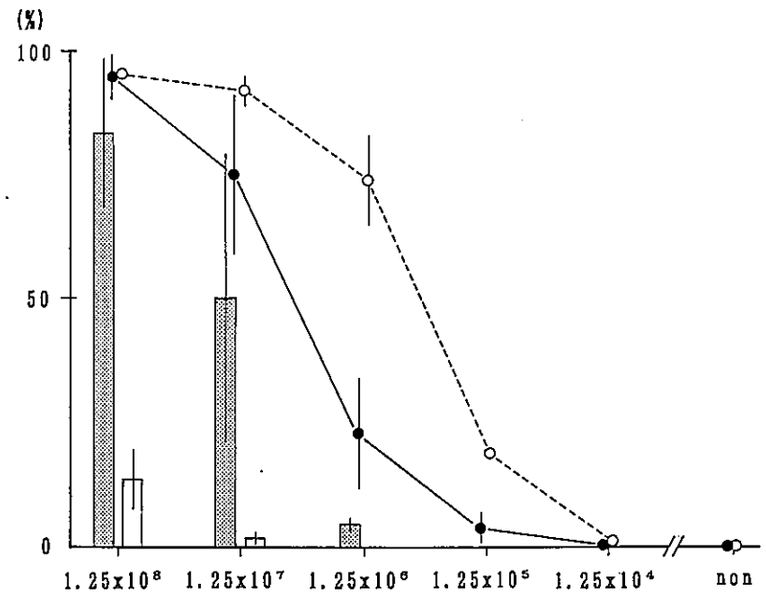


Fig. 5. Relationship between sperm concentration and activated or polyspermic oocytes in intact and naked metaphase I oocytes of *Mytilus edulis*.
Intact or naked metaphase I oocytes were treated with sperm at various concentrations. Ten minutes after addition of sperm, they were fixed in Carnoy's fixative, and stained with DAPI. The number of sperm pronuclei in each oocyte was determined as a criterion of monospermic or polyspermic fertilization. The vertical axis represents the percentage of fertilized oocytes among total and polyspermic oocytes. The horizontal axis represents the final concentration of sperm (spermatozoa/ml) in egg suspensions. "NON" shows no sperm addition as a control. Clear circles: percentage fertilization in intact oocytes; Solid circles: percentage fertilization in naked oocytes; Clear bar; percentage polyspermic fertilization in intact oocytes; Dotted bar; percentage polyspermic fertilization in naked oocytes. Each bar shows the standard error (n=5).

examined (Fig. 6).

Intact oocytes after addition of 10^7 spermatozoa/ml of egg suspension at metaphase I reinitiated meiotic division and reached metaphase II within 15 min. These oocytes were reinseminated with 10^8 spermatozoa/ml (final concentration). The number of polyspermic oocytes did not increase. Addition of sperm in CaSW did not improve the polyspermic fertilization ratio. The ratio of polyspermic fertilization was similar to that of the non-reinseminated control oocytes. Intact fertilized oocytes did not incorporate extra sperm at metaphase II.

When oocytes were treated with actinase for 10 min after addition of only 10^7 spermatozoa/ml (final concentration) at metaphase I, oocytes proceeded to metaphase II while the egg envelope was dissolving. When these naked metaphase II

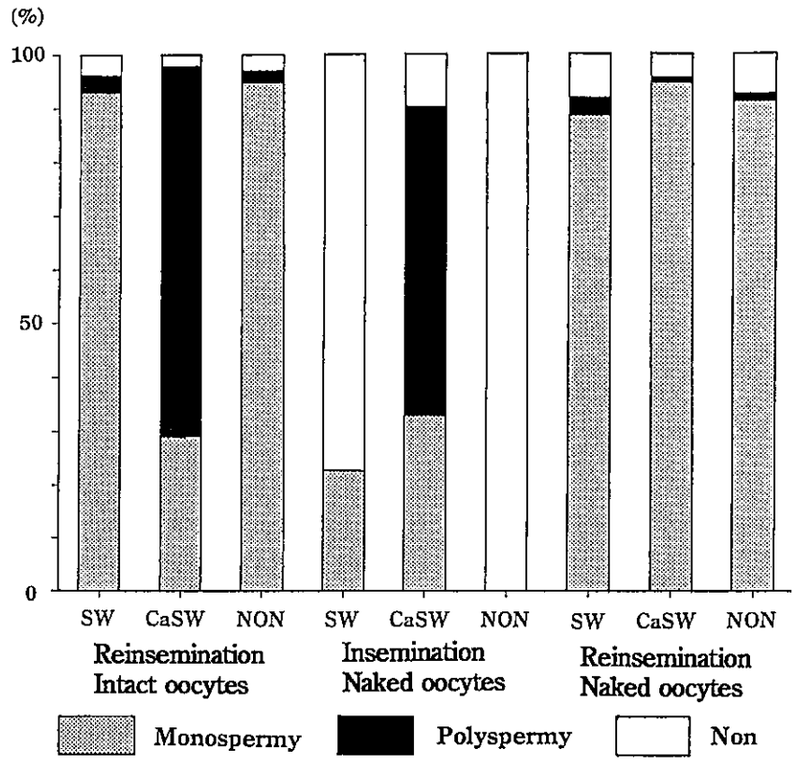


Fig. 6. Reinsemination of naked metaphase II oocytes of *Mytilus edulis* in seawater or excess Ca^{2+} seawater.
First three columns: After light insemination (sperm concentration, 10^6/ml) at metaphase I, oocytes were treated with actinase to dissolve the egg envelope, washed with seawater and reinseminated with 10 fold concentrated sperm in seawater (SW) or excess Ca^{2+} seawater (CaSW). Sperm were not added at metaphase II as a control (NON). Next three columns: Oocytes were treated with actinase without chelators and the naked metaphase II oocytes were obtained. Sperm (10^7/ml) were added to egg suspension in seawater (SW) or excess Ca^{2+} seawater (CaSW). Sperm were not added to control oocytes (NON). Last three columns: reinsemination of intact oocytes was examined at the same sperm concentrations as those in the first three columns. After light insemination at metaphase I, oocytes were reinseminated in seawater (SW) or excess Ca^{2+} seawater (CaSW). No second addition of sperm was performed for control oocytes (NON). These oocytes were fixed at 10 min after addition of sperm and the number of sperm pronuclei in the cytoplasm was observed by epifluorescence microscopy as described in Materials and Methods (Average of three experiments in each column).

oocytes were mixed with a high concentration of sperm (10^8 spermatozoa/ml, final concentration), the rate of polyspermic fertilization did not increase. When oocytes were reinseminated in CaSW, the ratio of polyspermic fertilization was increased

remarkably. Most of the control oocytes, without re-addition of sperm at metaphase II, were monospermic.

Actinase treatment of metaphase I oocytes in chelator-free medium induced parthenogenetic meiotic division and naked metaphase II oocytes were obtained. The fertility of these oocytes was low when mixed with 10^7 spermatozoa/ml (final concentration); however, addition of sperm at the same concentration in CaSW increased both the polyspermic and monospermic fertilization ratios.

These data show that the acrosome reaction-inducing activity of the egg plasma membrane at metaphase II is low; however, the egg plasma membrane still has an ability to receive acrosome-reacted sperm even after fertilization.

Discussion

Pronase P induces the resumption of meiosis in *Crassostrea gigas* (Osanai and Kyozuka, 1988). Increase of intracellular Ca^{2+} concentration is important for the reinitiation of meiosis (Guerrier *et al*, 1982; Moreau *et al*, 1984). In *Mytilus edulis*, increase of intracellular Ca^{2+} is observed when resumption of meiosis is induced by fertilization (Deguchi and Osanai, 1994). Actinase in CaMgFSW containing 4 mM EGTA did not induce meiosis resumption in *Mytilus* oocytes. High concentrations of chelator may inhibit increase of intracellular Ca^{2+} during actinase treatment.

Removal of the egg envelope from oocytes at metaphase I induced polyspermic fertilization. The ratio of polyspermic fertilization and the number of sperm entering the oocytes were correlated with the sperm concentration added to the egg suspension. These results suggest two possibilities. One is that there is no functional polyspermy blocking mechanism on the naked egg surface (egg plasma membrane) of *Mytilus*, as suggested previously by Dufresne-Dubé *et al*. (1983). In the case of sea urchins, after the removal of the vitalline coat by protease treatment, monospermic fertilization is still maintained, because of the electric polyspermy blocking mechanism of the egg plasma membrane (Jaffe, 1976) and the formation of the hyaline layer on it (Endo, 1964; Runnström, 1966). The existence of an electric polyspermy blocking mechanism of the plasma membrane of *Mytilus edulis* is unknown; however, it has been shown to operate temporarily for several minutes, in sea urchin eggs (Jaffe, 1976). A permanent polyspermy blocking mechanism may not exist on the egg plasma membrane, as reported for *Dentalium vulgare* (Moreau *et al*., 1989). Another possibility is that treatment with a proteolytic enzyme and Ca^{2+}-chelator damaged the egg plasma membrane, causing the removal of some components required for the establishment of a polyspermy block. These naked oocytes retained the normal ability for resumption of meiosis induced by sperm. Therefore, the egg plasma membrane and/or its Ca^{2+} channels were not damaged extensively by the actinase treatment.

Egg membrane lysin from spermatozoa dissolves egg envelopes species-specifically. The fertilizing sperm make a hole for their passage through the egg envelope by means of their lysin (cf. Dan, 1967; Hoshi, 1985). Egg membrane lysin has been isolated and purified from abalone (Haino-Fukushima and Usui, 1986). The lysin caused swelling and loosening of the fibrous structures in the egg envelope around the fertilizing sperm (Usui and Haino-Fukushima, 1991). Egg membrane lysin has also been isolated from *Mytilus* spermatozoa (Hauschka, 1963). From our observation of spèrm entry in intact oocytes, the egg envelope around the fertilizing sperm was not typically dissolved. Lysin did not seem to work efficiently to aid penetration of the sperm head through the egg envelope. The development of a cytoplasmic protrusion is important for sperm entry, as in some other bivalves (Hylander and Summers, 1977; Kyozuka and Osanai, 1985). In *Mytilus*, it may not be necessary for egg membrane lysin to make a hole to enable the sperm head to penetrate the egg envelope. Scanning electron microscopy showed that the surface of the egg envelope was changed at the binding site of the acrosomal process. Thus, lysin may aid the penetration of the acrosomal process through the egg envelope.

Naked oocytes at metaphase II rarely incorporated excess sperm at both fertilization and re-fertilization. However, numerous sperm could penetrate into the oocyte in seawater containing excess Ca^{2+}. Excess Ca^{2+} in seawater induces the acrosome reaction of sperm (Tamaki and Osanai, 1985). Therefore, the naked egg surface (egg plasma membrane) of the metaphase II oocytes may have a low ability to induce the acrosome reaction but still be able to incorporate acrosome-reacted sperm. If sperm passed through the egg envelope as a result of their lysin activity and reached the plasma membrane itself, polyspermic fertilization might occur in a high percentage of oocytes. Some blocking of sperm penetration of the egg envelope may exist as a mechanism to prevent polyspermic fertilization.

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