RESPONSE OF OOCYTES TO MEIOSIS-INDUCING AGENTS IN PELECYPODS

BULL. MAR. BIOL. STN. ASAMUSHI, TÔHOKU UNIV., 18(2), 45-56, 1988

RESPONSE OF OOCYTES TO MEIOSIS-INDUCING AGENTS IN PELECYPODS[1)]

KENZI OSANAI and RITSU KURAISHI[2)]

Marine Biological Station, Tôhoku University, Asamushi, Aomori 039-34, Japan

The response of germinal vesicle stage oocytes (GV oocytes) to agents inducing germinal vesicle breakdown (GVBD) was examined in three species of pelecypods. GV oocytes of *Crassostrea gigas* responded to spermatozoa, sea water (pH 8.3), serotonin and calcium ionophore, and underwent GVBD. GV oocytes of *Tapes philippinarum* were respondent not to spermatozoa and sea water, but to serotonin and calcium ionophore. GV oocytes of *Mytilus edulis* were insensitive to all agents mentioned above.

Pelecypod oocytes are said to recieve spermatozoa and resume meiosis (1) at the germinal vesicle stage or (2) at the first metaphase of meiosis (LONGO, 1983). In the first case the oocytes undergo germinal vesicle breakdown (GVBD) after fertilization and proceed to polar body formation and embryogenesis. *Spisula solidissima* oocytes belong to this type (ALLEN, 1953). *Mytilus edulis* is the second case. *Mytilus* oocytes are spawned at the first metaphase and then inseminated (IWATA, 1949).

The oyster, *Crassostrea gigas*, is an intermediate type. Oyster oocytes are able to receive spermatozoa both at the germinal vesicle stage and the first metaphase. In nature, oyster oocytes are spawned and fertilized at the first metaphase (WADA and ARAKAWA, 1983). The oocytes obtained by dissecting the ovary, are at the germinal vesicle stage. The germinal vesicle stage oocytes (GV oocytes) are also fertilized by insemination, undergo meiosis without first metaphase arrest and proceed to embryogenesis. (OSANAI, 1985).

The response of pelecypod oocytes to parthenogenetic agents often differs among species examined. The difference may have some relation with oocyte fertility. This consideration led us to examine the response of oocytes to meiosis-resuming agents in three pelecypod species.

1) Contribution from the Marine Biological Station, Tôhoku University No. 538.

2) 長内健治・倉石　立

Materials and Methods

Species

Crassostrea gigas (oyster), *Tapes philippinarum* and *Mytilus edulis* (marine mussel) were used as materials. *C. gigas* and *M. edulis* were collected near the Marine Biological Station, Asamushi, Aomori. *T. philippinarum* was gotten from commercial markets.

Oocytes

Crassostrea GV oocytes were obtained by dissecting ovaries in acid sea water, pH 6. Acid sea water was used to avoid spontaneous GVBD (Cf. Osanai, 1985). The oocytes were washed three or four times with acid sea water. GV oocytes of *T. philippinarum* were obtained by dissecting ovaries in filtered natural sea water (pH 8.3), in which they did not undergo spontaneous GVBD. In *M. edulis*, the most of ovarian oocytes discharged by the dissecting method cytolized in sea water. Oocyte suspension was centrifuged with a hand centrifuge. The supernatant was aspirated off and the sediment was resuspended in fresh sea water. After repeatedly washed, small amount of GV oocytes remained sedimented. The procedures for obtaining mature *Mytilus* oocytes (first metaphase oocytes) were as follows (Iwata, 1951). Pieces of dissected ovaries were exposed to 0.5 M potassium chloride for a few minutes and then placed in sea water. After 1 hr, they were transferred to a Petri dish containing fresh sea water. Oocytes discharged from the ovarian pieces were collected and washed by the hand centrifuge method.

Sperm

Testes were dissected in small amount of sea water. Sperm suspension was centrifuged with a hand centrifuge to sediment testis fragments and aggregated spermatocytes. The supernatant was centrifuged at 3,000 rpm for 5 min. The sedimented sperm pellet was stocked in small amount of sea water and diluted prior to use.

Chemicals and Solutions

Serotonin (5-hydroxytriptamine) creatinine sulfate (Sigma) and calcium ionophore A23187 (Sigma) were used for artificial induction of meiosis resumption. Serotonin (10 mM) was dissolved in distilled water or artificial sea water and stocked in a refrigerator. Calcium ionophore (10 mM) was dissolved in dimethyl sulfoxide (DMSO, Wako) or the mixture of DMSO and ethanol (1 : 3) as a stock solution. The stock solutions were diluted with experimental media prior to use.

Natural sea water was used after the filtration through a filter paper, Whatman No. 2. Artificial sea water was prepared according to the modified Herbst's procedure (Osanai, 1975). Bivalent cation-free sea water was prepared by replacing $CaCl_2$ by NaCl and $MgSO_4$ by Na_2SO_4. Acid sea water (pH 6) was prepared by adding about 0.6 ml of 1 N HCl to 500 ml of filtered sea water. Acid artificial sea water and acid Ca, Mg-free sea water were adjusted to pH 6 with 2-(N-morpholino) ethane-sulfonic acid monohydrate-NaOH buffer (1 mM).

Cytology

Meiotic asters and spindles were observed by immunofluorescence microscopy according to the method described in the previous paper (Osanai and Kyozuka, 1988). Prior to serotonin treatment, the chorion was removed by exposing GV oocytes to 0.02% trypsin (Sigma) in acid Ca, Mg-free sea water containing 1 mM ethyleneglycol-bis-(β-aminoethylether) N, N′-tetra-acetic acid (EGTA, Sigma) for 10 min. The dechorionated GV oocytes were incubated in sea water containing 1 μM serotonin for 0–25 min. After the rinsing with Ca, Mg-free sea water containing EGTA they were incubated in an extraction medium containing glycerin and Triton X-100 and then fixed in cold methanol. Fixed oocytes were adhered to a polylysin-coated glass coverslip and stained with fluorescein isothiocyanate-labelled anti-mouse IgG (goat) (Tago) and 4′, 6 diamino-2-phenylindoldihydrochloride (DAPI). The specimens were mounted on a glass slide with glycerin and examined with a fluorescence microscope (Nikon Optiphoto EF).

Results

C. gigas

Crassostrea oocytes discharged by dissecting the ovary are at the germinal vesicle stage. The GV oocytes are normally fertilized by insemination. GVBD proceeds often only by suspending GV oocytes in sea water. The spontaneous GVBD is dependent on external pH and does not occur in acid sea water (pH less than 6.5). Serotonin induces GVBD even in acid sea water. In sea water containing serotonin (more than 1 μM) the oocytes undergo GVBD and changes until the first metaphase of meiosis, at which they are arrested. The first metaphase oocytes are able to be fertilized and develop by insemination (Osanai, 1985).

Oocytes Maturation in Sea Water Containing Serotonin

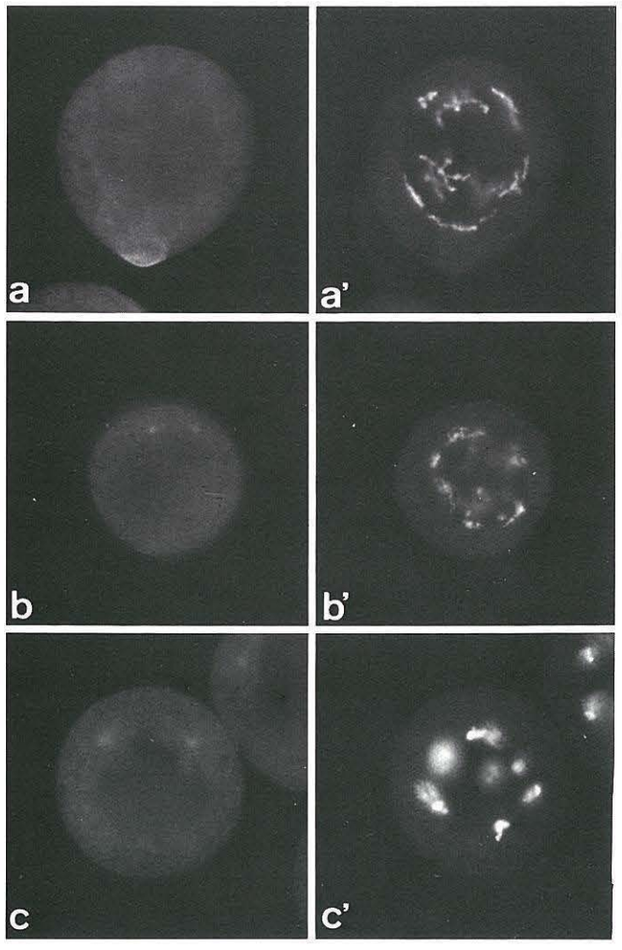
Our previous work (Osanai, 1985) shows that GVBD in sea water is pH dependent, but serotonin induces GVBD even in acid sea water. GVBD occurs also in Ca, Mg-free sea water (pH 8.3). We examined whether GVBD with serotonin required external calcium ions and GVBD in Ca, Mg-free sea water was pH dependent.

Oyster GV oocytes were incubated in acid artificial sea water (pH 6) or acid Ca, Mg-free sea water (pH 6) with or without 10 μM serotonin. GVBD occurred in acid Ca, Mg-free sea water containing serotonin, but did not in acid Ca, Mg-free sea water

Table 1.
GVBD of *C. gigas* oocytes in acid sea water

Incubation medium	GVBD (%)
Artificial sea water (ASW), pH 6	0
10 μM serotonin in ASW, pH 6	50.0
Ca, Mg-free sea water, pH 6	0
10 μM serotonin in Ca, Mg-free SW, pH 6	54.7

GV oocytes were incubated in treatment media for 2 hrs and 38–50 min at 24°C.



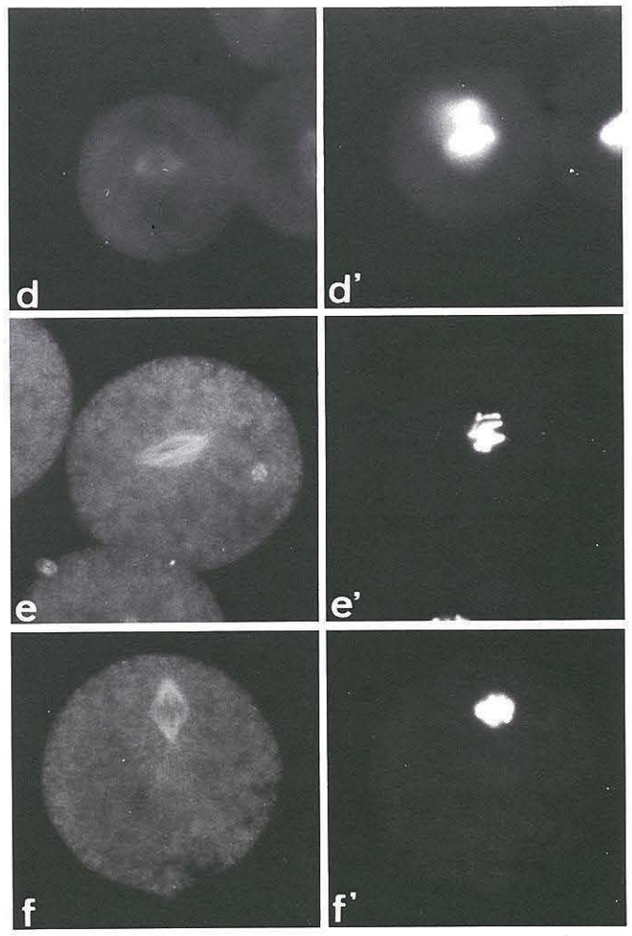


Fig. 1. Successive changes from the germinal vesicle stage to the first metaphase in *Crassostrea gigas* oocytes. a, a′: Germinal vesicle stage. Dechorionated GV oocytes were incubated in sea water containing 1 μM serotonin for 5 min (b, b′), 10 min (c, c′), 15 min (d, d′), 20 min (e, e′) and 25 min (f, f′). a-f: Stained with anti-tubulin antibody. a′-f′: Stained with DAPI. ×550.

alone (Table 1). This result suggests that serotonin-induced and spontaneous GVBDs are independent of external calcium ions.

The successive changes in egg maturation from the germinal vesicle stage to the first metaphase in sea water containing serotonin were traced by fluorescence microscopy. Dechorionated oocytes were incubated in sea water containing 1 μM serotonin for 5–25 min and fixed after extraction.

In the GV oocytes, we could not find tubulin accumulating spots (the premeiotic

asters) (Fig. 1a, a′). After the incubation for 5 min a pair of small asters appeared on one side of the germinal vesicle (Fig. 1b, b′). At 10 min after incubation the astral centers began to move toward the opposite poles along the outer surface of the germinal vesicle, in which chromosomes were condensing (Fig. 1c, c′). At 15 min the germinal vesicle disappeared. Condensed chromosomes distributed in the central area of the oocyte (Fig. 1d, d′). At 20 min the spindle with metaphase chromosomes developed in the center of the oocyte (Fig. 1e, e′). At 25 min after incubation, the meiotic spindle migrated to the one side of the oocyte surface (the animal pole). The spindle axis became perpendicular to the oocyte surface and parallel with the animal-vegetal pole axis. Chromosomes arrange on the metaphase plate (Fig. 1f, f′).

Induction of GVBD with Calcium Ionophore

Schuetz (1975) reported that calcium ionophore A23187 induces GVBD and the reinitiation of the meiotic process in *Spisula solidissima* oocytes. We examined whether A23187 induced GVBD also in *Crassostrea* oocytes.

The oocytes underwent GVBD in sea water (pH 8.3) containing 10 μM calcium ionophore and some of them formed polar bodies. GVBD was inhibited in acid sea water containing calcium ionophore (Table 2). This result seems to show that calcium ionophore does not induce GVBD, but stimulates meiosis-resumption from the first metaphase after GVBD.

T. philippinarum

Fertility of Tapes Oocytes

GV oocytes obtained directly from the ovary were inseminated in sea water (pH 8.3). They did not show any sighn of fertilization. When 0.2 ml of sea water containing 1 mM serotonin was injected into the base of the foot, the shellfishes discharged first metaphase oocytes (Fig. 2b). These oocytes were fertilized by insemination and began to develop.

Table 2.
GVBD of *C. gigas* oocytes in sea watar containing calcium ionophore A23187

Incubation medium	GVBD (%)
10 μM A23187 in pH 8.3 sea water	86.6
10 μM A23187 in pH 6.0 sea water	5.4

GVBD was counted after incubation for 60 min at 22°C.

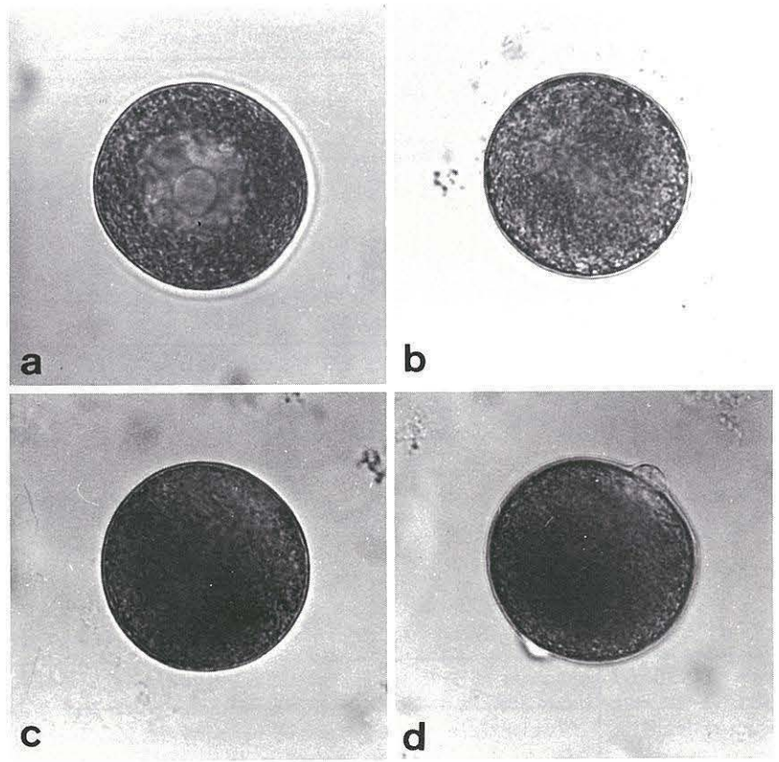


Fig. 2. Oocytes of *Tapes philippinarum*.
a: GV oocyte obtained from the dissected ovary. b: Oocyte spawned by the injection of serotonin. c: First metaphase oocyte, which was incubated sea water containing 0.1 mM serotonin for 100 min. d: Polar body formation in the oocyte incubated in 50 μM calcium ionophore in sea water for 110 min. ×500.

In Vitro Induction of GVBD

GV oocytes were incubated in sea water (pH 8.3) containing various concentrations of serotonin. The oocytes underwent GVBD in more than 1 μM serotonin. Spontaneous GVBD in sea water was hardly observed (Table 3).

Figure 3 shows the percentage of GVBD as a function of time after the serotonin incubation. In *Tapes* oocytes GVBD began about 30 min after the initiation of serotonin treatment and reached to a maximum after 100 min. After GVBD the oocytes remained in the first metaphase (Fig. 2c).

Induction of Meiosis with Calcium Ionophore

Tapes GV oocytes were incubated in sea water containing calcium ionophore

Table 3.
GVBD with serotonin in *T. philippinarum* oocytes

Concentration of serotonin (μM)	GVBD (%)		
	(A)	(B)	(C)
0	0	0	9.9
100	39.5	45.8	98.0
10	39.1	49.4	91.7
1	38.2	30.4	87.9
0.1	15.3	28.0	54.7
0.01	5.5	—	—

Counted after incubation for 128-159 min (A, B) or 65-87 min (B) at 21-23°C.

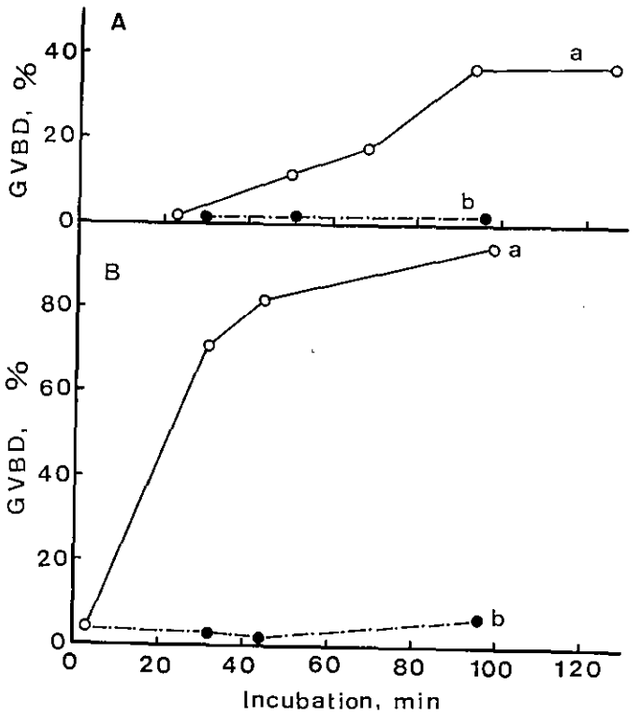


Fig. 3. GVBD with serotonin in *T. philippinarum* oocytes. GV oocytes were incubated in sea water containing 10 μM serotonin (a) or sea water (b) at 18°C (A) or 21°C (B).

Table 4.
GVBD of *T. philippinarum* oocytes in sea water containing calcium ionophore A23187

Concentration (μM)	0	10	1	10^{-1}	10^{-2}
GVBD (%)	12.2	95.8	78.0	40.0	19.4

Counted after incubation for 60 min at 19°C.

Table 5.
Effect of external calcium on GVBD with calcium ionophore A23187 in *T. philippinarum* oocytes

Batch No.	(A)	(B)	(C)	(D)
A23187	1 μM	1 μM	10 μM	5 μM
in ASW	96.8%	86.5%	67.5%	86.5%
in Ca-free SW	44.4	50.8	7.5	18.4
in Ca, Mg-free SW	—	—	—	19.5
Control				
ASW	14.9%	3.1%	0%	0%
Ca-free SW	6.1	3.5	0	0
Ca, Mg-free SW	—	—	—	0

GV oocytes were rinsed with Ca, Mg-free sea water (SW) containing 1 mM EGTA and Ca-free SW and then incubated in treatment media. GVBD was counted 100-110 min (A), 120 min (B), 90 min (C) or 65-80 min (D) after incubation at 15-17°C, Numbers show the percentage of GVBD. ASW : Artificial sea water.

A23187 (0.01-10 μM). GVBD was induced by more than 0.1 μM A23187 (Table 4). Calcium ionophore-inducing GVBD seemed to require external calcium ions. The GVBD was inhibited in Ca-free sea water and Ca, Mg-free sea water (Table 5). In 5 μM calcium ionophore GVBD began after 40 min incubation (Fig. 4). Some oocytes formed polar bodies by 100 min after the incubation (Fig. 2d). In *Tapes* oocytes calcium ionophore not only induces GVBD, but also stimulates polar body formation.

M. edulis

Response of GV Oocytes to Meiosis-Resuming Agents

Mytilus GV oocytes did not begin GVBD by insemination. Spontaneous GVBD did not occur in natural sea water. When GV oocytes were exposed to serotonin in sea water, they underwent hardly GVBD. Calcium ionophore did not also induce GVBD (Table 6). These results show that *Mytilus* GV oocytes are insensitive to spermatozoa and meiosis-resuming chemical agents.

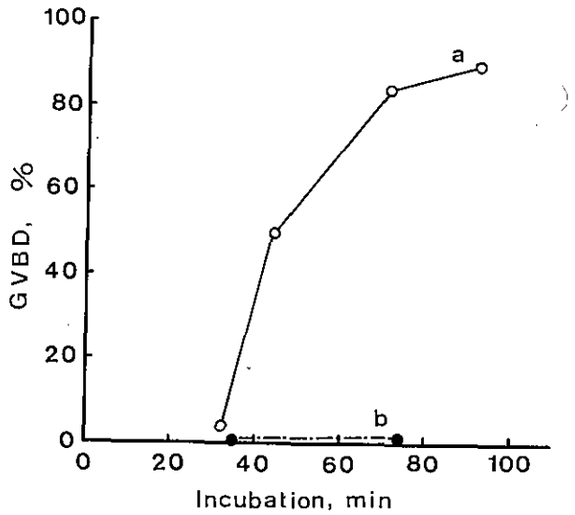


Fig. 4. GVBD with calcium ionophore A23187 in *T. philippinarum* oocytes. GV oocytes were incubated in sea water containing 5 μM A23187 (a) or sea water (b) at 17°C.

Table 6.
GVBD of *M. edulis* oocytes in sea water containing serotonin or calcium ionphore A23187

Treatment media	GVBD (%)
sea water	1.2
100 μM serotonin	5.2
10 μM serotonin	4.6
10 μM A23187	3.9
1 μM A23187	1.5

Incubated for 2.5 hrs at 19°C.

Response of First Metaphase Oocytes

First metaphase oocytes obtained with the KCl method initiated development by insemination. When first metaphase oocytes were incubated in sea water containing more than 1 μM calcium ionophore, they resumed meiosis and formed polar bodies (Table 7).

Discussion

The responses of GV oocytes of three pelecypod species to meiosis-resuming agents are summarized in Table 8. *Crassostrea* GV oocytes are respondent to spermatozoa, sea water and serotonin. *Tapes* GV oocytes respond not to spermatozoa and sea water, but to serotonin and calcium ionophore. *Mytilus* GV oocytes do not respond to all of agents described above. This result shows that there are three different types concerning respondence to meiosis-resuming agents. The fourth type may be the case of *Spisula* oocytes. The isolated immature oocytes (GV oocytes) undergo GVBD followed by polar body formation in serotonin in sea water. Serotonin-induced mature oocytes are not fertilizable (Hirai *et al.*, 1984). The difference of serotonin effect between *Spisula* and *Crassostrea* or *Tapes* may be related to the absence or the presence of first metaphase arrest.

Calcium ionophore induces not only GVBD, but also polar body formation. In *Crassostrea* oocytes, GVBD is dependent on external pH, but occurs in Ca-free sea water (pH 8.3) (Osanai, 1985). This suggests that GVBD requires not the influx of calcium ions, but the increase of intracelluar pH. Calcium ionophore-induced maturation in *Spisula* (Scheutz, 1975) and *Tapes* oocytes is inhibited in calcium deficient media. In *Spisula* and *Tapes* oocytes, calcium influx may be prerequisite for cytoplasmic alkalinization, which seems to be required for GVBD as in *Crassostrea* oocytes. The independency of external calcium in *Crassostrea* seems to be related with spontaneous GVBD in sea water. The level of calcium content may be higher in *Crassostrea* oocytes than in *Spisula* or *Tapes* oocytes.

Table 7.
Meiosis-resumption from the first metaphase in *M. edulis* oocytes with calcium ionophore A23187

Concentration of A23187 (μM)	Polar body formation (%)		
	(A)	(B)	(C)
0	0	0	0
10	50.7	60.7	74.1
1	58.6	—	—
0.1	12.2	—	—

* Polar bodies extruded on oocyte circumference were counted after the incubation for 2-2.5 hrs at 20-21°C. Actual percentages must be higher than the values shown in this table.

Table 8.
Response of GV oocytes to meiosis-resuming agents in pelecypods

Species	*C. gigas*	*T. philippinarum*	*M. edulis*
Fertility	+	−	−
GVBD in sea water	+	−	−
with serotonin	+	+	−
with A23187	(+)	+	−

References

ALLEN, R.D., 1953 Fertilization and artificial activation in the eggs of the surf-clam, *Spisula solidissima*. Biol. Bull., 105: 213-239.

HIRAI, S., T. KISHIMOTO, S.S. KOIDE and H. KANATANI, 1984 Serotonin induction of spawning and oocyte maturation in *Spisula*. Biol. Bull., 167: 518.

IWATA, K.S., 1949 Spawning of *Mytilus edulis*. (2) Discharge by electrical stimulation. Zool. Mag., 15: 443-446.

IWATA, K.S., 1951 Spawning of *Mytilus edulis*. (5) A method to obtain mature eggs from mantle peice. Zool. Mag., 17: 15-18.

LONGO, F.J., 1983 Meiotic maturation and fertilization. In *The Mollusca*, Vol. 3, edited by N.H. VERDONK *et al*. Academic Press, New York, 49-89.

OSANAI, K., 1975 Handling Japanese sea urchins and their embryos. In *The Sea Urchin Embryo*, edited by G. CZIHAK. Springer Verlag, Berlin, 26-40.

OSANAI, K., 1985 *In vitro* induction of germinal vesicle breakdown in oyster oocytes. Bull. Mar. Biol. Stn. Asamushi Tôhoku Univ., 18: 1-9.

OSANAI, K. and K. KYOZUKA, 1988 Pronase P induces the resumption of meiosis in oyster oocytes. Bull. Mar. Biol. Stn. Asamushi Tôhoku Univ., 18: 37-43.

SCHUETZ, A.W., 1975 Induction of nuclear breakdown and meiosis in *Spisula solidissima* oocytes by calcium ionophore. J. Exp. Zool., 191: 433-440.