Spawning, in vitro Maturation, and Changes in Oocyte Electrophysiology Induced by Serotonin in Tivela stultorum

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Abstract. Spawning was induced in both male and female Pismo clams by injecting 0.4 ml of 5 mM serotonin into the gonad. Spawned oocytes had already matured to metaphase I of meiosis or were undergoing germinal vesicle breakdown at the time of release. Prophase-arrested oocytes scraped from the ovaries of uninjected clams were induced to undergo germinal vesicle breakdown in vitro by incubating them with 0.2—20 μM serotonin in seawater; the former concentration was optimal. In vitro matured oocytes were fertilizable and developed to larvae, whereas sperm penetrated prophase-arrested oocytes without activating them. Fertilization was more successful in slightly alkaline seawater (pH 8.5). The electrical response of oocytes to serotonin was studied by means of intracellular microelectrode recording. Resting potentials of prophase-arrested oocytes were between -60 and -80 mV and there was no immediate electrical response to perfusion with serotonin. However, about 10—15 min later (before germinal vesicle breakdown), membrane potentials usually began to drift slowly in the positive direction (net change by 40 min + 9 ? 6.6 mV (SD; n = 5), whereas resting potentials of oocytes perfused with seawater alone usually drifted in the negative direction (−3 ± 6.1 mV; n = 7). A dramatic increase in input resistance was consistently observed in oocytes induced to mature with serotonin, probably due to the inactivation of K+ channels, although this was not tested. Action potentials were always (7 out of 7 cases) present in maturing oocytes, but were detected only sometimes (7 of 14 cases) in prophase-arrested oocytes, presumably due to their lower input resistances.

Introduction

Tivela stultorum, the Pismo clam, is an important seafood in California and in Baja California, Mexico. However, populations have been seriously reduced by overcollecting (e.g., Morris et al., 1980). We were interested in developing a method for obtaining fertile gametes from the gonads to use for repopulation efforts. There are few previous studies of Pismo clam reproduction. Coe (1947) observed that normal development was not obtained when ovarian eggs were fertilized, but that spawned oocytes fertilized in the laboratory would develop to normal larvae. He mentioned that a spawning clam could stimulate neighbors to spawn, but that attempts to induce spawning in the laboratory were only “sometimes” successful. Fitch (1950) also observed neighbor-stimulated spawning, and stated he could induce spawning by raising the water temperature a few degrees. However, neither author presented data.

Because serotonin induces spawning, oocyte maturation, or both in a variety of bivalves (e.g., Matsutani and Nomura, 1982; Gibbons and Castagna, 1984; Crawford et al., 1986; Alcazar et al., 1987; Osanai and Kuraishi, 1988; Hirai et al., 1988; Ram et al., 1993), we examined its effect on these processes in Tivela. Conditions for successful fertilization of matured ovarian oocytes were also explored. Furthermore, because serotonin is a common neurotransmitter, we studied its effects on electrical properties of the oocyte plasma membrane to better understand its mechanism of action during oocyte maturation.

Materials and Methods

Clams collected from San Quintín or San Ignacio, Baja California, Mexico, from July through November were

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maintained at 16°C in a seawater aquarium with a biological filter. For spawning tests, clams were placed individually in dishes containing 1.5 l of seawater for 1 h then injected intragonadally with 0.4 ml of serotonin (5-hydroxytryptamine creatinine sulfate complex, Sigma, St. Louis, MO), 5 mM in seawater with 5 mM Tris-HCl, pH 8. Controls were injected with 0.4 ml buffered seawater. Oocytes were also obtained directly from the ovaries by gently scraping their surfaces with a Pasteur pipet moistened with seawater. Oocyte suspensions in seawater were filtered through 120-μm nylon mesh (Small Parts, Inc., Miami Lakes, FL) to remove fragments of ovarian tissue, then poured into 60-μm mesh to trap the oocytes and wash them free of the milky contaminating body fluid. The oocytes were then washed several times in fresh seawater, and suspensions were maintained at 16°C. For some experiments vitelline coats were removed by vigorously pipeting oocyte suspensions through 60- or 120-μm mesh. Millipore-filtered (0.45 μm) natural seawater was used in all the experiments.

Induction of oocyte maturation in vitro was tested with various concentrations of serotonin in seawater (see Results). Germinal vesicle breakdown (GVBD) was assayed by ordinary light or phase-contrast microscopy in living or formaldehyde-fixed (5.5% in seawater) oocytes compressed under a coverslip, and progression to meiotic metaphase I was scored in fixed oocytes stained with acetoorcein (method in Stephano and Gould, 1987) or bisbenzimide (method in Stephano and Gould, 1995).

Sperm were obtained by scraping the surface of testes as described above, then filtered through 60-μm mesh to remove tissue fragments. They were washed several times in seawater by centrifugation at 5000 × g (4°C, 5 min), and concentrated suspensions were stored on ice. Only batches with good motility (checked by dark-field microscopy) were used. Fertilization was carried out at either pH 8 (unmodified seawater) or 8.5 (adjusted with 0.5 M NaOH). Samples were fixed in 5.5% formaldehyde in seawater and stained for chromosomes and pronuclei with bisbenzimide.

Electrophysiological measurements were performed as described by Jaffe et al. (1979). Oocytes were immobilized against ridges scratched in the bottoms of plastic petri dishes and were penetrated with a single 30–60 MΩ electrode filled with 3 M KCl at 100X with a Wild (Wild Heerbrugg Instruments, Farmingdale, NY) stereomicroscope equipped with a water-cooled (16°C) stage. The dishes containing oocytes were grounded through an agar bridge and electrode resistances were subtracted with the bridge balance in the amplifier (BioDynex AM-2, Santa Monica, CA). In some experiments, serotonin solutions were added to the bath with a pipet (final concentration 2 μM). In other experiments solution changes were carried out in a perfusion chamber (total volume 0.4 ml) constructed with paraffin and containing a plastic baffle (see Jaffe et al., 1979) to ensure that the entering solution flowed along the bottom of the dish where the oocytes were. Solutions (≥4 ml 2 μM serotonin or seawater alone) were added at one end of the chamber through a fine glass pipet connected to a syringe while excess liquid was removed through an aspirator pipet at the other end of the chamber.

**Results**

**Spawning induction**

After serotonin injection (see Methods), 10 of 21 clams (7 females and 3 males) spawned from 12 to 52 min later (average 32 min), whereas only 1 of 23 seawater-injected controls spawned (at approximately 100 min). Fourteen of the seawater-injected controls were then injected with serotonin 2–3 h later, and 5 of them (3 females and 2 males) spawned 38–85 min later. Spawning was preceded by characteristic behavior beginning about 15 min after injection: the shell valves opened periodically and then rapidly closed, expelling a jet of water from the exhalent siphon. This behavior was also observed in the control clam that spawned, but was not observed in any clams that did not spawn. Most of the spawned oocytes had already matured or were undergoing GVBD, and spermatozoa were motile within a few seconds of spawning.

**Maturation in vitro**

Oocytes removed from the ovaries without spawning induction were typically in meiotic prophase. At first they tended to be pear-shaped, then rounded up during incubation in seawater (Fig. 1). Fewer than 20% underwent GVBD during prolonged incubation in seawater (e.g., “C”, Fig. 2). The effects of incubating ovarian oocytes with varying concentrations of serotonin are illustrated in Figure 2. Concentrations inducing the highest percentage of GVBD were 0.2 to 20 μM, with the former concentration typically slightly better. At higher concentrations of serotonin, fewer oocytes matured (Fig. 2). Similar results were obtained in 15 additional experiments.

The procedure used for removing vitelline coats (VCs; see Methods) did not impair the ability of the oocytes to respond to serotonin. In four experiments with different egg batches, the percentage of GVBD was 82.5% ± 9.95% (SD; n = 4) in VC+ oocytes and 87.3% ± 13.23% in VC− oocytes exposed to 2 μM serotonin (P > 0.2, 2-tailed t test). The percentage of spontaneous GVBD following VC removal (15.25% ± 11.24%) was also not significantly different from that in intact oocytes (6.50% ± 4.51% (P > 0.02).

**REFERENCES**

Fertilization of oocytes matured in vitro

When prophase-arrested oocytes removed from the ovary without exposure to serotonin were inseminated, bisbenzimid staining revealed that sperm entered but the oocytes failed to respond: GVBD did not occur and development was not initiated. However, following maturation in vitro with serotonin, fertilization induced normal development. When fertilization was carried out at pH 8, rather high sperm concentrations were required; therefore we tried fertilizing oocytes at a higher pH, since in some species sperm undergo the acrosome reaction more readily when the pH is raised (e.g., Decker et al., 1976). Although we did not investigate the mechanism with Tivela, this strategy was successful. In two experiments with insemination at pH 8.5, development to normal blastulae occurred in 38% and 14% of mature oocytes inseminated with 100 sperm/oocyte, in 73% and 70% inseminated with 1000 sperm/oocyte and in 72% and 84% inseminated with 10,000 sperm/oocyte. The corresponding percentages for oocytes inseminated at pH 8 were 1.5 and 2.3, 47 and 35, and 65 and 52 (n > 130 for each sample). Development to “D” larvae was common although development beyond blastula was not quantitated. Sperm were obtained directly from the gonad and did not require serotonin treatment to fertilize eggs.

Electrophysiological response to serotonin

Resting potentials of prophase-arrested oocytes were typically between -60 and -80 mV, with no significant difference between intact (-68 ± 8 mV SD; n = 8) and denuded (-69 ± 10 mV; n = 10) oocytes. Upon application of serotonin, no immediate electrical response was observed. However, about 10 to 15 min later, resting potentials usually began to drift slowly in the positive direction. By about 40 min after serotonin application, the potential had drifted +17, +15, +9, and 0 mV in 4 oocytes with vitelline coats, and +15, +9, +7, and 0 mV in 4 denuded oocytes (mean +9 ± 6.6 mV). All of these oocytes had undergone GVBD. The positive drift was not observed in oocytes that were treated with seawater alone; in fact the resting potentials of these oocytes tended to drift to more negative values with time (-13, -6, -5, -2, -2, 0, and +7 in 7 oocytes with and without vitelline coats; mean -3 ± 6.1). This difference between the two treatments was significant (P < 0.01)

A dramatic change consistently observed in oocytes induced to mature with serotonin was an increase in input resistance. An example is shown in Figure 3. The I-V relation for this oocyte shortly after electrode penetration was almost a straight line with a slope resistance of 35 MΩ from -0.075 to -0.33 nA (r = 0.992; Fig. 3A, B). Ten minutes later, after perfusion with seawater alone, the slope resistance had decreased to about 3 MΩ (-0.4 to +0.4 nA, r = 0.958; Fig. 3C), and this situation persisted through 50 min, when the oocyte was perfused with serotonin. By 15 min after serotonin perfusion, the resistance had increased markedly (83 MΩ for the linear segment from -0.075 to -0.33 nA shown in Fig. 3A; r = 0.998), and an action potential appeared as an “on” response to a +0.035 nA current pulse (Fig. 3D), and as an “off” response following a -0.33 nA pulse (Fig. 3E). GVBD occurred at 19 min after serotonin perfusion.
MATURATION IN TIVELA

325

Figure 3. (A) Voltage responses to current pulses applied to an oocyte before (○) and after (●) serotonin treatment. The data shown by (○) were obtained 7-9 min after electrode penetration, serotonin was added by perfusion at 50 min, and the data shown by (●) were obtained 15 min later. GVBD occurred 19 min after serotonin addition. Slope resistances were 35 MΩ (-0.075 to -0.33 nA) before serotonin, and 83 MΩ (-0.075 to -0.33 nA) after serotonin. (B)-(E). Samples of voltage (upper) and current (lower) records at various times during the experiment: (B) 9 min after electrode penetration; (C) 25 min later, after seawater perfusion (see text); (D) and (E) 16 min after serotonin perfusion—(D) shows the action potential "on" response to +0.035 nA and (E) shows the "off" response to -0.33 nA.

The resistance increase occurred in every oocyte exposed to serotonin. For example, in 4 other oocytes with VC, the slope resistances rose from 7, 18, 135, and 179 MΩ before serotonin, to 43, 87, 286, and 284 MΩ afterwards. Similar results were obtained with denuded oocytes: input resistances rose in 6 of 6 after serotonin addition and declined in 4 of 4 after seawater addition. As mentioned above, resistances tended to decline after seawater perfusion.

The resistance increase began after a short delay. Resistances were still low in 3 of 3 oocytes tested from 1 to 1.5 min after serotonin perfusion was completed, then a substantial increase was noticeable at 2 min in one of these oocytes (the others were not tested at this time). GVBD occurred after the resistance increase in 4 of 4 oocytes for which the timing was observed carefully.

In some, but not all, starfish species examined, capacitance measurements indicate a substantial loss of membrane surface area during maturation (Moody and Bosma, 1985; Simoncini and Moody, 1990). We did not investigate capacitance changes in Tivela oocytes, so no conclusion is possible. However, an "anecdotal" calculation based on time constant and resistance measurements before and after serotonin treatment in the oocyte of Figure 3, revealed essentially no change: at 9 min after electrode penetration a current pulse of -0.4 nA produced a voltage change of -7 mV with a time constant of 0.032 s (C = 1.8 x 10^{-9} F), and at 18 min after serotonin perfusion -0.078 nA produced a -9 mV shift with a time constant of 0.2 s (C = 1.7 x 10^{-9} F).

As mentioned above, an action potential is present in Tivela oocytes. The threshold potential was -45 to -50 mV and peak potentials ranged from +20 to +35 mV; a typical example is shown in Figure 4A. Action potentials were always detected after serotonin treatment (7 of 7 oocytes), but sometimes (7 of 14 oocytes) were not observed in prophase-arrested oocytes, presumably be-
cause membrane resistances were so low that the applied current pulses failed to depolarize the membrane to the voltage threshold. For example in the oocyte of Figure 3, an action potential occurred as an “off” response to negative current injection at 3 min after penetration, then a spontaneous action potential occurred as the resting potential passed below −45 mV during recovery from electrode damage (not shown). Subsequently, action potentials were not detected with the current pulses used until after serotonin treatment (Fig. 3). When there was a robust action potential in an oocyte before serotonin treatment (Fig. 4B), it became even more prominent at about the time of GVBD (Fig. 4C). Later, more current was required to elicit the action potential and the duration decreased (not shown). Similar results were observed in the oocyte of Figure 3. By 20 min after GVBD the resting potential had drifted to −50 mV, the slope resistance had declined to 47 MΩ, and +0.11 nA was required to elicit an action potential with a peak amplitude of only −2 mV. These results suggest that oocyte excitability may be maximal at about the time of GVBD, but more data are required for a conclusion.

Sometimes spontaneous action potentials also occurred as the membrane potential crossed the threshold voltage during its positive shift in response to serotonin. In one oocyte, as the resting potential drifted to −50 mV about 25 min after serotonin addition, spontaneous action potentials of about 2-s duration began to recur at intervals ranging from 7 to 20 s; a total of 52 were recorded during 9.6 min until the electrode unfortunately came out. Figure 4D shows a typical action potential from this train.

These results show that serotonin causes a decrease in the oocyte plasma membrane conductance accompanied by a small positive shift in the resting potential and increased membrane excitability. The possible mechanisms involved and biological significance are discussed below.

Discussion

The above results show that serotonin can induce spawning and oocyte maturation in Pismo clams and that maturation is accompanied by changes in the electrical properties of the oocyte plasma membrane so that the input resistance is increased and an action potential is more readily elicited.

As mentioned in the introduction, injection of serotonin into the gonad or other tissue (adductor muscle, foot, etc.) has been shown to induce spawning in a number of bivalve molluscs. Valve opening and closing prior to spawning was also observed by Galtsoff (1938) in oysters. Matsutani and Nomura (1984; Kadam and Koide, 1989; Martínez and Rivera, 1994). However, whether serotonin is a natural trigger during the spawning process, and if so, whether it acts directly on the oocyte, on somatic cells in the ovary, or on neurons is still unknown. In Tivela, ovarian oocytes must have serotonin receptors because oocytes with no follicle cells attached respond to serotonin in seawater. Furthermore, the optimal concentration of serotonin for GVBD and maturation to metaphase I is low (200 nM), supporting the hypothesis that serotonin is a natural trigger. However, it remains to be shown that serotonin is released in the ovary at the time of spawning, and that blocking this release or inhibiting serotonin binding to oocytes in the ovary prevents maturation.

Serotonin has also been shown to induce GVBD followed by arrest in metaphase I in immature oocytes removed from the ovary of the oyster Crassostrea gigas (Osanai and Kuraishi, 1988) and the clam Ruditapes philippinarum (Osanai and Kuraishi, 1988; Guerrier et al., 1993). (These oocytes also have no accessory cells attached.) However, higher concentrations of serotonin are required for maximum percentages of GVBD: on the order of 10 μM in Crassostrea, and 1 μM in Ruditapes (Osanai and Kuraishi, 1988; Guerrier et al., 1993). As in Tivela, prophase-stage oocytes of Ruditapes do not develop when fertilized (Osanai and Kuraishi, 1988; Guerrier et al., 1993). Oyster oocytes, on the other hand, can be fertilized and will complete meiosis and develop normally when they are inseminated at any stage from prophase to metaphase I (e.g., Imai et al., 1950). In the clam Spisula solidissima, GVBD in isolated oocytes is also induced by serotonin (10 μM), but the biological significance is unclear because the treated oocytes (which complete meiosis and form a pronucleus instead of arresting at metaphase I) are reported to be unfertilizable (Hirai et al., 1984, 1988). Furthermore, Spisula oocytes with intact germinal vesicles are readily fertilized and will develop (e.g., Allen, 1953); when spawning is induced by injecting clams with serotonin, most of the spawned oocytes have intact germinal vesicles and undergo GVBD after fertilization (Hirai et al., 1988). Unlike Tivela oocytes, those
of Spisula were reported to be less responsive to serotonin following VC removal (Krantic et al., 1993). However, this difference could be due to damage, because the Spisula VCs were removed by a rather harsh treatment, 1 M glycerol in 0.2 M phosphate buffer.

In summary, although in all of these species serotonin may experimentally induce adults to spawn and isolated oocytes to undergo GVBD, there are significant variations in both sensitivity and response, and the roles of serotonin in the natural induction of these processes remain to be elucidated.

How does serotonin induce the reinitiation of meiosis and the electrical changes in the Tivela oocyte membrane? A variety of serotonin receptors have been identified and characterized in neurons and other somatic cells of both vertebrates and invertebrates (e.g., reviews by Hen, 1992; Frazer and Hensler, 1994). Many are coupled to G-proteins and others are ligand-gated ion channels (e.g., Hen, 1992; Frazer and Hensler, 1994; Shih et al., 1995). In Rudistapes oocytes, a transient increase in inositol-3,4,5-triphosphate (IP3) peaked at 3 min after serotonin application, and GTP and GTP γS induced GVBD in a majority of injected oocytes (Gobet et al., 1994), consistent with, but not proving, the involvement of a G-protein-linked receptor.

In our studies there was no immediate electrical response to serotonin, suggesting that the hormone may not act via a ligand-gated channel. Rather, a decreased conductance developed slowly. Although we did not investigate which channels were being inactivated to cause the conductance decrease, the most likely candidates are K+ channels, as shown in starfish oocytes where maturation is accompanied by decreased membrane conductance (Shen and Steinhardt, 1976; Miyazaki, 1979; Moody and Lansman, 1983; Simoncini and Moody, 1990). Voltage-clamp analysis showed that the decrease in conductance was primarily due to inactivation of inwardly rectifying K+ channels (so-called because they are activated by hyperpolarizing rather than depolarizing membrane potentials) (Moody and Lansman, 1983; Moody and Bosma, 1985; Simoncini and Moody, 1990). Ca2+ action potentials are present in immature starfish oocytes, and with the decreased conductance, less current was required to elicit them and their duration was longer (Shen and Steinhardt, 1976; Moody and Lansman, 1983; Moody and Bosma, 1985; Simoncini and Moody, 1990). The action potentials in Tivela oocytes responded in the same way to the decreased conductance during maturation (see Fig. 4).

In some other species, the increased membrane resistance and more prominent action potentials in oocytes at the stage when they are fertilized strengthen the electrical polyspermy block. Examples among marine invertebrates are in sea urchin (Jaffe, 1976; Jaffe and Robinson, 1978), starfish (Miyazaki and Hirai, 1979; Miyazaki, 1979), and the marine worm Urechis (Gould-Somero et al., 1979; Holland and Gould-Somero, 1981). Although Tivela might be expected to have an electrical polyspermy block, this remains to be shown.

Whether trains of spontaneous action potentials (see Fig. 4D and related text) might also occur during in vivo maturation of Tivela oocytes is an intriguing question. For example, if these are Ca2+ action potentials they might cause increases in intracellular Ca2+ that could play a role in inducing maturation. Indeed, Schlichter (1983a, b) observed that maturing Rana pipiens oocytes fired trains of spontaneous Na+ action potentials during the period from metaphase I to 1st polar body formation, and when these were prevented, maturation was delayed.

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Literature Cited


