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Activation by serotonin of starfish eggs expressing the rat serotonin 1c receptor

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Starfish oocytes were injected with mRNA for the serotonin 1c receptor or with rat brain poly A^+ mRNA, incubated to allow expression of the membrane protein, then matured to eggs by addition of 1-methyladenine. Applying serotonin to these eggs caused cortical granule exocytosis like that occurring at fertilization. Because the serotonin 1c receptor specifically activates a G-protein, these results provide support for the hypothesis that sperm activate eggs by way of a receptor-G-protein interaction. The starfish oocyte may be a generally useful system for expression of exogenous mRNA for membrane proteins.

Introduction

The activation of eggs by sperm at fertilization has been proposed to be analogous to the activation of somatic cells by a neurotransmitter or hormone (Kline *et al.*, 1988; Jaffe, 1990). According to this hypothesis, the sperm may carry on its surface an agonist-like molecule that interacts with a receptor in the egg membrane, leading through the activation of a G-protein and production of inositol trisphosphate ($InsP_3$) to release of calcium from intracellular stores. Calcium then causes several early responses to fertilization, such as ion channel opening and cortical granule exocytosis.

In support of this hypothesis, frog oocytes have been injected with specific mRNA for muscarinic m1 acetylcholine receptors to express these receptors in the egg membrane. After application of acetylcholine, these eggs show several responses that normally occur at fertilization (Kline *et al.*, 1988). Because m1 receptors act by way of G-proteins to stimulate phosphatidylinositol metabolism and produce calcium-dependent responses (Bonner *et al.*, 1987; Jones *et al.*, 1988; Shapiro *et al.*, 1988), these results indicate that in the frog egg, receptor-mediated activation of a G-protein is sufficient to mimic early responses to sperm.

The possible participation of a G-protein in fertilization of echinoderm eggs has been suggested by the activation of sea urchin eggs by injection of guanosine-5′-0-(3-thiotriphosphate) (GTP-γ-S) (Turner *et al.*, 1986; Swann *et al.*, 1987) or cholera toxin (Turner *et al.*, 1987). In this paper, we show that echinoderm eggs, like frog eggs, can be stimulated by way of a receptor that activates a G-protein to produce responses like those occurring at fertilization. For this purpose, we used the serotonin 1c receptor (Julius *et al.*, 1988). The results also demonstrate the expression of a membrane protein in an echinoderm oocyte after injection of exogenous mRNA, thus providing a potentially useful expression system for other membrane proteins.

Results

Cortical granule exocytosis in starfish eggs was stimulated by serotonin after injection of serotonin 1c receptor mRNA

Serotonin 1c receptors were introduced into starfish (*Asterina miniata*) eggs by injection of mRNA synthesized from the corresponding cDNA. Starfish eggs are normally fertilized at first meiotic metaphase, a stage occurring ~30–60 min after application of the maturation-inducing hormone, 1-methyladenine, to the prophase-arrested immature oocytes. Once meiosis has been reinitiated, however, the lifetime of the egg is brief; therefore, to allow time for expression of mRNA, we injected the mRNA into immature oocytes. After incubation of the mRNA-injected oocytes, we applied 1-methyladenine to cause maturation, and then tested for responses to serotonin.

Figure 1 shows a starfish egg that had been injected 30 h previously with serotonin 1c re-

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ceptor mRNA, before (Figure 1a) and after (Figure 1b) applying 0.1 μM serotonin. The occurrence of cortical granule exocytosis was detected by the resulting elevation and modification of the vitelline envelope to produce the fertilization envelope (Figure 1b). The exocytosed granules were visible in the space between the fertilization envelope and the egg plasma membrane (Figure 1b). Oocytes that had been incubated 2–3 d lost their vitelline envelopes, and therefore activation of such eggs did not cause elevation of a fertilization envelope; however, exocytosis in response to serotonin was detected by the appearance of cortical granules on the external surface of the plasma membrane (see Figure 3b below).

Eggs tested 30–80 h after injection of 30 pg of serotonin 1c receptor mRNA all underwent exocytosis within 1–4 min after applying 0.1 μM serotonin (Figure 2). This time course of exocytosis was similar to that observed after applying sperm to eggs in the injection chamber; some of the delay resulted from time for serotonin or sperm to move to the eggs in the space between the coverslips. Eggs that had been injected with 7 pg of serotonin 1c receptor mRNA also underwent exocytosis, but a higher serotonin concentration (10 μM) (not shown) or a longer incubation period (80 h) (Figure 2) was required to obtain responses in all eggs. Control eggs that had not been injected with serotonin 1c receptor mRNA (Figure 2), or that had been injected with nonspecific RNA (see Figure 4 below), did not respond to serotonin.

These results were obtained from 56 oocytes from two starfish; all of these oocytes showed normal morphology. Injection of the same amounts of the same RNA stock into oocytes from two other starfish was toxic to the oocytes. The reason for this toxicity is not understood, but it was not due to the injection itself and did not appear to be due to overexpression of RNA, because dilutions of the stock to levels that were nontoxic did not result in serotonin responsiveness. This RNA stock was not toxic when injected into *Xenopus* oocytes.

Cortical granule exocytosis in starfish eggs was stimulated by serotonin after injection of poly A$^+$ rat brain mRNA

The experiments described above involved the injection of synthetic mRNA derived from a clone for the rat serotonin 1c receptor. Frog oocytes have also been used for expression of mixtures of mRNAs extracted from tissues, for purposes including screening for gene cloning. To examine the potential of starfish oocytes for such a use, we injected the oocytes with poly A$^+$ RNA from rat brain, which includes sequences for the serotonin 1c receptor (Julius *et al.*, 1988). Oocytes injected with 100–200 pg of poly A$^+$ RNA and subsequently matured and tested with 10 μM serotonin underwent cortical granule exocytosis. The response was similar to that obtained with the specific serotonin 1c receptor mRNA, except that more mRNA was required (Figures 3 and 4). Eggs injected with 1000 pg of sea urchin egg poly A$^+$ RNA, used as a nonspecific control mRNA, did not undergo exocytosis in response to serotonin (Figure 4). These results were obtained from 94 oocytes

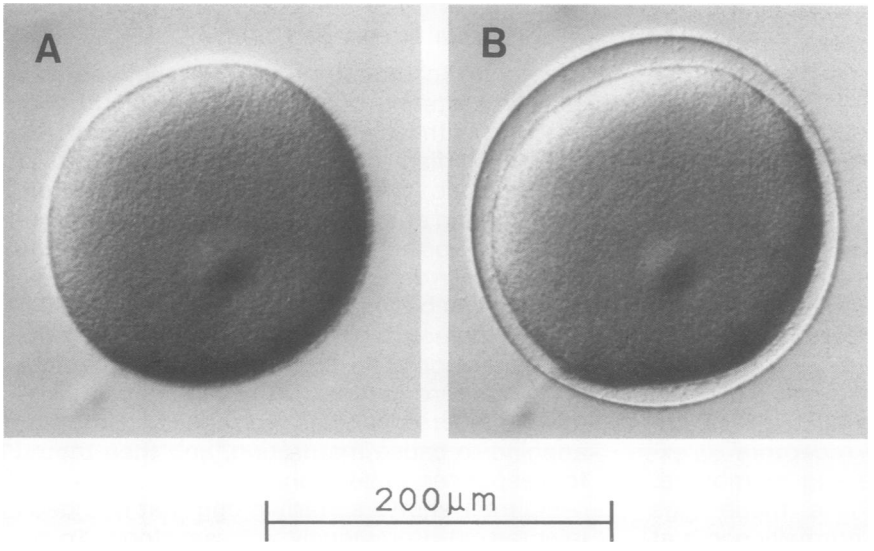

Figure 1. **Fertilization envelope elevation in a starfish egg expressing serotonin 1c receptors, in response to serotonin.** (A) The egg before applying serotonin. This egg was injected 30 h previously with 7 pg of serotonin 1c receptor mRNA and matured with 1-methyladenine. (B) The egg 10 min after applying 0.1 μM serotonin. The fertilization envelope has elevated, and cortical granules are visible in the space between the egg plasma membrane and the fertilization envelope.

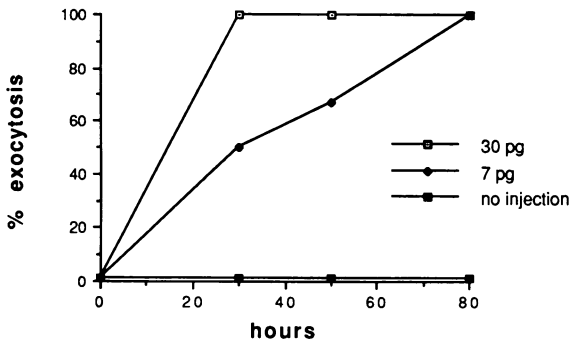

Figure 2. **Exocytosis of cortical granules in response to serotonin, from starfish eggs expressing serotonin 1c receptors, as a function of time after injection of serotonin 1c receptor mRNA.** Oocytes were injected with 7 or 30 pg of mRNA; control oocytes were not injected with mRNA. After the indicated times, oocytes were matured with 1-methyladenine and exposed to 0.1 μM serotonin. The occurrence of cortical granule exocytosis was detected by observation of fertilization envelope elevation or the appearance of cortical granules outside of the egg, visible 1–4 min after serotonin addition. The graph combines results from 40 injected eggs and 43 noninjected eggs from two starfish.

from four starfish; injection of rat brain RNA into oocytes from one of the four animals produced some toxicity, but oocytes from the other three animals showed normal morphology.

Discussion

Taking advantage of the specific action of the serotonin 1c receptor to activate G-proteins in other cells (Litosch and Fain, 1985; Julius *et al.*, 1988), we used this receptor to activate G-proteins in starfish egg membranes. In response, the eggs underwent cortical granule exocytosis like that normally occurring at fertilization. This observation adds to the available evidence that fertilization of echinoderm eggs may be mediated by a receptor-G-protein interaction (see Jaffe, 1990). The present results extend previous findings that GTP-γ-S (Turner *et al.*, 1986; Swann *et al.*, 1987) or cholera toxin (CTX) (Turner *et al.*, 1987) injection activates sea urchin eggs. The serotonin 1c receptor is a more specific agent for activating G-proteins than GTP-γ-S, which could influence any GTP-dependent reaction in the cell. CTX, although relatively specific for G-proteins, is not usually effective in modifying those G-proteins associated with activation of processes involving $InsP_3$ and Ca (but see McCloskey, 1988). A rise in cyclic AMP (cAMP) was excluded as an explanation of the activation of sea urchin eggs by CTX, because injection of 1–4 mM cAMP-S, a hydrolysis-resistant analogue of cAMP, did not cause exocytosis (Turner *et al.*, 1987). However, other indirect effects of CTX are possible. The serotonin 1c receptor, on the other hand, is known in other systems to act by way of a G-protein to stimulate the production of $InsP_3$ and release of Ca. It is therefore the most specific means yet used to activate the G-protein that is proposed to function at fertilization.

These results also demonstrate that mRNA for membrane proteins can be expressed in an echinoderm oocyte. Sea urchin eggs have been used previously to express mRNA for globin (Colin and Hille, 1986); however, compared with the sea urchin egg, the starfish oocyte has the desirable characteristic of having a longer life-

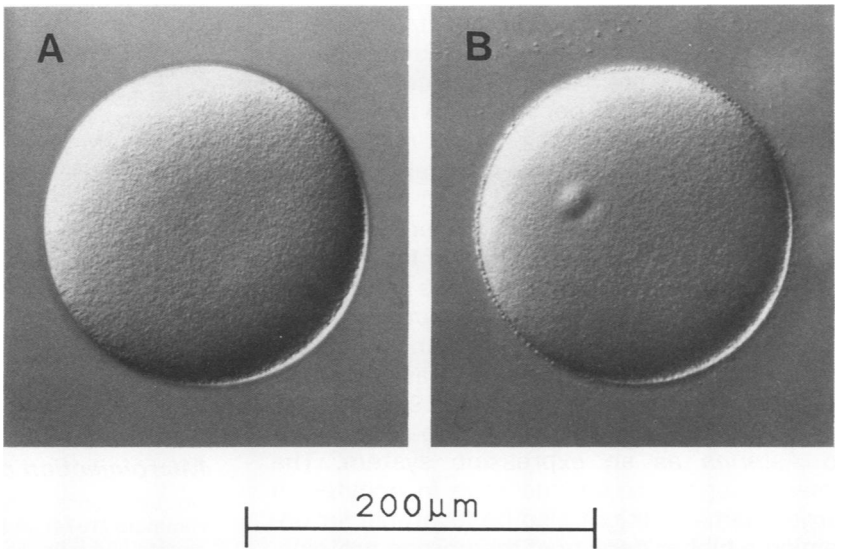

Figure 3. **Cortical granule exocytosis from a starfish egg injected with rat brain poly A^+ RNA and exposed to serotonin.** (A) A control egg that was not injected with RNA. The egg was matured with 1-methyladenine and photographed 15 min after applying 10 μM serotonin. No cortical granule exocytosis was observed. (B) An egg injected 74 h previously with 100 pg of rat brain poly A^+ RNA, matured with 1-methyladenine, and then exposed to 10 μM serotonin. The egg was photographed 15 min later; cortical granules were visible around the egg surface.

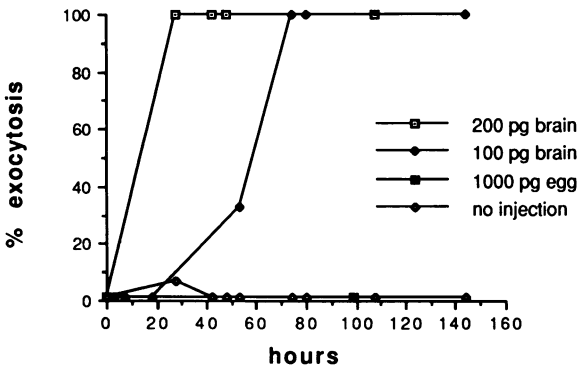

Figure 4. **Exocytosis of cortical granules in response to serotonin, from starfish eggs previously injected with rat brain poly A^+ RNA, as a function of time after injection.** Oocytes were injected with 100 or 200 pg of rat brain poly A^+ RNA, or as a control, 1000 pg of sea urchin egg poly A^+ RNA; additional control oocytes were not injected with RNA. After the indicated times, oocytes were matured with 1-methyladenine and exposed to 10 μM serotonin. The occurrence of cortical granule exocytosis was detected by observation of fertilization envelope elevation or the appearance of cortical granules outside of the egg. The graph combines results from 94 injected eggs and 226 noninjected eggs from three starfish.

time in culture. Starfish oocytes may be useful for expressing other membrane receptors or ion channels, as an alternative to the *Xenopus* oocyte, which has been used extensively for this purpose (Dascal, 1987). Some RNAs, such as poly A^+ RNAs from crustacea and insects, are relatively weakly expressed in *Xenopus* oocytes (Saito *et al.*, 1987). The starfish oocyte would be an advantageous expression system for studies of certain membrane proteins, such as chloride channels, because these channels are endogenous in the *Xenopus* oocyte (Dascal, 1987) but not in the starfish oocyte (Hagiwara and Jaffe, 1979). *Asterina* oocytes can be removed from the animal and defolliculated more simply than *Xenopus* oocytes, and they are optically clear. On the other hand, *A. miniata* with full-grown oocytes can be routinely collected from the west coast of the United States only from approximately May to October (see Methods). Also, the smaller diameter of the *Asterina* oocyte (160–190 μm) results in less protein production per oocyte than with *Xenopus;* thus, for some purposes, species of starfish with larger oocytes (~1 mm in species such as *Mediaster* and *Leptasterias* [Hagiwara *et al.*, 1975; Moody, 1985; Strathmann, 1987]) might be preferable to *Asterina* as an expression system. The smaller surface-to-volume ratio in oocytes of larger diameter might also be favorable for obtaining a higher density of membrane proteins.

Methods

Preparation and culture of oocytes

Specimens of *Asterina (Patiria) miniata* were obtained from Bodega Marine Laboratory (Bodega Bay, CA) or from Marinus (Long Beach, CA), and were maintained in sea water at 15°C. Animals containing full-grown oocytes can be collected from these locations from approximately May through October; the results described in this paper were obtained with oocytes from starfish collected not more than 2 mo before use. The possibility of obtaining good-quality oocytes from animals maintained in aquaria for longer periods remains to be examined.

Oocytes were obtained by cutting a slit between the arms and pulling out a fragment of ovary; the same starfish could be used several times. The ovary was placed in ice-cold calcium-free sea water (440 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 26 mM $MgSO_4$, 2 mM $NaHCO_3$, pH 8.0) and cut up with scissors; this released oocytes surrounded by follicle cells. The follicle cells were removed by washing the oocytes several times in ice-cold calcium-free sea water over a period of ~20–30 min, followed by several washes in filtered natural sea water. The resulting preparation of oocytes was maintained at 20°C for use throughout the day. Only animals yielding oocytes having diameters of 160–190 μm were used; oocytes smaller than this diameter do not respond to 1-methyladenine. For reasons that are not understood, oocytes from some individual animals did not survive well after mRNA injection, so it was useful to test oocytes from more than one starfish. It was also important to start with a preparation of oocytes free of follicle cells, mature eggs, or other debris that could contaminate the culture.

Oocytes were loaded into microinjection/culture chambers as described by Kiehart (1982). The oocytes were held between two coverslips separated by two layers of double-stick tape to make a space of ~200 μm. To prevent bacterial growth, 50 μg/ml gentamycin (GIBCO, Grand Island, NY) was added to the filtered natural sea water in the chamber. Any debris present in the chamber was removed by "vacuum cleaning" with a microinjection pipette (see below). The chambers were stored in a moist container at 18–20°C.

Preparation of mRNA

The serotonin 1c receptor cDNA, in the vector Bluescript, was kindly provided to us by Dr. David Julius (Julius *et al.*, 1988). To make synthetic mRNA encoding this receptor, the template was first linearized with Not 1 and then transcribed with T7 polymerase (Stratagene, La Jolla, CA). Five to ten micrograms of linearized template was used for each transcription reaction. Standard components (Promega, Madison, WI) were used in the synthesis, except that 0.5 mM of the capping analogue $m^7G(5')ppp(5')G$ (Pharmacia, Piscataway, NJ) was used in addition to 50 μM GTP (Konarska *et al.*, 1984). The reaction was terminated by phenol/chloroform extraction followed by ethanol precipitation. After resuspension in RNAase-free water, the amount of RNA was estimated by comparison with RNA molecular size markers of known concentration on agarose gels. The concentration of the serotonin 1c receptor RNA used for microinjection was ~0.2 μg/μl. Rat brain poly A^+ RNA was prepared as described by Cooperman *et al.* (1987) and used for microinjection at a concentration of ~0.5 μg/μl.

Microinjection of mRNA

Microinjections were made using methods described by Hiramoto (1974) and Kishimoto (1986). The microinjection pipette had a tip diameter of ~5 μm and an oil-filled con-

striction to slow the flow of injection solutions. Pressure was applied to the pipette by means of an oil-filled tube connected to a micrometer syringe. Injected volumes were 1–10% of the total oocyte volume of ~3 nl and were standardized by measuring the length of the column of solution drawn into the pipette. By drawing up an equivalent volume of oil and then expelling the oil drop and measuring its diameter, a precise calibration was obtained.

mRNA was taken up into the micropipette from a loading capillary in which the mRNA stock was held between columns of oil. The micropipette was reloaded from this reservoir for each injection. During injection, oocytes were maintained in filtered natural sea water, except for experiments in which very large injection volumes (10% of the oocyte volume) were used; in such cases, the oocytes were kept in 80% sea water during injection (Kishimoto, 1986).

Activation of eggs with serotonin

After injection of mRNA and culture for sufficient time to allow mRNA expression, the solution in the culture chamber was replaced with natural sea water containing 1 μM 1-methyladenine (Sigma, St. Louis, MO), in order to stimulate reinitiation of meiosis. Twenty to forty minutes after addition of the hormone, the nuclear envelope disappeared; eggs were tested with serotonin 40–80 min after applying 1-methyladenine. Serotonin (5-hydroxytryptamine, creatinine sulfate complex, Sigma) was dissolved at a concentration of 0.1–10 μM in sea water. The solution in the egg chamber was replaced with the serotonin solution, and responses of the eggs were monitored with a 20× objective and differential interference optics (Carl Zeiss, New York).

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