Analysis of the Breakdown of Cortical Granules in Echinoderm Eggs by Microinjection of Second Messengers

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ABSTRACT. Breakdown of cortical granules was investigated by microinjecting Ca-EGTA buffers at various concentrations of Ca^{2+} , inositol 1, 4, 5-trisphosphate (IP_3), guanosine triphosphate (GTP) and guanosine 5'-O-thiotriphosphate ($\text{GTP}\gamma\text{S}$) into echinoderm (*Clypeaster japonicus*, *Temnopleurus hardwicki*, and *Scaphechinus mirabilis*) eggs using a video microscope. The breakdown started in the region near the injection site and then propagated over the entire surface of the egg. The cortical granules did not synchronously break down side by side, but instead underwent random breakdown, in the same manner as that upon fertilization. The reaction times of the breakdown of the cortical granule after the injection of Ca-EGTA buffers, IP_3 , and $\text{GTP}\gamma\text{S}$ were 0.4 sec, 1.0 sec, and 2.7 sec, respectively, in *C. japonicus* eggs, and were in the same order in *T. hardwicki* and *S. mirabilis* eggs. It is inferred from this order of reaction times that GTP-binding proteins, IP_3 and Ca^{2+} act in this sequence upon fertilization in echinoderm eggs.

Upon fertilization in echinoderm eggs, the fertilization envelope elevates through the breakdown (exocytosis) of cortical granules. In sea urchin eggs, cortical granules are distributed just beneath the plasma membrane of the egg and measure 0.4–1.2 μm in diameter (7, 8, 17). After the cortical granule breakdown, the fertilization envelope is elevated by colloid osmotic pressure generated by a component of cortical granule inclusion which protrudes into the space between the plasma membrane and the vitelline coat (11). Subsequently, another component of the inclusion hardens the fertilization envelope (7, 8, 21).

Sperm incorporation triggers the cortical granule breakdown, which starts at the site of sperm attachment and propagates over the entire surface of the egg in a wavelike manner. It is thought that the stimulation of sperm incorporation is transmitted by second messengers to the cortical granules and that Ca^{2+} and inositol 1, 4, 5-trisphosphate (IP_3) are candidates for the second messengers acting in the eggs as follows. A transient increase of Ca^{2+} in the egg cytoplasm occurs upon fertilization (5, 6, 9, 14, 16, 18, 23, 24, 27). The role of Ca^{2+} in the eggs has been understood by the fact that intracellular Ca^{2+} increase induced by microinjecting Ca-EGTA buffers (10) and treating with calcium ionophore (2, 22) caused fertilization envelope elevation and egg activation. It is reported that IP_3 is produced at fer-

This paper is dedicated to the memory of the late Dr. M. Yoshida, who, for a number of years, served as director of Ushimado Marine Biological Station of Okayama University.

tilization (3), and that microinjection of IP_3 into sea urchin eggs causes elevation of the fertilization envelope (25, 26). In medaka and golden hamster eggs, injection of IP_3 induced an immediate Ca^{2+} transient (14, 16). Furthermore, there is some evidence that GTP-binding protein (G-protein) plays a crucial role in fertilization. Microinjection of guanosine 5'-O-thiotriphosphate (GTP γ S), an activator of G-protein, caused a Ca^{2+} transient (16) and fertilization envelope elevation (25) while guanosine 5'-O-(2-thiodiphosphate) (GDP β S), an inhibitor of G-protein, inhibited fertilization envelope elevation at fertilization (25). Turner, Jaffe and Fein (25) proposed a hypothesis for the roles of these second messengers as to their function in living eggs, as follows: 1) the sperm incorporation activates GTP-binding protein (G-protein), 2) the activated G-protein stimulates phospholipase C, which produces IP_3 from triphosphoinositide, 3) the produced IP_3 releases Ca^{2+} from the endoplasmic reticulum or other Ca^{2+} storage sites, and 4) the released Ca^{2+} causes fertilization envelope elevation.

In the present study, we observed and recorded using a video microscope the process of the cortical granule breakdown induced by microinjection of Ca-EGTA buffers, IP_3 , GTP and GTP γ S into echinoderm eggs. We determined the reaction times of the breakdown of the cortical granule in order to investigate the sequence of steps in which the above agents act.

MATERIALS AND METHODS

Eggs. Eggs were obtained from the sea urchin, *Temnopleurus hardwicki* and sand dollars, *Scaphechinus mirabilis* and *Clypeaster japonicus* by intracoelomic injection of artificial sea water (ASW; Jamarin U; Jamarin Laboratory, Osaka) containing 1 mM acetylcholine. The eggs were washed three times with ASW and kept at 15°C before use.

Microinjection. Microinjection was carried out at $20 \pm 1^\circ C$ in the case of *S. mirabilis* and at $25 \pm 1^\circ C$ in the cases of *T. hardwicki* and *C. japonicus* using an injection apparatus devised by us (Fig. 1). With this apparatus we could inject a fixed volume of a solution into an egg within 0.15 sec using a braking micropipette (12). The injection solution was sucked into the tip of the micropipette and ejected into the egg by applying high pressure supplied by a compressed air bomb through a pressure regulator (cf. 4, 12, 13, 19). The supplied pressure was adjusted to 2.8×10^5 Pa by the pressure regulator. Pressure applied to the back orifice of the micropipette was controlled by a pair of solenoidal valves (SV_1 , SV_2), which were operated for 0.1 sec or 0.15 sec by a pulse generator. The generator also started a videotimer for recording the time after the injection. The micropipette was filled with silicone oil, as shown in Fig. 1. The shaded area in the figure connecting to the micropipette was filled with degassed water while the clear spaces in syringe S_2 and between SV_2 and the air bomb contained air.

The injection procedure was carried out as follows. Before injection, SV_1 was open and SV_2 and a valve V were closed. First, 6–7 μ l of injection solution was sucked into the micropipette filled with silicone oil by applying a negative pressure supplied by syringe S_1 ; a small volume of silicone oil for the plug of the micropipette was then sucked in. V was then opened. A 20 ml syringe (S_2) whose plunger moved smoothly up and down was set vertically as shown in Fig. 1 to keep the pressure slightly positive by putting an appropriate weight (about 4×10^4 Pa) on the plunger to prevent any outside medium from invading the micropipette by the capillarity. Then, the tip of the micropipette was inserted into the egg with a micromanipulator (MO-102, Narishige Scientific Instrument Laboratory, Tokyo). When the switch of the pulse generator was turned on, an electric pulse operated SV_1 and SV_2 through a relay system. SV_1 was closed for a short time and then open. At the same time, SV_2

Fig. 1. Schematic diagram of the videomicroscope and the microinjection apparatus. A differential interference microscope was used for observation. A videocamera mounted on it is connected to a videorecorder and a videomonitor through a video timer. Turning on the switch, the pulse generator generates a single pulse, which starts the video timer. The pulse also opens a solenoidal valve (SV₂) through a relay for a short period, and simultaneously closes the other valve (SV₁). Injection solution in a micropipette is ejected by air at high pressure supplied by the air bomb through the opened SV₂. The syringe, S₂ (a 20 ml glass syringe), whose plunger moves smoothly up and down is set vertically on the table. Shortly after injection, this syringe can depress high pressure through SV₂ to stop the injection but apply a small positive pressure to an injection micropipette in order to prevent sea water from invading it by capillarity by putting appropriate weight on the plunger.

was open and then closed. Thus the solution was injected into the egg together with the small volume of silicone oil by a high-pressure pulse applied to the micropipette through SV₂. Then the high pressure in the micropipette was released through SV₁ and the pressure in the micropipette recovered to the original small positive pressure. The volume of the solution injected per egg was 6–7 pl corresponding to 1% of the egg volume.

Injection solution. IP₃ (Sigma Chemical Company, St. Louis, USA), GTP (Sigma Chemical Company, St. Louis, USA) and GTPγS (Boehringer Mannheim Biochemicals, Indianapolis, USA) were dissolved in a buffer containing 100 mM potassium aspartate and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate (HEPES: Wako Pure Chemical Industry Ltd., Tokyo) (pH 6.8 or 7.0) (see ref. 25). Ca-EGTA buffer solutions were prepared by mixing 0.1 M Ca-EGTA and 0.1 M EGTA (pH 6.8 or 7.0) at various ratios following the

method of Hamaguchi and Hiramoto (10).

Observation and measurements. The breakdown of cortical granules was observed in the image field (a rectangle area of $50 \times 65 \mu\text{m}$ at most) of a differential interference microscope (Nikon Optiphot; Nikon Corp., Tokyo) with a $100 \times / 1.25$ Nikon DIC objective and recorded by a videosystem including a videocamera (WV-1550; Matsushita Electric Industrial Co., Ltd., Osaka), a video timer (VTG-33; FOR-A Co. Ltd., Tokyo), a videocassette recorder (NV 8950; Matsushita Electric Industrial Co., Ltd., Osaka) and a videomonitor (WV 5360; Matsushita Electric Industrial Co. Ltd.) (Fig. 1). Reaction time of the breakdown of the cortical granule was determined as the time interval between the start of the injection and the time when one of the cortical granules in the image field disappeared completely, which was found by reviewing the recorded images of cortical granules at the egg surface and the time superimposed on them. A $20 \times / 0.50$ Nikon DIC objective was used to determine whether or not the fertilization envelope was elevated after the injection.

RESULTS

Relationship between the reaction time and the distance of the injection site from the cell surface. To examine the difference in cortical granule breakdown by the depth of the injection site from the egg surface, a Ca-EGTA buffer ($3.0 \mu\text{M}$ of Ca^{2+}) was microinjected into *S. mirabilis* eggs at various distances from the egg surface. The reaction times of the breakdown of the cortical granule after the injection are shown in Fig. 2. The reaction times for the distances of $5 \mu\text{m}$ and $10 \mu\text{m}$ from the egg surface were almost the same (0.3 ± 0.1 sec and 0.3 ± 0 sec, respectively), while the reaction time increased gradually as the distance increased from 10 to $50 \mu\text{m}$. This result is reasonably understood because the injected solution of 6–7 pl may reach the cell surface immediately after the injection since the volume of the solution occupies a spherical space of 11–12 μm in radius. In the present study, unless

Fig. 2. Cortical granule breakdown at the various injected sites. This figure shows the mean reaction times of the breakdown of the cortical granule after injection when Ca-EGTA buffer ($3.0 \mu\text{M}$ of Ca^{2+} concentration) was microinjected into *Scaphechinus mirabilis* eggs by changing the distance from the egg surface to the tip of the micropipette.

Fig. 3. The time course of cortical granule breakdown shown after microinjecting a Ca-EGTA buffer ($5.7 \mu\text{M}$ of Ca^{2+} concentration) into a *Clypeaster japonicus* egg. **a.** Cortical granules before injection. **b.** The first breakdown of a cortical granule 0.45 sec after the injection. The arrowhead indicates the cortical granule which was broken first. **c.** Most of cortical granules had broken down 5.45 sec after the injection.

otherwise stated, the site of microinjection was $10 \mu\text{m}$ from the egg surface.

Cortical granule breakdown by microinjection of Ca-EGTA buffers. The cortical granules in *Clypeaster japonicus* eggs, which are $1.0\text{--}1.2 \mu\text{m}$ in diameter (Fig. 3a), are larger in size and smaller in number than those in *Scaphechinus mirabilis* and *Temnopleurus hardwicki*. Figure 3 shows the time sequence of the breakdown of cortical granules in a *C. japonicus* egg by the microinjection of the Ca-EGTA buffer solution ($5.7 \mu\text{M}$ Ca^{2+}). This concentration was effective enough to cause cortical granule breakdown. The first breakdown of a cortical granule, indicated by the arrowhead in Fig. 3a, was recognized at 0.45 sec after the injection, as shown in Fig. 3b, and most of the cortical granules around it broke down within a few seconds after the injection (Fig. 3c).

In a highly magnified image, the breakdown of the cortical granules appeared similar to the explosion of a bomb, as described by Endo (7, 8). The breakdown occurred sporadically on the egg surface at a high magnification, but at low magnification it was viewed as spread over the entire surface of the egg from the region close to the injection site. The above phenomena were almost the same as those upon fertilization. The breakdown of cortical granules by the microinjection of the Ca^{2+} buffer occurred in a similar manner in *T. hardwicki* and *S. mirabilis* eggs.

The reaction times of the breakdown of the cortical granule at various Ca^{2+} concentrations are shown in Table 1. In *S. mirabilis* and *C. japonicus* eggs, the reaction time increased as the Ca^{2+} concentration of Ca-EGTA buffers decreased. In *S. mirabilis*, no cortical granule breakdown in four out of six eggs was observed by the injection of Ca-EGTA buffer at $0.3 \mu\text{M}$ Ca^{2+} , while cortical granule breakdown was observed by microinjection of the Ca-EGTA buffer at Ca^{2+} concentrations of $0.8 \mu\text{M}$ or more. The fertilization envelope elevated in the cases of $1.4 \mu\text{M}$ and $3.0 \mu\text{M}$ Ca^{2+} injection. In *C. japonicus* eggs, all cortical granules within the circle of ca. $30 \mu\text{m}$ in diameter of the image field were broken down at the concentration of $0.6 \mu\text{M}$ but the fertilization envelope did not elevate at that concentration. This

TABLE 1. REACTION TIME OF THE BREAKDOWN OF THE CORTICAL GRANULE AFTER INJECTION OF Ca-EGTA BUFFER

species	injected Ca^{2+} [μM]	reaction time [sec]
<i>C. japonicus</i>	0.6	8.0 ± 2.9 (n=3)
	1.7	2.1 ± 0.6 (n=3)
	2.8	0.6 ± 0 (n=2)
	5.7	0.3 ± 0.1 (n=5)
<i>T. hardwicki</i>	5.7	0.6 ± 0.1 (n=10)
<i>S. mirabilis</i>	0.3	* (n=6)
	0.8	1.7 ± 0.4 (n=6)
	1.4	0.5 ± 0.1 (n=5)
	3.0	0.3 ± 0 (n=6)

* No breakdown of the cortical granule was observed in four cases, but the reaction times of the remainder were 5.5 sec and 8.1 sec.

suggests that the breakdown of cortical granules occurred only near the injection site.

Cortical granule breakdown by injection of IP_3 . IP_3 solutions at various concentrations were microinjected into eggs of *S. mirabilis*, *T. hardwicki* and *C. japonicus*. As shown in Table 2, the breakdown of the cortical granule occurred with reaction times of 0.5–0.7 sec in *S. mirabilis* eggs and 1.0 sec in *C. japonicus* and *T. hardwicki* eggs almost independently of the concentrations of IP_3 used in the present study (0.1–20 μM). However, the fertilization envelope was elevated after the injection of IP_3 at 5 μM or more, but did not elevate after the injection of IP_3 at 1 μM or less.

Cortical granule breakdown by injection of $\text{GTP}\gamma\text{S}$ and GTP. $\text{GTP}\gamma\text{S}$ solutions at various concentrations were injected into echinoderm eggs. As shown in Table 3, the breakdown of cortical granules was evoked by $\text{GTP}\gamma\text{S}$ in all these species examined. It is noted that the reaction time is independent of the concentrations of $\text{GTP}\gamma\text{S}$ above the threshold concentration, although the threshold is different in each species. However, the fertilization envelope did not elevate after injection of $\text{GTP}\gamma\text{S}$ in the above three species, even when the breakdown of cortical granules was observed.

Injection of GTP at concentrations of 1–50 mM into *C. japonicus* eggs caused cortical granule breakdown, but did not cause elevation of the fertilization envelope.

TABLE 2. REACTION TIME OF THE BREAKDOWN OF THE CORTICAL GRANULE AFTER INJECTION OF IP_3

species	IP_3 [μM]	reaction time [sec]
<i>C. japonicus</i>	10	1.0 ± 0.1 (n=2)
	20	1.0 ± 0.2 (n=6)
<i>T. hardwicki</i>	20	1.0 ± 0.1 (n=3)
<i>S. mirabilis</i>	0.1	0.5 ± 0.1 (n=4)
	1	0.7 ± 0.2 (n=9)
	5	0.6 ± 0.1 (n=5)
	10	0.7 ± 0.5 (n=10)
	20	0.5 ± 0.1 (n=7)

TABLE 3. REACTION TIME OF THE BREAKDOWN OF THE CORTICAL GRANULE AFTER INJECTION OF GTP γ S

species	GTP γ [mM]	reaction time [sec]
<i>C. japonicus</i>	1	1.7 $\pm$ 0.1 (n=2)
	10	3.2 $\pm$ 0.2 (n=2)
	20	2.4 $\pm$ 0.7 (n=4)
<i>T. hardwicki</i>	1	** (n=2)
	20	1.9 $\pm$ 0.3 (n=8)
<i>S. mirabilis</i>	0.1	** (n=2)
	1	2.0 $\pm$ 0.9 (n=5)
	10	1.4 $\pm$ 0.4 (n=9)
	20	1.8 $\pm$ 0.5 (n=6)
	50	2.0 $\pm$ 1.0 (n=12)

** No breakdown of the cortical granule was observed.

The mean reaction time of the breakdown of the cortical granule after injection of GTP (1–50 mM) was 2.4 $\pm$ 0.7 sec (n=6), and it was almost equal to that time (2.3 $\pm$ 1.3 sec) after injection of GTP γ S (1–20 mM) in *C. japonicus*.

Comparison among the reaction times of the breakdown of the cortical granule by injections of Ca²⁺, IP₃ and GTP γ S. When the eggs of three species of echinoderm were injected with solutions of Ca²⁺ buffers, IP₃ and GTP γ S, the reaction times after injection were independent of the concentration of GTP γ S and IP₃ but dependent on Ca²⁺ concentration, as described in the previous three sections (see Tables 1–3). The mean reaction times of the breakdown of the cortical granule after injection of IP₃ and GTP γ S above the threshold concentration (0.1–20 μ M for IP₃ and 10–50 mM for GTP γ S) were calculated; the results are illustrated in Fig. 4.

Fig. 4. Summary of injection of Ca²⁺, IP₃, and GTP γ S into echinoderm eggs of three species. This figure summarizes the mean reaction times of the breakdown of the cortical granule after injections of Ca-EGTA buffers (2.8–5.7 μ M for Ca²⁺), IP₃, and GTP γ S above threshold concentrations (0.1–20 μ M for IP₃ and 10–50 mM for GTP γ S).

Because Ca^{2+} concentration in the egg cytoplasm increased up to a few μM upon fertilization (2.5, 2.5–4.5, 4–9, and 3.5 μM in refs. 9, 23, 24, and 18, respectively), it is reasonable that the reaction times after injection at Ca^{2+} concentrations of 2.8–5.7 μM are thought to be the same as those after the increase in Ca^{2+} at fertilization. Therefore, their means were calculated and are represented in Fig. 4. In all three species of echinoderm used in this study, the reaction times were shortened in the order of injection of $\text{GTP}\gamma\text{S}$, IP_3 and Ca-EGTA buffer. These results indicate that GTP-binding protein, IP_3 and Ca^{2+} may react in this order in the egg during fertilization, if the corresponding reactions are activated successively in the egg cytoplasm by them.

DISCUSSION

In the present study, a video microscope, which is a differential interference microscope equipped with a videocamera and connected to a videomonitor and a videorecorder, was used to observe and record the breakdown of cortical granules in echinoderm eggs after injection of Ca-EGTA buffers, IP_3 and $\text{GTP}\gamma\text{S}$. Using this equipment the magnification can be increased up to $10,000\times$ and the microscopic image can be slowly reviewed many times, and therefore, the observation of the breakdown has been simplified in this experiment. The mean reaction times of the breakdown of the cortical granule by injection of Ca-EGTA buffers, IP_3 and $\text{GTP}\gamma\text{S}$ were determined to increase in this order and to be 0.4 sec, 1.0 sec and 2.7 sec, respectively, in *C. japonicus*; minor differences in these values existed in other species. These results may indicate that $\text{GTP}\gamma\text{S}$, IP_3 , and Ca^{2+} participate in sequential reaction steps leading to the breakdown of cortical granules. Turner et al. (25) concluded that they successively activated corresponding reaction steps in the egg cytoplasm in a different sea urchin species (see INTRODUCTION).

Upon fertilization, many events, such as sperm attachment, depolarization of membrane potential, and sperm-egg membrane fusion, take place sequentially before cortical granule breakdown after insemination. It is reported that the breakdown has been detected 3–5 sec after insemination (1), 23 sec after sperm attachment (20), and 6–8 sec after the onset of depolarization of the membrane (15). In the present study, cortical granule breakdown was detected 1.9–2.7 sec after the injection of $\text{GTP}\gamma\text{S}$, which is shorter than the reported values upon fertilization. The difference between 1.9–2.7 sec and the reported values may be due to the difference between the time required for the sperm to attach to, enter the egg, and activate Na^+ channels and the time required for activating G-protein if it is assumed that both $\text{GTP}\gamma\text{S}$ and sperm trigger cortical granule breakdown through activating the G-protein in the membrane.

Yoshimoto et al. (27) reported that the wave of intracellular Ca^{2+} release was demonstrated in eggs of sand dollars (*C. japonicus* and *S. mirabilis*) when the egg was inseminated. In the present study, we confirmed that cortical granule breakdown appeared to be the corresponding Ca^{2+} increase. However, at a high magnification using our videosystem we observed that cortical granules broke down at random and in nonspecific location. It is interesting how such an increase of Ca^{2+} evokes this type of random cortical granule breakdown.

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