Spatiotemporal characteristics and mechanisms of intracellular Ca\(^{2+}\) increases at fertilization in eggs of jellyfish (Phylum Cnidaria, Class Hydrozoa)

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Abstract

We have clarified, for the first time, the spatiotemporal patterns of intracellular Ca\(^{2+}\) increases at fertilization and the Ca\(^{2+}\)-mobilizing mechanisms in eggs of hydrozoan jellyfish, which belong to the evolutionarily old diploblastic phylum, Cnidaria. An initial Ca\(^{2+}\) increase just after fertilization took the form of a Ca\(^{2+}\) wave starting from one cortical region of the egg and propagating to its antipode in all of four hydrozoan species tested: *Cytaeis uchidae*, *Cladonema pacificum*, *Clytia* sp., and *Gonionema vertens*. The initiation site of the Ca\(^{2+}\) wave was restricted to the animal pole, which is known to be the only area of sperm–egg fusion in hydrozoan eggs, and the wave propagating velocity was estimated to be 4.2–5.9 μm/s. After a Ca\(^{2+}\) peak had been attained by the initial Ca\(^{2+}\) wave, the elevated Ca\(^{2+}\) gradually declined and returned nearly to the resting value at 7–10 min following fertilization. Injection of inositol 1,4,5-trisphosphate (IP\(_3\)), an agonist of IP\(_3\) receptors (IP\(_3\)R), was highly effective in inducing a Ca\(^{2+}\) increase in unfertilized eggs; IP\(_3\) at a final intracellular concentration of 12–60 nM produced a fully propagating Ca\(^{2+}\) wave equivalent to that observed at fertilization. In contrast, a higher concentration of cyclic ADP-ribose (cADPR), an agonist of ryanodine receptors (RyR), only generated a localized Ca\(^{2+}\) increase that did not propagate in the egg. In addition, caffeine, another stimulator of RyR, was completely without effect. Sperm-induced Ca\(^{2+}\) increases in *Gonionema* eggs were severely affected by preinjection of heparin, an inhibitor of Ca\(^{2+}\) release from IP\(_3\)R. These results strongly suggest that there is a well-developed IP\(_3\)R-, but not RyR-mediated Ca\(^{2+}\) release mechanism in hydrozoan eggs and that the former system primarily functions at fertilization. Our present data also demonstrate that the spatial characteristics and mechanisms of Ca\(^{2+}\) increases at fertilization in hydrozoan eggs resemble those reported in higher triploblastic animals.

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Keywords: Fertilization; Hydrozoan egg; Calcium; IP\(_3\); cADPR; Caffeine; *Cytaeis*; *Cladonema*; *Clytia*; *Gonionema"

Introduction

An increase in intracellular free Ca\(^{2+}\) is considered a universally occurring phenomenon in fertilized oocytes or eggs of animals. Such Ca\(^{2+}\) increases are indispensable for releasing oocytes or eggs from the cell cycle arrest at a certain species-specific stage and allowing them to start development (Jaffe et al., 2001; Nixon et al., 2000; Whitaker and Patel, 1990). In addition, it is known in most species that the Ca\(^{2+}\) increases at fertilization alter the physical and/or chemical properties of extracellular matrix around the oocytes or eggs, which contributes to the prevention of polyspermy as well as to the protection of developing embryos (Gould and Stephano, 2003; Wessel et al., 2001).

Based on indirect evidence from various animals, Jaffe (1983, 1985) proposed an interesting hypothesis that there is a great difference in the Ca\(^{2+}\) source and pattern at fertilization between deuterostomes and protostomes: a point-source Ca\(^{2+}\) wave is produced by a Ca\(^{2+}\) release from internal stores in deuterostome oocytes or eggs, whereas a synchronous Ca\(^{2+}\) elevation is caused by a Ca\(^{2+}\) influx from...
external medium in protostomes. Since then, spatiotemporal patterns of Ca²⁺ changes at fertilization have been monitored directly and mechanisms underlying the Ca²⁺ changes have been investigated in several species of deuterostomes and protostomes. In deuterostomes such as sea urchins, starfish, ascidians, fish, amphibians, and mammals, an initial large Ca²⁺ increase appearing just after fertilization indeed takes the form of a Ca²⁺ wave propagating from a sperm entry point to its antipode (reviewed in Dumolland et al., 2002; Iwao, 2000; Sardet et al., 1998; Stricker, 1999). It is likely that the propagating Ca²⁺ wave is regulated by a Ca²⁺ release from the endoplasmic reticulum (ER), and that this release is chiefly mediated by inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃R) on the ER membrane (Iwao, 2000; McDougall et al., 2000; Miyazaki et al., 1993; Runft et al., 2002; Stricker, 1999). In some deuterostomes such as sea urchins and starfish, another Ca²⁺ release pathway, which is regulated by ryanodine receptors (RyR), also exists in oocytes or eggs (Stricker, 1999), although contribution of this mechanism to the initial Ca²⁺ wave is still controversial (Leckie et al., 2003; Kuo et al., 2000; Kuroda et al., 2001). In oocytes of nemertean worms (Stricker, 1996, 1999), bivalves (Deguchi and Morisawa, 1997, 2003), and echinuran worms (Stephano and Gould, 1997), all of which belong to protostomes, an initial Ca²⁺ increase just after fertilization is a centripetal Ca²⁺ wave from the entire cortex. This Ca²⁺ pattern, called “cortical flash”, appears to result from a Ca²⁺ influx regulated mainly by voltage-dependent Ca²⁺ channels on the plasma membrane (Deguchi and Morisawa, 2003; Deguchi et al., 1996; Stephano and Gould, 1997; Stricker, 1996). It is, therefore, possible that different sources and mechanisms are used in deuterostomes and protostomes to generate an initial main Ca²⁺ increase at fertilization. However, it is known that small cortical flash precedes the main Ca²⁺ wave in some deuterostomes such as sea urchins and starfish (Carroll et al., 1997; Shen and Buck, 1993). Conversely, no such cortical flash is detected in the initial phase of fertilization in protostomes such as the annelidan worm Chaetopterus (Eckberg and Miller, 1995). Furthermore, in cases where multiple Ca²⁺ spikes (Ca²⁺ oscillations) occur following the initial Ca²⁺ increase, each of the later Ca²⁺ spikes takes the form of a propagating Ca²⁺ wave and is basically regulated by an IP₃R-mediated Ca²⁺ release in many protostomes as well as in deuterostomes (Sardet et al., 1998; Stricker, 1999). Thus, there is another view that characteristics of fertilization-induced Ca²⁺ signals are more or less conserved throughout the animal kingdom (Stricker, 1999; Thomas et al., 1998).

Hydrozoan jellyfish belong to the evolutionarily old diploblastic phylum, Cnidaria, which is thought to have branched before the divergence of higher triploblastic animals including deuterostomes and protostomes (Collins, 2002; Gilbert, 2003; Philippe et al., 1994). Spawned eggs of hydrozoans are arrested and fertilized at pronuclear stage; it is known that immature or maturing oocytes cannot accept sperm until the completion of two meiotic divisions (Freeman, 1987). Freeman and Ridgway (1993) monitored intracellular Ca²⁺ changes at fertilization in two hydrozoan species (Mitrocomella and Phialidium), using the unique features of the eggs that contain endogenous Ca²⁺-sensitive photoproteins (Freeman and Ridgway, 1987). Their study demonstrated that fertilized eggs of the two species exhibit a single monotonous Ca²⁺ increase lasting for a few minutes. However, precise information about the spatiotemporal properties of Ca²⁺ increases at fertilization has not yet been obtained from any hydrozoan species (Stricker, 1999).

In the hydrozoan Phialidium, application of Ca²⁺-ionophore in Ca²⁺-free seawater (Freeman and Ridgway, 1987) and direct injection of IP₃ (Freeman and Ridgway, 1991) both cause a Ca²⁺ increase in unfertilized eggs. In contrast, application of excess K⁺ seawater, which is known to cause membrane depolarization and a resulting Ca²⁺ influx via voltage-dependent Ca²⁺ channels, induces no Ca²⁺ change in unfertilized eggs, although the same treatment is effective in triggering a Ca²⁺ increase in early embryos (Freeman and Ridgway, 1987, 1993). Consistent with the data, an electrophysiological analysis shows that a current injection into early embryos, but not into unfertilized eggs, can cause a Ca²⁺-dependent action potential (Freeman and Ridgway, 1987). In a different hydrozoan species, Hydractinia, it is known that only a slight membrane depolarization, which cannot contribute to electrical polyspermy block, takes place in fertilized eggs (Berg et al., 1986). The indirect evidence described above supports the importance of Ca²⁺ release from internal stores, rather than external Ca²⁺ influx, in fertilized hydrozoan eggs. However, detailed analysis of the Ca²⁺-mobilizing mechanisms involved in eggs and used at fertilization has not yet been done in hydrozoans. Here, we provide the first spatiotemporal information about intracellular Ca²⁺ changes in fertilized hydrozoan eggs; a Ca²⁺ increase starts from the sperm entry site, which is situated at the animal pole, and propagates to its antipode in a wave-like fashion. Our data support the conclusion that a Ca²⁺ release from IP₃-sensitive stores is the primary mechanism responsible for the Ca²⁺ increases at fertilization, as is the case for most of the higher triploblastic animals.

Materials and methods

Biological materials

Four hydrozoan species, Cladonema pacificum (Order Anthomedusae), Cytaeis uchidae (Order Anthomedusae), Clytia sp. (Order Leptomedusae), and Gonionema vertens (Order Limnomedusae), were used in this study. The genus Phialidium mentioned above has been considered as a synonym of Clytia (e.g., Inouye and Tsuji, 1993), but this genus name is also used in this paper, according to the original descriptions in the references cited. Sexually mature medusae of Cladonema and Gonionema, which are
commonly found in the area where *Sargassum* and other algae are abundant (Hirai and Kakinuma, 1957; Kakinuma, 1971), were collected in Sendai Bay (Miyagi Prefecture) during their breeding season from June to July. Colonies of *Cytaeis* living on the shells of the gastropod *Niotha livescens* (Hirai and Kakinuma, 1973) and colonies of *Clytia* sticking to the blades of the eelgrass *Zostera marina* (Nishihira, 1968) were collected in Mutsu Bay (Aomori Prefecture) from July to September and in October, respectively. Most of the collected colonies of the two species had many medusa buds just before liberation; the newly-liberated medusae were obtained within a few weeks of collection. The methods of regulation of life cycle have been reported in *Cladonema* (Hirai and Kakinuma, 1957), *Phialidium* (*Cllytia*) (Roosen-Runge, 1970), and *Gonionema* (Kakinuma, 1971). Essentially according to the methods, we obtained the second and third generations of the medusae even in the off-season. In *Cytaeis*, it is known that the colonies separated from the host gastropod tend to produce medusae within a few months (Hirai and Kakinuma, 1973). We maintained some strains of *Cytaeis* colonies “asexually” by repetitive transplantation to plastic culture dishes to obtain medusae throughout the year.

The newly-liberated, growing, and adult medusae of the four species were placed into suitably-sized plastic containers containing filtered seawater (FSW) and maintained at 18–23°C. *Artemia* nauplii (brine shrimp larvae) were used as food and supplied to the medusae almost every day. Within several hours of feeding, the medusae were transferred into fresh seawater. Under these culture conditions, the newly-liberated medusae began to spawn eggs or sperm within 2 weeks (*Cytaeis* and *Clytia*), 1 month (*Cladonema*), or 1 and 1/2 months (*Gonionema*). In all of the four species, sexually mature medusae survived for 1–3 months, during which they released eggs or sperm almost every day according to light–dark cycles. Before induction of spawning, female medusae were placed individually in holes of a 6-well or 12-well plastic culture plate, whereas male medusae were grouped together in much smaller places to obtain concentrated sperm suspensions. In *Cladonema*, the medusae that had been maintained under constant light were placed in the dark for 25–35 min; spawning began to occur within 45 min of the beginning of the dark stimulation. The same procedure was applied for *Gonionema*, although the time required for the medusae to initiate spawning was longer (~60 min). In *Cytaeis* and *Clytia*, in contrast, exposure to light following a sufficient dark period was used as a trigger for spawning. Mature eggs or sperm of *Cytaeis* and *Clytia* were usually released at 40–50 min and at 80–110 min, respectively, after the light stimulation. In all species, only freshly obtained eggs (within 30 min of spawning) were used because of the relatively short-lived ability of the hydrozoan eggs to be fertilized normally (data not shown).

To evaluate the results obtained from hydrozoans, we also used sea urchin eggs, where spatiotemporal patterns and mechanisms of sperm- and agonist-induced intracellular Ca$^{2+}$ changes have been well described (McDougal et al., 2000; Runft et al., 2002; Stricker, 1999). The sea urchin *Hemicentrotus pulcherrimus* was collected in Mutsu Bay from January to February and the gametes were obtained by injecting 0.53 M KCl into the coelomic cavity.

### Ca$^{2+}$ imaging

All experiments described below were carried out at 20–23°C. The fluorescent Ca$^{2+}$ indicators Calcium Green-1 10-kDa dextran (CGD) and Fura-2 were purchased from Molecular Probes (Eugene, OR, USA) and prepared at 100–200 μM and 500 μM, respectively, in an injection buffer containing 100 mM K aspartate and 10 mM HEPES (pH 7.0). In some experiments, CGD was further supplemented with 100 mg/ml heparin (3 kDa, Sigma). Hydrozoan and sea urchin eggs bathed in FSW were pressure-injected with the dye-containing solution as described elsewhere (Deguchi and Osanai, 1994). Estimated concentrations of the injected chemicals in the cytoplasm ranged from 1 to 3% of the original concentrations in pipets.

One to several dye-injected eggs were introduced into a measurement chamber, where they were slightly compressed by two coverslips adhered with a double-stick tape. To prevent sperm from sticking to the coverslips, they were coated with 40–50 mg/ml bovine serum albumin (BSA; fraction V, Sigma) before use. The targeted eggs in the chamber were observed with an inverted epifluorescence microscope (TMD-300; Nikon, Tokyo, Japan). Fluorescence of CGD-injected eggs was detected using an excitation filter (470–490 nm), a dichroic mirror (510 nm), and an emission filter (520–560 nm). Fura-2-injected eggs were excited at 340 nm and 380 nm alternately, by changing the excitation filters every 1 s, and the emission fluorescence at 520–560 nm was collected.

Fluorescence images of eggs in a chamber were captured with a silicon-intensified target tube (SIT) camera (C-2400; Hamamatsu Photonics, Hamamatsu, Japan) and continuously recorded on videotape. The eggs were inseminated or stimulated with various agents after the steady level of fluorescence had been confirmed. For insemination, sperm suspension diluted with FSW was added to a chamber, yielding a final sperm concentration of 10$^5$–10$^6$ sperm/ml. Caffeine (Sigma) dissolved in FSW was externally added to a chamber, resulting in a final concentration of 25 mM. In some experiments, this drug was applied to eggs also by direct injection (see below).

$IP_3$ (Dojindo, Kumamoto, Japan), cyclic ADP-ribose (cADPR; Calbiochem, San Diego, CA, USA), and caffeine were prepared in an injection buffer described above and quantitatively injected into the peripheral region of a single egg, which had been injected with CGD alone or CGD and heparin. For the second and quantitative injection, a glass micropipet without an internal filament was filled with silicon oil and connected to a microinjector. Each injectant was aspirated from the tip of the pipet; the volume of the
injectant, which was calculated by measuring the diameter of droplet injected into silicon oil, was restricted to 0.4–0.5% of the total egg volume (~250 pl in Cladonema; ~500 pl in Cytaeis; ~1800 pl in Clytia; ~350 pl in Gonionema). Following insertion of the pipet into the targeted egg, bright-field observation using red light (~650 nm) was performed simultaneously with the fluorescence recording, during which the solution was ejected from the tip of the pipet by pressure. When the second injection was performed in FSW, the injectant was frequently prevented from entering the cytoplasm of the targeted egg. To ensure the successful injection, FSW in a measurement chamber was replaced by low Ca2+ seawater, a 1:19 mixture of FSW and Ca2+-free seawater (prepared according to Osanai, 1975), and the second injection and the accompanying fluorescence recording were performed in this medium.

**DNA staining**

After the fluorescence measurement, we checked whether each of the inseminated eggs underwent the first cleavage on schedule and, in some cases, whether it further developed into a planula larva. Alternatively, the number and position of sperm nuclei entering the egg were examined by DNA staining. In Cytaeis and Clytia, the targeted egg with CGD fluorescence was stained in vivo with 25 μM Hoechst 33342 (Sigma) for 2 min before or after Ca2+ measurement; translucent eggs of the two species permitted us to observe the fused sperm nucleus in addition to the female pronucleus without fixation. The observation of the stained egg was performed 2–10 min after the initiation of Ca2+ increase, which was defined as the time of fertilization (see below). In Cladonema and Gonionema, each egg used for the Ca2+ recording was recovered from a chamber 2–5 min after fertilization. The egg was fixed in 5% formaldehyde in FSW after removal of non-fused sperm by mouth-pipetting in Ca2+-, Mg2+-free seawater (Osanai, 1975) containing 2 mM EDTA. Following the fixation for at least 3 h, the egg was stained with 10 μM 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) in deionized water for 2 min. The stained egg was finally cleared by incubation with 100% glycerol and then observed.

**Data analysis**

Fluorescence images of the targeted egg recorded on videotape were converted into digital images and processed using NIH Image (a public domain image processing software for the Macintosh computer) as described previously (Deguchi and Morisawa, 2003; Deguchi et al., 2000). Background fluorescence intensities, which were determined from non-injected eggs, were subtracted from all fluorescence images prior to the calculations described below. To investigate the temporal Ca2+ pattern at fertilization in a CGD-injected egg, fluorescence intensities (F) calculated in the whole region (two thirds of egg diameter) were normalized by dividing them by the resting value (F0) obtained from the images just before the first detectable Ca2+ increase following insemination (e.g., Fig. 1). To investigate the detailed spatiotemporal character-
Fig. 2. Ca\(^{2+}\) dynamics during the rising phase of initial Ca\(^{2+}\) increase just after fertilization in eggs of Cytaeis (A), Cladonema (B), Clytia (C), and Gonionema (D). The eggs for B, C, and D are identical to those for Figs. 1B, C, and D, respectively, whereas the egg for A is different from that for Fig. 1A. Sequential fluorescence images acquired every 0.5 s (A, B, and D) or 1 s (C) were normalized by dividing them by the resting image just before a Ca\(^{2+}\) increase in a pixel-to-pixel manner, and expressed as pseudocolor images. Since the acquisition of fluorescence images was terminated after the achievement of initial Ca\(^{2+}\) peak in respective eggs, the subsequent Ca\(^{2+}\) changes (e.g., the second Ca\(^{2+}\) transient in Fig. 1D) are not seen in these images. Right panels in A and C show the positions of nuclei, which were detected by simultaneous staining with Hoechst 33342, in the targeted eggs. In either case, the wave initiation site corresponds to the animal pole, where a female pronucleus (white arrowhead) and a more condensed sperm nucleus (white arrow) are situated. In the egg for A, two polar bodies (yellow arrowheads) are also visible near the animal pole.
istics of initial Ca\(^{2+}\) increase just after fertilization or agonist stimulation, sequential images were divided by the resting image in a pixel-to-pixel manner and the result was presented by sequential pseudocolor images of the egg (e.g., Fig. 2) or a chart in which F/F\(_0\) changes in three different points of the egg were calculated separately (e.g., Figs. 3 and 4). In a Fura-2-injected egg, fluorescence intensities at the excitation wavelengths at 340 nm (F340) and at 380 nm (F380) were measured in the whole region and the ratio values (F340/F380) every 2 s were determined.

**Results**

**Temporal patterns of intracellular Ca\(^{2+}\) changes at fertilization**

Unfertilized eggs obtained by natural spawning measured 100–110 \(\mu\)m in diameter in *Cytaeis*, 70–80 \(\mu\)m in *Cladonema*, 140–160 \(\mu\)m in *Clytia*, and 85–95 \(\mu\)m in *Gonionema*. None of these eggs had apparent autofluorescence interfering with the fluorescence measurements (data not shown). We first investigated changes in intracellular Ca\(^{2+}\), expressed as the normalized fluorescence value F/F\(_0\), at normal fertilization in CGD-injected eggs. Within several minutes of insemination, intracellular Ca\(^{2+}\), which had been maintained at a steady level, began to increase in eggs of all four species examined (Fig. 1). In this study, the time of the first detectable Ca\(^{2+}\) increase (zero time) was regarded as the time of fertilization. Following the achievement of the peak value, the elevated Ca\(^{2+}\) gradually declined toward the resting level without an additional Ca\(^{2+}\) spike in fertilized *Cytaeis* (6/6 cases; Fig. 1A) and *Clytia* eggs (6/6; Fig. 1C). A similar monotonic Ca\(^{2+}\) increase at fertilization was observed in *Cladonema* eggs, although there were always small fluctuations in Ca\(^{2+}\) level in the initial phase (6/6; Fig. 1B). In fertilized *Gonionema* eggs, a few sharp Ca\(^{2+}\) spikes occurred before the subsequent plateau phase (11/11; Fig. 1D). In spite of the small differences in the initial Ca\(^{2+}\) patterns, the increased Ca\(^{2+}\) returned nearly to the original levels at similar times (7–10 min after fertilization) in the four species (Fig. 1). The eggs that had displayed such Ca\(^{2+}\) changes underwent the first cleavage on schedule (at ~1 h after fertilization in *Cytaeis*, *Cladonema*, and *Clytia* and at ~1.5 h in *Gonionema*). Some of the embryos were retrieved from measurement chambers to follow the subsequent development; in these cases, most of them (3/6 in *Cytaeis*, 5/6 in *Cladonema*, 4/4 in *Clytia*, and 7/8 in *Gonionema*) developed to planula larvae by the next day.

**Spatial patterns of initial Ca\(^{2+}\) increase at fertilization**

In all of the four hydrozoan species tested, the rising phase of the initial Ca\(^{2+}\) increase just after fertilization took the form of a wave, which started at a single site of egg cortex and propagated to its antipode (Fig. 2). To analyze the spatial characteristics of the Ca\(^{2+}\) wave in more detail,
changes in F/F₀ were calculated separately in three points of the egg: the wave initiation site, the center, and the antipode where the increased Ca²⁺ finally arrived (Fig. 3A, inset). The peak F/F₀ values in the three points were not significantly different in *Cytaeis* (Fig. 3A and Table 1), *Cladonema* (Fig. 3B and Table 1), and *Gonionema* (Fig. 3D and Table 1), indicating that the Ca²⁺ wave propagates without considerable attenuation in these species. In *Clytia*,

Table 1  
Characteristics of Ca²⁺ changes at fertilization in hydrozoan and sea urchin eggs

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak amplitude (F/F₀)ᵃ</th>
<th>Wave velocity (µm/s)ᵇ</th>
<th>Time to peak (s)ᶜ</th>
<th>No. of eggs examined</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cytaeis uchidae</em></td>
<td>1.48 ± 0.07</td>
<td>5.6 ± 0.4</td>
<td>32.4 ± 2.5</td>
<td>6</td>
</tr>
<tr>
<td><em>Cladonema pacificum</em></td>
<td>1.96 ± 0.09</td>
<td>5.9 ± 0.2</td>
<td>15.7 ± 1.0</td>
<td>6</td>
</tr>
<tr>
<td><em>Clytia</em></td>
<td>1.59 ± 0.08</td>
<td>4.2 ± 0.5</td>
<td>53.0 ± 3.6</td>
<td>6</td>
</tr>
<tr>
<td><em>Gonionema vertens</em></td>
<td>2.04 ± 0.08</td>
<td>4.7 ± 0.3</td>
<td>20.6 ± 0.9</td>
<td>11</td>
</tr>
<tr>
<td><em>Hemicentrotus pulcherrimus</em> (sea urchin)</td>
<td>2.88 ± 0.07</td>
<td>4.5 ± 0.5</td>
<td>36.6 ± 1.6</td>
<td>7</td>
</tr>
</tbody>
</table>

Note. Each egg was injected with CGD and then inseminated.

ᵃ Peak value of F/F₀ in the wave initiation site, the center, and the antipode during an initial Ca²⁺ increase at fertilization. Mean ± SEM.
ᵇ Wave propagating velocity calculated from the difference in the time when half of the peak F/F₀ value was attained and the distance between the wave initiation site and the antipode. Mean ± SEM.
ᶜ Time required from the first detectable Ca²⁺ increase to the achievement of peak in the whole egg. Mean ± SEM.
in contrast, the peak heights tended to decrease gradually as the Ca\(^{2+}\) wave propagated (Fig. 3C and Table 1). The wave propagation velocity was calculated from the difference in the time when half of the peak F/F\(_0\) value was attained and the distance between the wave initiation site and the antipode; the mean velocities were 4.2–5.9 \(\mu\)m/s in the four hydrozoans (Table 1). These values were comparable to those recorded from the fertilized eggs of the sea urchin Hemicentrotus pulcherrimus (~100 \(\mu\)m in diameter) under the same experimental conditions (4.5 \(\mu\)m/s; Table 1). The time required from the first detectable Ca\(^{2+}\) increase to the Ca\(^{2+}\) wave propagated (Fig. 3C and Table 1). The wave propagated from the site of the female pronucleus (data not shown). These facts led us to assume that the initiation point of Ca\(^{2+}\) wave in fertilized hydrozoan eggs corresponds to the animal pole. We performed the following experiments to verify this notion. In translucent eggs of Clytia and Cladonema, the site of female pronucleus was easily detected under bright-field observation. In some but not all CGD-injected eggs, the female pronucleus was also recognized as a brighter spot in fluorescent images. In these cases, the Ca\(^{2+}\) wave at fertilization invariably propagated from the site of the female pronucleus (data not shown). Staining with Hoechst 33342 further demonstrated that both a female pronucleus and a sperm nucleus (monochrome images in Figs. 2A and C) are positioned at the same place as the initiation site of Ca\(^{2+}\) wave in the two species.

Similar attempts to visualize the nuclei using Hoechst 33342 were unsuccessful in Cladonema and Gonionema, because of the colored and opaque features of their eggs. Nevertheless, a Ca\(^{2+}\) wave did originate at a brighter fluorescent spot expected for the site of female pronucleus in some CGD-injected eggs (data not shown). In addition, when an egg that had been used for the Ca\(^{2+}\) measurement at fertilization was fixed after removal of the surrounding sperm and then stained with a different DNA-specific dye, DAPI, a female pronucleus and a sperm nucleus were observed in the same cortical region of the egg under the fluorescent microscope (4/4 in Cladonema and 3/3 in Gonionema; data not shown). These observations may serve as indirect evidence that the animal pole is also the site of sperm–egg fusion and initiation of Ca\(^{2+}\) wave in the two species.

Comparison of peak Ca\(^{2+}\) heights in fertilized eggs

Peak F/F\(_0\) levels during the initial Ca\(^{2+}\) increase at fertilization were somewhat different in four hydrozoan species (Figs. 1–3 and Table 1). Among the species, Gonionema eggs scored the highest F/F\(_0\) values (~2.0; Table 1), but the values were still lower than those recorded in sea urchin eggs (~2.9; Table 1). These results led us to think that peak Ca\(^{2+}\) concentrations at fertilization were lower in hydrozoan eggs than in sea urchin eggs. To compare the absolute changes in intracellular Ca\(^{2+}\) levels in the initial phase of fertilization, we injected a ratiometric Ca\(^{2+}\) indicator, Fura-2, into unfertilized eggs of Cladonema, Gonionema, and Hemicentrotus, and measured F340/F380 values before and after fertilization. Similar resting levels of F340/F380 were obtained from unfertilized eggs of Cladonema (0.52 ± 0.04, n = 4), Gonionema (0.61 ± 0.04, n = 4), and Hemicentrotus (0.53 ± 0.02, n = 6). Following insemination, Fura-2-injected eggs of the two hydrozoans exhibited initial oscillatory increases in F340/F380 (data not shown); the first and second peak values were 2.75 ± 0.02 and 1.94 ± 0.06, respectively, in Cladonema (n = 4) and 3.29 ± 0.34 and 2.40 ± 0.33, respectively, in Gonionema (n = 4). Hemicentrotus eggs displayed a single increase in F340/F380 with a higher peak value of 4.67 ± 0.14 (n = 6). The rank order of the peak height in Fura-2-injected eggs was the same as that in CGD-injected eggs, consistent with the view of smaller Ca\(^{2+}\) increases in fertilized hydrozoan eggs.

Ca\(^{2+}\) changes induced by IP\(_3\)

Although the fact that injection of IP\(_3\) can cause a propagating Ca\(^{2+}\) wave in eggs of the hydrozoan Phialidium has been mentioned in a review article (Freeman and Ridgway, 1991), detailed information about the IP\(_3\)-induced Ca\(^{2+}\) changes has not yet been obtained in any hydrozoan species. In this series of experiments, we investigated characteristics of Ca\(^{2+}\) changes in those eggs which were injected with a certain amount of IP\(_3\) (0.4–0.5% of the total volume) at various concentrations. Injection of 12 \(\mu\)M IP\(_3\) into unfertilized eggs (resulting in a final intracellular concentration of 48–60 nM) immediately caused a fully propagating Ca\(^{2+}\) wave from the injection site in Clytaeis (5/5; Fig. 4A), Cladonema (5/5; Fig. 4B), Clytia (5/5; Fig. 4C), and Gonionema (4/4; Fig. 4E). The amplitudes of Ca\(^{2+}\) increase in the wave initiation site (= injection site), the center, and the opposite site were all comparable to those obtained from fertilized eggs in Clytaeis (compare Figs. 3A and 4A; see also Tables 1 and 2), Cladonema (Figs. 3B and 4B; Tables 1 and 2), and Gonionema (Figs. 3D and 4E; Tables 1 and 2). In the case of Clytia, however, peak Ca\(^{2+}\) levels were much higher in the eggs injected with 12 \(\mu\)M IP\(_3\) than in fertilized eggs (Figs. 3C and 4C; Tables 1 and 2). The mean velocities of Ca\(^{2+}\) wave in the eggs injected with 12 \(\mu\)M IP\(_3\) were 16–25 \(\mu\)m/s, which far exceeded the values at fertilization (Tables 1 and 2).

Effects of lower concentrations of IP\(_3\) were tested in Cladonema, Clytia, and Gonionema. Injection of 3 \(\mu\)M IP\(_3\)
Hemicentrotus pulcherrimus
Gonionema vertens
Clytia sp.

12 1.90 ± 0.20
0.97 ± 0.02
1.88 ± 0.07
1.78 ± 0.10
1.55 ± 0.05
0.98 ± 0.02
1.90 ± 0.18
1.66 ± 0.06
0.53 ± 0.04
1.13 ± 0.10
2.17 ± 0.05
2.08 ± 0.11
1.61 ± 0.16
0.99 ± 0.01
2.61 ± 0.15
2.78 ± 0.33
1.79 ± 0.03
1.00 ± 0.03
1.47 ± 0.11
1.00 ± 0.00
1.82 ± 0.03
1.59 ± 0.08
1.22 ± 0.02
1.02 ± 0.02
2.04 ± 0.14
1.50 ± 0.07
1.17 ± 0.07
1.00 ± 0.01
2.03 ± 0.06
1.73 ± 0.10
1.15 ± 0.04
1.00 ± 0.01
2.48 ± 0.15
1.89 ± 0.13
1.22 ± 0.05
1.01 ± 0.01
1.44 ± 0.12
1.02 ± 0.00
1.87 ± 0.06
1.47 ± 0.14
1.05 ± 0.02
1.03 ± 0.00
2.07 ± 0.16
1.34 ± 0.08
1.03 ± 0.02
1.00 ± 0.00
1.98 ± 0.08
1.83 ± 0.18
1.03 ± 0.01
1.26 ± 0.06
1.54 ± 0.12
1.07 ± 0.04
1.02 ± 0.01
16.2 ± 1.6
24.9 ± 2.4
14.2 ± 1.9
–
16.0 ± 2.1
12.9 ± 1.3
–
–
18.7 ± 4.7
12.9 ± 2.5
–
–
18.9 ± 3.2
16.5 ± 2.4
–
–

Table 2
Characteristics of Ca2+ changes induced by injection of IP3 in hydrozoan and sea urchin eggs

Species | Concentration of IP3 (μM) | Peak amplitude (F/F0) | Wave velocity (μm/s) | No. of eggs examined
--- | --- | --- | --- | ---
Cytaeis uchida | 12 | 1.63 ± 0.20 | 1.47 ± 0.11 | 16.2 ± 1.6 | 5
0 | 0.97 ± 0.02 | 1.00 ± 0.00 | – | 4
Cladonema pacificum | 12 | 1.88 ± 0.07 | 1.82 ± 0.03 | 24.9 ± 2.4 | 5
3 | 1.78 ± 0.10 | 1.59 ± 0.08 | 14.2 ± 1.9 | 5
0.7 | 1.55 ± 0.05 | 1.22 ± 0.02 | – | 4
0 | 0.98 ± 0.02 | 1.02 ± 0.02 | – | 5
Cytaeis uchida | 12 | 1.90 ± 0.18 | 2.04 ± 0.14 | 16.0 ± 2.1 | 5
3 | 1.66 ± 0.06 | 1.50 ± 0.07 | 12.9 ± 1.3 | 5
0.7 | 1.53 ± 0.04 | 1.17 ± 0.07 | 12.9 ± 1.3 | 5
0 | 1.13 ± 0.10 | 1.00 ± 0.01 | 12.9 ± 1.3 | 5
Gonionema vertens | 12 | 2.17 ± 0.05 | 2.03 ± 0.06 | 18.7 ± 4.7 | 4
3 | 2.08 ± 0.11 | 1.73 ± 0.10 | 12.9 ± 2.5 | 5
0.7 | 1.61 ± 0.16 | 1.15 ± 0.04 | 12.9 ± 2.5 | 5
0 | 0.99 ± 0.01 | 1.00 ± 0.01 | 12.9 ± 2.5 | 5
Hemicentrotus pulcherrimus | 12 | 2.61 ± 0.15 | 2.48 ± 0.15 | 18.9 ± 3.2 | 5
3 | 2.78 ± 0.33 | 1.89 ± 0.13 | 16.5 ± 2.4 | 3
0.7 | 1.79 ± 0.03 | 1.22 ± 0.05 | 16.5 ± 2.4 | 3
0 | 1.00 ± 0.03 | 1.01 ± 0.01 | 16.5 ± 2.4 | 3

Note: Each egg, which had been injected with CGD, was given a second injection of IP3.

a Pipet concentration. Injection volume was 0.4–0.5% of each egg volume.

b Peak value of F/F0 in the injection site, the center, and the antipode during a Ca2+ increase following injection. In the case of no Ca2+ increase, the maximal value during a period of 20 s following injection was used. Mean ± SEM.

c See Table 1.

d Injection buffer alone was injected.

(12–15 nM in eggs) also produced a propagating Ca2+ wave; the increased Ca2+ reached the antipode without considerable attenuation in some eggs (3/5 in Cladonema; 1/5 in Clytia; 4/5 in Gonionema; Fig. 4F), whereas the Ca2+ heights gradually declined with the wave propagation in the remaining eggs (2/5 in Cladonema; 4/5 in Clytia; 1/5 in Gonionema; Fig. 4D). As a consequence, the peak Ca2+ levels especially in the antipode of the injection site varied widely (Table 2). It is worth noting that the peak Ca2+ levels induced by 3 μM IP3 were similar to those recorded at fertilization in Clytia eggs (compare Figs. 3C and 4D; see also Tables 1 and 2). A much lower concentration of IP3 (0.7 μM in pipets; 2.8–3.5 nM in eggs) still caused a considerable Ca2+ increase at the injection site, although the increased Ca2+ failed to reach the antipode (Fig. 4G and Table 2). A similar dose–response relationship was observed in IP3-injected eggs of the sea urchin Hemicentrotus (Table 2). Finally, none of the hydrozoan and sea urchin eggs exhibited a detectable Ca2+ increase following injection of buffer alone, except for a slight increase at the injection site in 2 out of 5 Clytia eggs (Table 2), indicating that our method of injection adopted in this study has no significant influence on intracellular Ca2+ levels by itself.

Ca2+ changes induced by cADPR and caffeine

We next examined whether cADPR, which is thought to be an endogenous modulator of RyR (Galione et al., 1991; Takasawa et al., 1993), would cause a Ca2+ increase in hydrozoan eggs. It was difficult to evaluate the effect of injection of 200 μM or higher cADPR, since the injection site frequently burst as soon as a high Ca2+ increase had been induced (data not shown). This phenomenon was observed only when the extremely high concentrations of cADPR, but not of IP3, were used. Injection of 50 μM cADPR (200–250 nM in eggs) constantly produced a Ca2+ increase in eggs of Cytaeis (3/3; Fig. 5A), Cladonema (4/4; Fig. 5B), Clytia (4/4; Fig. 5C), and Gonionema (5/5; Fig. 5D). However, the Ca2+ increase took the form of an incomplete wave that did not propagate to the antipode of the injection site (Figs. 5A–D; Table 3). Injection of 12 μM cADPR (48–60 nM in eggs) still produced a localized Ca2+ increase of lower amplitude in some Gonionema eggs (3/7; Fig. 5E), whereas the same operation generated no Ca2+ increase in the remaining eggs (4/7; Fig. 5F). This concentration of cADPR was completely without effect in Cladonema eggs (Table 3). In contrast to the weak response of hydrozoan eggs, a fully propagating Ca2+ wave was induced by cADPR at the same concentrations (50 and 12 μM in pipets) in Hemicentrotus eggs (Table 3).

Caffeine is also a useful drug known to activate a RyR-mediated Ca2+ release (McCarron et al., 2003; Ozawa, 2001). Exposure of unfertilized eggs to 25 mM caffeine induced a considerable Ca2+ increase in Hemicentrotus eggs (3/3; Fig. 6, inset), but not in any hydrozoan eggs (0/4 in Cytaeis; 0/5 in Cladonema; 0/5 in Clytia; 0/4 in Gonionema; Fig. 6). Direct injection of this drug (25 mM in pipets) into Gonionema eggs was also ineffective in causing a Ca2+ increase (0/5; data not shown).
Effect of heparin on Ca\(^{2+}\) increases at fertilization

The data obtained from agonist experiments led us to assume that Ca\(^{2+}\) increases at fertilization are dependent mainly upon a Ca\(^{2+}\) release pathway from IP\(_3\)-sensitive stores in hydrozoan eggs. In the final series of experiments, we examined the effect of heparin, which is a potent competitive inhibitor of the IP\(_3\)-induced Ca\(^{2+}\) release (Ghosh et al., 1988), on Ca\(^{2+}\) changes in Gonionema eggs. Preinjection of 100 mg/ml heparin greatly suppressed an IP\(_3\)-induced Ca\(^{2+}\) increase; injection of 12 \(\mu\)M IP\(_3\) into heparin-preinjected eggs resulted in an incomplete Ca\(^{2+}\) wave (Fig. 7A and Table 4), the characteristics of which were similar to those induced by 0.7 \(\mu\)M (Fig. 4G and Table 2) rather than 12 \(\mu\)M IP\(_3\) (Fig. 4E and Table 2) in the eggs without heparin. In contrast, the spatiotemporal pattern of cADPR-induced Ca\(^{2+}\) increase was not severely affected by the injection of heparin (compare Figs. 5D and 7B; see also Tables 3 and 4).

Spatiotemporal characteristics of sperm-induced Ca\(^{2+}\) increases were significantly altered in the eggs injected with 100 mg/ml heparin (Figs. 7C–E). An initial Ca\(^{2+}\) increase took place at a single site of the egg cortex, probably

Table 3
Characteristics of Ca\(^{2+}\) changes induced by injection of cADPR in hydrozoan and sea urchin eggs

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration of cADPR ((\mu)M)(^a)</th>
<th>Peak amplitude (F/F(_0))(^b)</th>
<th>Wave velocity ((\mu)m/s)(^c)</th>
<th>No. of eggs examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injection site</td>
<td>Center</td>
<td>Antipode</td>
<td></td>
</tr>
<tr>
<td>Cytiai uchidae</td>
<td>50</td>
<td>1.52 ± 0.16</td>
<td>1.19 ± 0.05</td>
<td>1.02 ± 0.00</td>
</tr>
<tr>
<td>Cladonema pacificum</td>
<td>50</td>
<td>1.43 ± 0.05</td>
<td>1.21 ± 0.07</td>
<td>1.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.99 ± 0.01</td>
<td>0.99 ± 0.01</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>Clytia sp.</td>
<td>50</td>
<td>1.56 ± 0.11</td>
<td>1.14 ± 0.09</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>Gonionema vertens</td>
<td>50</td>
<td>1.74 ± 0.05</td>
<td>1.24 ± 0.07</td>
<td>1.11 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.18 ± 0.10</td>
<td>1.05 ± 0.01</td>
<td>1.05 ± 0.01</td>
</tr>
<tr>
<td>Hemicentrotus pulcherrimus (sea urchin)</td>
<td>50</td>
<td>2.82 ± 0.19</td>
<td>2.64 ± 0.25</td>
<td>2.56 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.25 ± 0.17</td>
<td>2.21 ± 0.19</td>
<td>2.22 ± 0.21</td>
</tr>
</tbody>
</table>

Note: Each egg, which had been injected with CGD, was given a second injection of cADPR.

\(^{a}\) See Tables 1 and 2.

\(^{b}\) See Tables 1 and 2.

\(^{c}\) See Tables 1 and 2.
corresponding to the sperm entry site, but the elevated Ca\textsuperscript{2+} did not propagate to the antipode immediately; the state of localized Ca\textsuperscript{2+} elevation lasted for up to 1 min (9/9; Figs. 7D and E). In most cases (7/9), the localized Ca\textsuperscript{2+} elevation initially ceased (e.g., the state between 50 and 70 s in Figs. 7D and E), although a global Ca\textsuperscript{2+} increase spreading throughout the egg occurred subsequently (90–110 s in Figs. 7D and 7E). The remaining 2 eggs exhibited a global Ca\textsuperscript{2+} increase following a long-lasting localized Ca\textsuperscript{2+} elevation without showing such a "resting state" (data not shown). In either case, the time required from the first detectable Ca\textsuperscript{2+} increase to the achievement of peak Ca\textsuperscript{2+} in the whole egg was similarly prolonged; the value of time to peak was 92 ± 6 s (ranging from 73 to 121 s; Table 4) in heparin-injected eggs, which was more than four times longer than that for normally fertilized eggs (21 ± 1 s, ranging from 16 to 26 s; Table 1). When about half of the heparin-injected and inseminated eggs were fixed for the staining with DAPI following the Ca\textsuperscript{2+} measurements, a single sperm nucleus was invariably detected at the animal pole of each egg (4/4; a monochrome image in Fig. 7E), suggesting the establishment of monospermic fertilization under these conditions. The remaining eggs were maintained without being fixed for at least 2 h, but none of them (0/5) underwent the first cleavage during the period.

**Discussion**

*Spatiotemporal characteristics of Ca\textsuperscript{2+} increases at fertilization in hydrozoan eggs*

The present study provides new information about the spatiotemporal patterns of Ca\textsuperscript{2+} increases in fertilized hydrozoan eggs. In all of the four hydrozoan species tested, an initiation point of Ca\textsuperscript{2+} increase was restricted to one cortical region and the increased Ca\textsuperscript{2+} propagated toward the antipode in a wave-like fashion. The wave propagating velocity was estimated to be 4.2–5.9 \textmu\text{m/s}; these values are comparable to those calculated in fertilized oocytes or eggs of sea urchins (~5 \textmu\text{m/s}, Mohri and Hamaguchi, 1991; ~4.5 \textmu\text{m/s}, this study), starfish (~5 \textmu\text{m/s}, Stricker et al., 1994), ascidians (~6 \textmu\text{m/s}, Yoshida et al., 1998), frogs (5–10 \textmu\text{m/s}, Fontanilla and Nuccitelli, 1998), and newts (~6 \textmu\text{m/s}, Yamamoto et al., 2001). As is the case for these higher animals, the wave initiation site was found to correspond to the sperm entry site in hydrozoan eggs. A unique feature in hydrozoans was that the sperm entry site was restricted to the animal pole where a female pronucleus is situated. Previous studies demonstrate that the localized surface area around the animal pole is both structurally (Yamashita, 1987) and chemically (Freeman, 1996) different from the other cortical region in unfertilized hydrozoan eggs, and that the sperm–egg fusion only occurs at this area (Freeman, 1990; Freeman, 1996; Freeman and Miller, 1982; Yamashita, 1987). It is known that the zygote nucleus remains in this eccentric position around the animal pole even after the nuclear fusion (Freeman, 1990).

As mentioned above, it seems unlikely that electrical polyspermy block functions at fertilization in hydrozoan eggs (see Introduction). Consistent with the notion, our present study demonstrates that a cortical flash Ca\textsuperscript{2+} pattern, which is frequently seen as a result of membrane depolarization (e.g., Stephano and Gould, 1997), is absent in fertilized hydrozoan eggs. It is shown that hydrozoan eggs treated with Ca\textsuperscript{2+} ionophore become unable to accept sperm (Freeman and Miller, 1982; Freeman and Ridgway, 1993). There is a possibility that “fast” polyspermy block might be established by a Ca\textsuperscript{2+} increase in hydrozoan eggs, since the
restricted area of sperm–egg fusion would be fully covered with the initial Ca\textsuperscript{2+} increase just after fertilization.

During the first Ca\textsuperscript{2+} increase at fertilization, peak values of F/F\textsubscript{0} in CGD-injected eggs were lower in four hydrozoan species (1.4–2.0) than in the sea urchin Hemicentrotus (~2.9). Similarly, peak values of F340/F380 ratio in Fura-2-injected eggs were also lower in Cladonema (2.75) and Gonionema (3.29) than in Hemicentrotus (4.67), in spite of comparable resting levels in the three species (0.52, 0.61, and 0.53). In Fura-2-loaded cells, intracellular Ca\textsuperscript{2+} concentrations can be calculated from the equation $K(R - R_0)/(R_S - R) = [\text{Ca}^{2+}]$, where $R_0$ is the ratio at 0 Ca\textsuperscript{2+} and $R_S$ is the ratio at saturating Ca\textsuperscript{2+} (Grynkiewicz et al., 1985). In Hemicentrotus eggs, resting and peak Ca\textsuperscript{2+} concentrations at fertilization have been estimated to be ~100 nM and ~3 μM, respectively (Mohri and Hamaguchi, 1991). Assuming that
the F340/F380 value of 0.53 corresponds to 100 nM and 4.67 to 3 μM and using the K value of 4.5 × 10⁻⁶ (Deguchi and Osanai, 1994), peak Ca²⁺ concentrations in fertilized Cladonema and Gonionema eggs would be 1.3 μM and 1.7 μM, respectively. Peak Ca²⁺ concentrations at fertilization may be much lower in Cytaeis and Clytia eggs, although this was not confirmed by the measurements with Fura-2. Nevertheless, these estimated values in hydrozoan eggs are within the range of those reported for other marine invertebrates (see Stricker, 1999). All of the above results suggest that the spatial patterns and the amplitudes of the intracellular Ca²⁺ increases at fertilization are well conserved between diploblastic hydrozoans and triploblastic animals.

In spite of the small differences in the initial Ca²⁺ changes, the following phase of Ca²⁺ elevation was rather similar in the four hydrozoans; the elevated Ca²⁺ declined gradually toward the original level without showing marked fluctuations in Ca²⁺ level. In this respect, the overall pattern of Ca²⁺ changes at fertilization in hydrozoan eggs was far from so-called Ca²⁺ oscillations reported in such animals as nemertean worms (Stricker, 1996; Stricker and Smythe, 2003), bivalves (Deguchi and Osanai, 1994), Chaetopterus (Eckberg and Miller, 1995), ascidians (Speksnijder et al., 1990; Yoshida et al., 1998), and mammals (Deguchi et al., 2000; Miyazaki et al., 1986) and should be classified into a “single Ca²⁺ increase” as observed in the echinuran worm Urechis (Stephano and Gould, 1997), the bivalve Macrura (Deguchi and Morisawa, 2003), starfish (Iwasaki et al., 2002; Stricker et al., 1994), amphibians (Fontanilla and Nuccitelli, 1998; Yamamoto et al., 2001), fish (Gilkey et al., 1978), and sea urchins (Shen and Buck, 1993; this study). Since sperm-induced Ca²⁺ oscillations are detected only in the animals where oocytes or eggs are fertilized at the first or second mehardes but not in the animals where fertilization takes place at the first prophase or pronuclear stage, there is a possibility that the single or oscillatory Ca²⁺ changes might be determined in a cell cycle-dependent manner (Levasseur and McDougall, 2000). However, a single Ca²⁺ increase does occur in fertilized starfish, amphibians, and fish oocytes in spite of the fertilization stage of the first or second mehardes. Another possibility is that the sperm-induced Ca²⁺ patterns are affected by differences in ER structure in oocytes or eggs; ER clusters are rapidly reorganized following fertilization in starfish (Jaffe and Terasaki, 1994) and frog oocytes (Terasaki et al., 2001) and sea urchin eggs (Jaffe and Terasaki, 1993), all of which display a single Ca²⁺ increase, whereas there is no obvious structural change in ER immediately after fertilization in nemertean worm (Stricker and Smythe, 2003; Stricker et al., 1998) and mouse oocytes (FitzHarris et al., 2003) with Ca²⁺ oscillations.

Mechanisms of Ca²⁺ increases involved in hydrozoan eggs and used at fertilization

In the present study, we clarified the precise concentration-dependent Ca²⁺ patterns induced by two Ca²⁺-mobilizing agents, IP₃ and cADPR, in hydrozoan eggs; these drugs were applied by quantitative injection performed in low Ca²⁺ seawater (see Materials and methods). Although the rate of successful injection was greatly reduced, similar Ca²⁺ patterns were also observed even in FSW (data not shown). Thus, it seems unlikely that lowering external Ca²⁺ concentration substantially influences the effects of these drugs on intracellular Ca²⁺ changes. A Ca²⁺ release pathway mediated by IP₃R in oocytes or eggs has unexceptionally been detected in higher triploblastic animals, even in the species where this mechanism does not appear to function at fertilization, such as Urechis (Stephano and Gould, 1997) and Macrura (Deguchi and Morisawa, 2003). In the four hydrozoan species tested, injection of 3 or 12 μM IP₃ (12–15 nM or 48–60 nM in eggs) induced a propagating Ca²⁺ wave equivalent to that observed at fertilization. A lower concentration of IP₃ (0.7 μM in pipets; 2.8–3.5 nM in eggs) still produced a considerable Ca²⁺ increase in the injection site, although the elevated Ca²⁺ did not reach the antipode, resulting in an incomplete Ca²⁺ wave. A similar dose–response relationship was observed in eggs of the sea urchin Hemicentrotus under the same experimental conditions. In addition, the final intracellular concentrations of IP₃ to cause a submaximal Ca²⁺ increase in our experiments (3–15 nM) are comparable to those reported in other animals including starfish (1–10

Table 4

<table>
<thead>
<tr>
<th>Stimulation(a)</th>
<th>Peak amplitude (F/F₀)(b)</th>
<th>Time to peak (s)(c)</th>
<th>No. of eggs examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initiation site</td>
<td>Center</td>
<td>Antipode</td>
</tr>
<tr>
<td>Fertilization</td>
<td>1.79 ± 0.07</td>
<td>1.58 ± 0.07</td>
<td>1.59 ± 0.08</td>
</tr>
<tr>
<td>12 μM IP₃</td>
<td>1.66 ± 0.09</td>
<td>1.30 ± 0.06</td>
<td>1.09 ± 0.02</td>
</tr>
<tr>
<td>50 μM cADPR</td>
<td>1.79 ± 0.10</td>
<td>1.28 ± 0.05</td>
<td>1.10 ± 0.03</td>
</tr>
</tbody>
</table>

*Note.* Each egg, which had been injected with CGD and heparin (pipet concentration: 100 mg/ml), was inseminated or given a second injection of IP₃ or cADPR.

a See Tables 1 and 2.
b See Tables 1 and 2.
c See Tables 1 and 2.
d Not determined.
developed IP3-induced Ca\textsuperscript{2+} release mechanism similar to and Levasseur, 1998), newts (~10 nM, Yamamoto et al., 2001), and mice (2.5–10 nM, Oda et al., 1999). It is, therefore, likely that hydrozoan eggs possess a well-developed IP3-induced Ca\textsuperscript{2+} release mechanism similar to that involved in oocytes or eggs of the higher triploblastic animals.

It has been demonstrated that cADPR stimulates a RyR-mediated Ca\textsuperscript{2+} release pathway and generates a substantial Ca\textsuperscript{2+} increase in starfish (Moccia et al., 2003) and bovine oocytes (Yue et al., 1995) as well as sea urchin eggs (Buck et al., 1994; Galione et al., 1993; Lee et al., 1993). In the above cases where cADPR is effective, caffeine is also effective in producing a Ca\textsuperscript{2+} increase, although this drug requires much higher concentrations than cADPR to induce a similar Ca\textsuperscript{2+} increase (Buck et al., 1994; Galione et al., 1991; Stricker et al., 1994; Yue et al., 1995). In this study, we found that a cADPR-induced Ca\textsuperscript{2+} increase does occur in all of the four hydrozoan species examined. However, a relatively high concentration of ADPR (50 μM in pipets, 200–250 nM in eggs) caused only an incomplete Ca\textsuperscript{2+} wave not propagating to the antipode of the injection site. In addition, a slight dilution of cADPR (12 μM in pipets, 48–60 nM in eggs) resulted in a considerable reduction or even a complete loss of the induction of Ca\textsuperscript{2+} increase. These results are not due to the quality of cADPR used or our handling of this drug, since expected results were obtained from Hemicentrotus eggs injected with the same cADPR. Similarly, caffeine caused a substantial Ca\textsuperscript{2+} increase in Hemicentrotus eggs, but not in hydrozoan eggs. We interpret these results as indicating that RyR-sensitive Ca\textsuperscript{2+} stores in hydrozoan eggs, if any, are less developed than those in sea urchin eggs, and that Ca\textsuperscript{2+} can be released from the stores only when a relatively high concentration of cADPR is present.

Heparin has been used as an inhibitor of IP\textsubscript{3}-induced Ca\textsuperscript{2+} release in various cell types including oocytes or eggs (reviewed by Miyazaki et al., 1993; Stricker, 1999), although some nonspecific effect of this drug on other Ca\textsuperscript{2+} release pathways has also been reported (e.g., Shen and Buck, 1993). In Gonionema eggs, Ca\textsuperscript{2+} changes at fertilization were severely affected by the presence of heparin; the first Ca\textsuperscript{2+} increase following insemination remained restricted to a localized region of egg cortex for up to 1 min, without propagating toward the antipode. A similar localized Ca\textsuperscript{2+} elevation has been observed in heparin-injected and inseminated frog oocytes (Fontanilla and Nuccitelli, 1998; Nuccitelli et al., 1993), where an IP\textsubscript{3}-R-mediated Ca\textsuperscript{2+} release system functions predominantly and RyR is absent (Iwao, 2000). In the case of Gonionema, however, the localized Ca\textsuperscript{2+} elevation was followed by a global Ca\textsuperscript{2+} increase spreading throughout the egg. It is known that several sperm can enter heparin-injected oocytes or eggs (Mohri et al., 1995; Nuccitelli et al., 1993; Whalley et al., 1992) and that such polyspermy overcomes an inhibitory effect of heparin on sperm-induced Ca\textsuperscript{2+} increases (Miyazaki et al., 1993; Mohri et al., 1995). However, such a situation may be ruled out in Gonionema, since only a single sperm nucleus could be detected in all of the heparin-injected and inseminated eggs. The possibility of acceleration of external Ca\textsuperscript{2+} influx in heparin-injected eggs, which is pointed out in bivalves (Deguchi et al., 1996), is also unlikely, since removal of external Ca\textsuperscript{2+} during the localized Ca\textsuperscript{2+} elevation did not suppress the subsequent global Ca\textsuperscript{2+} increase in Gonionema eggs (data not shown). In fertilized sea urchin eggs, an initial increase in intracellular IP\textsubscript{3} concentration just before the onset of Ca\textsuperscript{2+} wave is estimated to be 200–300 nM (Kuroda et al., 2001), whereas a much greater IP\textsubscript{3} production seems to occur within the subsequent 2 min (Kuroda et al., 2001; Lee and Shen, 1998). Assuming a similar large and persistent IP\textsubscript{3} increase during the initial few minutes of fertilization in Gonionema eggs and considering that heparin is a competitive inhibitor unable to block the effect of more concentrated IP\textsubscript{3} (e.g., permission of a Ca\textsuperscript{2+} increase around the IP\textsubscript{3} injection site; see Fig. 7A), the failure of complete inhibition of sperm-induced Ca\textsuperscript{2+} increases in heparin-injected eggs would be explained. There is a possibility that the use of more specific and potent antagonists of IP\textsubscript{3}-induced Ca\textsuperscript{2+} release, such as “IP\textsubscript{3} sponge” (Iwasaki et al., 2002), might inhibit the Ca\textsuperscript{2+} increases at fertilization more dramatically. In any case, weak effects of RyR agonists on Ca\textsuperscript{2+} changes eliminate the view that RyR mainly contributes to sperm-induced Ca\textsuperscript{2+} increases in hydrozoan eggs. Taken collectively, our present data strongly suggest that an IP\textsubscript{3}-R-mediated Ca\textsuperscript{2+} release is the chief mechanism functioning at fertilization in hydrozoan eggs, although we cannot completely rule out the possibility of supplementary role of RyR in the regulation of initial Ca\textsuperscript{2+} increase (Kuo et al., 2000) or later phase of Ca\textsuperscript{2+} elevation (Leckie et al., 2003).

IP\textsubscript{3} is produced by members of phospholipase C (PLC) enzyme family, the roles of which at fertilization have been well investigated in echinoderms, ascidians, frogs, and mammals (reviewed by Runft et al., 2002). In echinoderms and ascidians, several lines of evidence strongly suggest that activation of PLC\textsubscript{y} in oocytes or eggs is a prerequisite step leading to a Ca\textsuperscript{2+} increase at fertilization (Carroll et al., 1997, 1999; Giusti et al., 2000; Kinsey and Shen, 2000; Runft and Jaffe, 2000; Runft et al., 2004; Shearer et al., 1999). PLC\textsubscript{y} also seems to play an essential role in the initiation of Ca\textsuperscript{2+} increase in fertilized frog oocytes (Sato et al., 2000), although the mechanism underlying the activation of this enzyme is probably different from that involved in echinoderms and ascidians (Runft et al., 1999). In mammals, diffusion of a newly characterized PLC, PLC\textsubscript{z}, from sperm to oocyte is the most likely mechanism underlying the Ca\textsuperscript{2+} oscillations at fertilization (Kouchi et al., 2004; Saunders et al., 2002; Yoda et al., 2004), although functional PLC\textsubscript{y} is present in oocytes (Mehlmann et al., 1998; Sette et al., 1998, 2002) and might function at fertilization (Sette et al., 1998, 2002). Starfish, frog, and mammalian oocytes also possess functional PLC\textsubscript{z} (Runft et
al., 1999; Shilling et al., 1994; Williams et al., 1998), although this enzyme seems unlikely to contribute to Ca\(^{2+}\) increases at fertilization (Runft et al., 1999; Williams et al., 1998). It is, therefore, likely that the PLC subtypes used at fertilization, as well as the way of their activation, considerably varied among the higher triploblastic animals. Future studies in the evolutionarily old metazoans, hydrozoans, will be important to understand how IP\(_3\)-regulated Ca\(^{2+}\) release mechanisms working at fertilization have changed in the course of animal evolution. In addition, hydrozoan eggs offer a unique opportunity to investigate the mechanisms of initiation of Ca\(^{2+}\) wave at fertilization, since the initiation site is restricted to the animal pole, which can be predicted before fertilization in some species.

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