The Intracellular Calcium Increase at Fertilization in Urechis caupo Oocytes: Activation without Waves

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The intracellular Ca\(^{2+}\) (Ca\(_i\)) increase at fertilization of the marine worm Urechis caupo (Echiura) was studied with conventional and confocal epifluorescence microscopy in oocytes microinjected with calcium green dextran or dually labeled with the calcium-insensitive dye tetramethylrhodamine dextran. Calcium green fluorescence was also measured with a photomultiplier system while the oocyte membrane potential was recorded and manipulated. The results show that Ca\(_i\) rises simultaneously around the oocyte cortex and peaks slightly later in the nucleoplasm. The Ca\(_i\) rise coincides with the initiation of the fertilization potential and we conclude that it is due primarily to external Ca\(^{2+}\) entering through the voltage-gated Ca\(^{2+}\) action potential channels that open during the fertilization potential because: (1) current clamping the oocyte membrane potential to positive values in the absence of sperm produces a similar Ca\(_i\) increase, (2) external Ca\(^{2+}\) is required, (3) and the confocal images are consistent with this mechanism. External application of sperm acrosomal peptide (P23) also caused a Ca\(_i\) increase that was inhibited in the presence of CoCl\(_2\). Ca\(_i\) and pH\(_i\) (measured with BCECF dextran) were manipulated in experiments employing microinjection of BAPTA (to chelate Ca\(_i\)), external application of NH\(_4\)Cl (to increase pH\(_i\)) and CoCl\(_2\) (to block Ca\(^{2+}\) channels), and fertilization of eggs in pH 7 seawater (Ca\(_i\) increase without pH\(_i\) increase). The results showed that increases in both Ca\(_i\) and pH\(_i\) are required for GVBD; neither alone is sufficient. However, although nuclear and cytoplasmic Ca\(^{2+}\) levels tended to parallel each other in oocytes fertilized at pH 7, and during the initial Ca\(_i\) response in oocytes fertilized at pH 8, there was a disproportionate fluorescence increase in the nucleoplasm of the latter prior to GVBD which could not be explained by any artifact we tested, suggesting there may be a selective increase in nuclear Ca\(^{2+}\) associated with GVBD. Finally, electrophysiological experiments with BAPTA-injected oocytes showed that the opening of the fertilization potential Na\(^{+}\) channels was Ca\(^{2+}\)-independent, (although they did not close at the normal time). These and earlier results suggest that Urechis sperm may activate oocytes by interacting directly with the Na\(^{+}\) channels or associated receptors.

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INTRODUCTION

Increases in intracellular Ca\(^{2+}\) appear to be universal mediators of egg activation in animal species (reviewed by Jaffe, 1985; Nuccitelli, 1991; also see Freeman and Ridgway, 1993; Deguchi and Osanai, 1994a,b; Eckberg and Miller, 1995; Stricker, 1996). In fish, frog, mammalian, ascidian, echinoderm (references in Jaffe, 1991), and nemertean (Stricker, 1996) oocytes, one or more waves of intracellular Ca\(^{2+}\) release are initiated at the site of sperm penetration and sweep over the egg. In shrimp oocytes, Ca\(^{2+}\) waves are initiated at activation by Mg\(^{2+}\) instead of sperm (Lindsay and Clark, 1992). In the vertebrate and echinoderm species, one function of Ca\(^{2+}\) waves is to stimulate cortical vesicle exocytosis and help prevent polyspermy (e.g., Gilkey, 1981; Kline, 1988; Kline and Stewart-Savage, 1994; Whitaker and Steinhardt, 1985; Jaffe and Gould, 1985). In ascidian, nemertean, and shrimp eggs there is no cortical vesicle exocytosis associated with Ca\(^{2+}\) waves at fertilization or activation (Jaffe and Gould, 1985; Stricker, 1996; Lindsay and Clark, 1992); however, in ascidians cortical contraction waves reorganize cytoplasmic components for normal development (Roegiers et al., 1995). Cortical contraction waves also occur in nemertean oocytes, but their function is not yet known (Stricker, 1996). The precise function(s) of the Ca\(^{2+}\) wave in shrimp oocytes also has not been clarified (Lindsay and Clark, 1994).

At fertilization in Urechis, there is no wave of cortical granule exocytosis, the vitelline coat elevates irregularly beginning at many sites simultaneously, and no other wave-
like phenomena such as cortical contractions have been detected (Tyler, 1932; Gould-Somero and Holland, 1975). Furthermore, extracellular Ca$^{2+}$ is required for activation and there is a substantial net uptake of Ca$^{2+}$ during the first few minutes of activation (Johnston and Paul, 1977; Jaffe et al., 1979). These observations, together with the fact that Ca$^{2+}$ action potential channels open during the fertilization potential (Jaffe et al., 1979), suggest that the mechanism for increasing Ca during activation may be quite different in Urechis. Therefore, the present study was undertaken to determine the spatial and temporal characteristics of intracellular Ca$^{2+}$ (Ca) changes at fertilization and to test the hypothesis that the Ca$^{2+}$ required for activation enters primarily through the action potential channels.

Another important characteristic of oocyte activation in Urechis is the requirement for an increase in intracellular pH (Gould and Stephano, 1993; Stephano and Gould, 1997). When oocytes are inseminated in acidified (pH 7) seawater, the pH doesn't increase and activation fails to occur, although sperm penetrate, fertilization potentials are normal, and Ca$^{2+}$ uptake is the same as that at pH 8 (Paul, 1975; Johnston and Paul, 1977; Gould-Somero et al., 1979). However, direct measurements are required to determine whether Ca increases are really the same at pH 7 and pH 8. Therefore we measured Ca following insemination at pH 7 and performed additional experiments to determine whether either a pHi or a Ca increase alone was sufficient to activate oocytes.

Finally, we were interested in whether the Na$^{+}$ channels responsible for the fertilization potential (Jaffe et al., 1979) were opened by sperm independently of an increase in Ca. Since the fertilization potential is the first known response of the oocyte to sperm (Jaffe et al., 1979), this question is important for understanding the mechanism of the initial sperm interaction with the oocyte plasma membrane. These channels open locally at the site of sperm interaction (Gould-Somero, 1981), suggesting that they are not Ca$^{2+}$-dependent since Ca$^{2+}$ entry through the Ca$^{2+}$ action potential channels would be global, not local. However, an alternative possibility is that this global Ca$^{2+}$ increase is insufficient and the sperm induces an additional local Ca$^{2+}$ increase to open the Na$^{+}$ channels. Observations with the confocal microscope and electrophysiological experiments following microinjection with a Ca$^{2+}$ chelator were performed to distinguish between these alternatives.

**Materials and Methods**

**Animals and Gametes**

Adult worms were maintained at 15-16°C in a natural seawater aquarium with biological filter, and gametes were obtained as described previously (Gould, 1967). Oocytes were washed and stored in filtered (0.45 μm) natural seawater at 16°C and sperm were stored “dry” at 4°C.

**Microinjection**

Following one wash with Ca$^{2+}$-free artificial seawater (484 mM NaCl, 10 mM KCl, 27 mM MgCl$_2$, 29 mM MgSO$_4$, 2.4 mM NaHCO$_3$, 2 mM EGTA, pH 7) oocytes were resuspended in 0.75 mM glucose, 5 mM EGTA, pH 7, and then immediately recently-fused (with a hand centrifuge) and gently suspended in natural seawater, adjusted to pH 7 with HCl. The glucose-EGTA treatment softens the vitelline coat and was kept brief to avoid dissolving it completely, which made the oocytes fragile and prone to lysis. pH 7 solutions were used to inhibit artificial activation, which sometimes occurs after the glucose-EGTA treatment. After approximately 30 hr at pH 7 the oocytes were resuspended in pH 8 seawater, 2 mM Tris–HCl, and stored at 16°C until use. Oocytes were not damaged by this treatment; 100% activated and developed to larvae, and fertilization potentials were normal (e.g., Fig. 8).

Microinjections were performed using the methods described by Kiehart (1982). The microscope (American Optical) was equipped with a custom made hollow aluminum stage (CICESE, Ensenada) through which chilled (15°C) water flowed. The microinjection chamber was constructed with one thickness of Scotch double-stick tape and the coverslip fragment used for the shelf was slanted so the oocyte would be trapped in the front edge of the shelf rather than against the tape at the back of the shelf. The microinjection needle was advanced until it formed an indentation in the oocyte surface and then held there until the oocyte spontaneously allowed itself to be penetrated. Following injection of the oil drop at the tip of the pipet and the microinjection solution, positive pressure was maintained as the pipet was withdrawn. Injected volumes were <1% of the oocyte volume as measured by the oil drop method (Kiehart, 1982). The coverslip with the microinjection chamber attached was then pushed toward the center of the slide so the oocyte would not be so near the edge, and another coverslip fragment was added to cover the top of the chamber left exposed by this maneuver.

Fluorescent probes injected were the dextran (10 kDa) conjugates of calcium green-1, BCECF [bis(carboxyethyl)-carboxyfluorescein], and tetramethylrhodamine (Molecular Probes, Eugene OR), dissolved in 220 mM KCl, 2.4 mM MgCl$_2$, 27 mM MgSO$_4$, 2.4 mM NaHCO$_3$, 2 mM EGTA, pH 7 (Gould, 1967). Oocytes were washed and stored for 4-5 days at approximately 50-200 μM. Stock solutions (0.5 M) were made of BAPTA (0.5 M Na$_2$BAPTA) and Ca-BAPTA (CaCl$_2$ crystals added to 0.5 M K$_2$BAPTA for a final concentration of 0.5 M) were prepared and mixed in the proportion 10:1. IP$_3$ (D-myoinositol-1,4,5-triphosphate, hexapotassium salt, and NPE-caged IP$_3$ (Molecular Probes) were dissolved in 220 mM KCl (1 and 5 mM, respectively).

**Fluorescence Measurements by Conventional Microscopy**

Oocytes in microinjection chambers were transferred to the water-cooled stage of an Olympus BH2 epifluorescence upright microscope and observed with a 60× oil immersion objective (SPlanApo, N.A., 1.4). The light source was a 12-V 100-W halogen lamp connected to a 3- to 12-V, 10-A stabilized power supply (LEP Ltd., Hawthorne NY) and was operated at 6 V. Excitation wavelengths (485 nm for calcium green; 440 and 495 nm for BCECF) were selected with custom 10-nm bandpass filters (Omega Optical, Brattleboro VT) and emissions were monochromatic dichroic beam-splitter and barrier filter combinations (calcium green, 530 DF 25; BCECF, 535 DF 25). With BCECF, the excitation intensity at 495 nm was reduced with 0.3 or 0.6 neutral density filters to increase the relative signal at 440 nm so more reliable ratios could be calculated.
lateral. Measurements were made with a photomultiplier (R 928, Hamamatsu Corp., San Jose CA) mounted on the phototube of the microscope and recorded on a chart recorder. F/F₀ ratios were calculated for calcium green, where F₀ was the baseline fluorescence before adding sperm or other agents. For BCECF, the ratios of the emission intensities at ex 495/ex 440 nm were calculated. Sperm suspensions (diluted 1:100–1:10,000 in seawater at pH 7 or 8, 10 mM Tris–HCl) or other solutions were added to the front of the chamber while fluorescence was recorded. Other solutions included sperm peptide P23 (Gould and Stephano, 1991; 1 mg/ml in seawater + 10 mM Tris–HCl, pH 8); 50 mM NH₄Cl ± 10–20 mM CoCl₂, pH 8; BAPTA/CoBAPTA 10:1 (see Microinjection), 0.1 mM in seawater; 10–20 mM CoCl₂ in seawater, pH 8. Phenol red was sometimes added to the solutions to check for the efficiency of exchange in the chambers.

Standard curves for BCECF in oocyte lysates were performed with mixtures that contained 60% oocytes, 0.1 mM digitonin, 15 mM K+Hepes buffers, and 5 mM BAPTA/CoBAPTA pH or dH₂O. Drops were placed on slides and measurements were made with the Photon Technology International (PTI, Brunswick, NJ) RatioMaster Ratio Fluorescence Microscope Spectrometer system attached to an Olympus BH2 epifluorescence microscope. Excitation was with monochromatic light at 440 and 495 nm, and emission intensities (dichroic DC 515RLPXT02, emission filter 535DF35; Omega Optical) were recorded with the PTI Felix software.

Confocal Microscopy

Most of the studies were performed with the Bio-Rad MRC-600 system using a krypton–argon laser at 1 or 10% transmittance. The microscope (Olympus BH2) was equipped with a cooling stage set at about 16°C. Oocytes were viewed with a 60× oil immersion objective (SPlanapo, N.A. 1.4). A focal plane was selected to include a clear image of the germinal vesicle and scans were in either the "slow" (2-sec scans at 5-sec intervals) or "normal" (1-sec scans at 3-sec intervals) modes. The two-channel mode was used for the dual emission studies with calcium green and rhodamine, the laser intensity was 1%, and 1-sec scans were at 3-sec intervals. Average pixel values in boxes drawn in various regions of the oocyte were computed using the COMOS (Bio-Rad) software or NIH Image. With COMOS, pixel intensities ranged from 0 (low) to 255 (high) (Figs. 5A, 7A, and 7B, Table 2), whereas with NIH Image the range was 255 (low) to 0 (high) (Figs. 5B, 7C, and 7D). The pseudo-colored images (Fig. 4) were produced with Adobe Photo Shop.

Real time video images of calcium green dextran-injected oocytes were collected with a Noran Odyssey confocal microscope (Nikon Diaphot 200 with a 60× Planapo NA 1.4 oil immersion objective) using the 488-nm excitation and BP515 barrier filters. Images were stored on videotape and analyzed with NIH Image.

Caged IP₃ Release

With the conventional epifluorescence microscope, calcium green fluorescence was measured with the fluorescein filter set (ex BP490, DM500 + O515) and oocytes were illuminated with UV light by sliding in the bisbenzamide filter set (ex UG1, DM400 + L420) for 3 sec while recording continued. With the Bio-Rad MRC600, the caged IP₃ was released by firing a camara flash attachment (Sunpak, Olympus) close to the preparation between scans.

Simultaneous Electrophysiological and Fluorescence Measurements

Following microinjection, oocytes were transferred to 10 × 30 mm plastic petri dishes containing seawater and placed on a water-cooled stage affixed to an Olympus IMT-2 inverted microscope. Electrophysiological methods were modifications of those described in Jaffe et al. (1979). The dish containing the oocytes was connected to ground via an agarose–seawater bridge and oocytes were penetrated with 30- to 60-Mohm KCl-filled electrodes pulled from Kwik-fill glass capillaries (1B150, World Precision Instruments, New Haven CT) with a Sutter Instrument Co. P87 micropipet puller. Oocytes were immobilized against a strip of double-stick tape in the bottom of the dish and electrode penetration was facilitated by a brief (1–2 msec) increase in capacitance compensation. The amplifier (Axoclamp 2A, Axon Instruments Inc., Foster City CA) was operated in the bridge mode and the electrode resistance was electronically balanced before passing current pulses and checked afterward for drift. Current pulses were generated with a Grass SD9 (Quincy MA) stimulator connected to the amplifier. Current and membrane potential were monitored with an oscilloscope (Tektronix) and recorded on a chart recorder (Gould Brush 220).

Fluorescence measurements were with a photomultiplier system (PTI 710 Photomultiplier Detection System and D-104 Microscope Photometer) attached to the microscope. The oocyte was viewed with a 40× objective (ULWD CPlan40PL). Light from the Olympus high-pressure Hg lamp (HBO 100W/2) was passed through an IMT2-DMB dichroic mirror unit (BP490 nm exciter filter, DM500 nm dichroic filter, and AFC + 515 nm barrier filter) and emission data were collected as counts/sec at 1-sec intervals with the PTI Felix software. Event markers were used to synchronize the electrophysiological and photomultiplier tube recordings.

For electrophysiology without fluorescence measurements (e.g., Fig. 10), methods were the same as described above except that the oocytes were viewed at 50× on the water-cooled stage of a stereomicroscope (Wild Heerbrugg Instruments, Inc., Farmingdale NY).

RESULTS

Caᵢ Increase at Fertilization

Following insemination in normal (pH 8) seawater, Caᵢ rises rapidly to an initial peak followed by a plateau phase that lasts for several minutes (Figs. 1A and 1B). In experiments described below (Fig. 8 and related text) the Caᵢ rise began at the same time as the initiation of the fertilization potential. The rise time from base to peak ranged from 4 to 16 sec in different oocytes and fluorescence ratios (F₀/Fₚ) at the peak ranged from 1.11 to 1.74 (Fig. 2).

Fluorescence during the plateau was variable, but typically rose to values that were ≥ the initial peak fluorescence. The plateau phase was present in all of the oocytes that activated following insemination at pH₈ = 8 (n = 33). In two oocytes that failed to activate following insemination, the initial peak was normal but Caᵢ returned to preinsemination values and remained there. The decline in fluorescence marking the end of the plateau usually began between 5 and 7 min after fertilization and was correlated with...
FIG. 1. (A and B) Ca\textsubscript{i} increase after insemination at pH 8 in calcium green dextran microinjected oocytes which did (B) and did not (A; recording continued for 32 min) show Ca\textsubscript{i} oscillations after GVBD. The initial peak is relatively higher in (A) because the plane of focus was off center favoring the cortical signal. Both oocytes activated (GVBD). Oocytes were observed by phase contrast at breaks in the recordings (B, C, E, F). (C) Suppression of the Ca\textsubscript{i} increase in an oocyte microinjected with BAPTA. Sperm were added at the arrow and recording was continued for 25 min. The oocyte did not activate. (D) Absence of a pH\textsubscript{i} increase following insemination of an oocyte injected with BAPTA. FR is the emission (535 nm) ratio of excitation 495/440 nm (see Materials and Methods). After 28 min the germinal vesicle was still intact. (E) Ca\textsubscript{i} increase in an oocyte inseminated at pH 7. Irregular oscillations during the plateau phase were sometimes observed in oocytes fertilized at both pH 7 and 8. Recording was continued for 35 min, during which time the germinal vesicle remained intact. When the oocyte was perfused with pH 8 seawater and reinseminated, there was another Ca\textsubscript{i} increase followed by GVBD (not shown). (F) Ca\textsubscript{i} increase in an oocyte exposed to sperm peptide P23. The nuclear envelope was intact (discrete border) during the first transmitted light observation, but had faded (blurred border) by the second observation (arrow). F/F\textsubscript{0} of the initial rise was 1.57. The oocyte formed two polar bodies. (G) Inhibition of the P23-induced Ca\textsubscript{i} increase in the presence of 10 mM CoCl\textsubscript{2} (F/F\textsubscript{0} = 1.01). The oocyte did not activate. Following washing to remove the Co\textsuperscript{2+}, more P23 was added and the oocyte responded with a Ca\textsubscript{i} increase and activated (F). (H) Oocyte fertilized at pH 8 and then perfused with BAPTA (added at the arrows; see Materials and Methods). GVBD occurred. Measurements in this figure were made with a photomultiplier attached to the camera port of an Olympus upright epifluorescence microscope (see Materials and Methods). The time scale in A applies to all of the recordings except D.
Intracellular Calcium Increase at Fertilization

When oocytes were inseminated, there was no Ca\textsuperscript{i} increase and the oocytes failed to activate (3/3 cases; Fig. 1C). However, the pH\textsubscript{i} increase that normally occurs during egg activation was also inhibited (3/3 oocytes; Fig. 1D). Since a pH\textsubscript{i} increase is required for GVBD (Gould and Stephano, 1993; Stephano and Gould, 1997), these experiments are inconclusive with respect to the Ca\textsuperscript{i} requirement. Furthermore, the BCECF fluorescence ratios were low, suggesting that the oocytes might even be acidi®ed following BAPTA injection, a possibility that is not generally considered when BAPTA is introduced into cells. To rule out a possible artifactual effect of BAPTA on BCECF sensitivity, we performed standard curves in vitro with oocyte lysates buffered to different pH values (see Materials and Methods). However, BCECF was sensitive to pH in the lysates containing BAPTA (slope FR/pH = +9.47, r = 0.998, for the pH range 6.9±7.4; in fact fluorescence intensities in the BAPTA-containing lysates were even higher than those in control lysates for reasons that aren't clear). These results indicate that chelating intracellular Ca\textsuperscript{2+} may acidify oocyte cytoplasm in vivo.

Therefore we tried a different strategy to test for a Ca\textsuperscript{i} requirement without inhibiting the pH\textsubscript{i} rise. If oocytes are exposed to 50 mM NH\textsubscript{4}Cl, both pH\textsubscript{i} and Ca\textsuperscript{i} increase and they activate (Fig. 3A, Table 1). When the Ca\textsuperscript{i} increase was eliminated by adding CoCl\textsubscript{2} to the seawater (Fig. 3B), the pH\textsubscript{i} increase still occurred but the oocytes failed to activate (Table 1). Similar results were obtained in another experiment where fluorescence ratios were 3.65 ± 0.06 (5) with NH\textsubscript{4}Cl alone (98% GVBD) and 5.24 ± 0.05 (5) with NH\textsubscript{4}Cl + CoCl\textsubscript{2} (0% GVBD). Oocytes in NH\textsubscript{4}Cl + CoCl\textsubscript{2} were not damaged, since 100% activated (polar body formation) following dilution 30-fold with a sperm suspension. These results show that a Ca\textsuperscript{i} increase is necessary for GVBD; a pH\textsubscript{i} increase alone is not sufficient.

FIG. 2. Fluorescence ratios (F/F\textsubscript{o}) at the initial peak of the Ca\textsuperscript{i} increase in oocytes fertilized at pH 8 and 7. Although the mean F/F\textsubscript{o} was greater at pH 8 (1.45 ± 0.17, n = 29) than pH 7 (1.29 ± 0.13, n = 14; P < 0.01), all of the ratios at pH 7 overlapped, showing that an insufficient Ca\textsuperscript{i} increase doesn't explain the failure of oocytes to activate at pH 7. Data from both confocal and standard epifluorescence microscopy are included.

GVBD. (see Nuclear Calcium below). Following the plateau, Ca\textsuperscript{i} either oscillated (Figs. 1B and 7C) or remained steady (Fig. 1A). Among 18 oocytes observed through 15 min after the initial Ca\textsuperscript{i} rise, 50% showed at least one oscillation. Although it was difficult to score the experimental eggs for sperm penetration (the Kiehart chambers are not optimal for obtaining long-term development and oocytes tended to stick in them so they were difficult to remove), at least 4 of the eggs were monospermic since they developed to normal 2-cell or blastula stages. The general characteristics of their responses were similar to those of the rest of the eggs (e.g., peak F/F\textsubscript{o} values were 1.11, 1.31, 1.38, and 1.58).

The sperm acrosomal peptide P23 also induces a Ca\textsuperscript{i} increase (Fig. 1F). Similar results were obtained with 3 other oocytes (av F/F\textsubscript{o} = 1.55 ± 0.29; n = 4). Therefore, fusion with a sperm is not required.

Is the Ca\textsuperscript{i} Increase Required for GVBD?

We attempted to answer this question by microinjecting oocytes with the Ca\textsuperscript{2+} chelator BAPTA (10:1 K\textsubscript{4}BAPTA/Ca-BAPTA; see Materials and Methods), which should buffer Ca\textsuperscript{2+} to about 0.1 \mu M (Kline et al., 1986). (Assuming uniform distribution in an oocyte water volume of 440 pl, Jaffe et al., 1979, microinjection of 2.2 pl BAPTA solution would result in an internal concentration of 2.5 mM.) When these

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TABLE 1
A pH Increase without a Ca Increase Is Insufficient to Activate Oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activation (% GVBD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 50 mM NH₄Cl</td>
<td>98</td>
</tr>
<tr>
<td>50 mM NH₄Cl + 10 mM CoCl₂</td>
<td>0</td>
</tr>
<tr>
<td>10 mM CoCl₂</td>
<td>0</td>
</tr>
<tr>
<td>Untreated control</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ \text{(pH)} \text{ Fluorescence ratio} \]

\[ \text{(Ca)} \text{ Fluorescence increase (F/F₀)} \]

B. 50 mM NH₄Cl 4.56 ± 0.03 (6)
50 mM NH₄Cl + 20 mM CoCl₂ 4.33 ± 0.05 (6)
20 mM CoCl₂ 3.10 ± 0.05 (6)
Untreated 3.15 ± 0.05 (4)

C. 50 mM NH₄Cl 1.69 ± 0.12 (3)
50 mM NH₄Cl + 20 mM CoCl₂ 1.02, 0.99

\( ^{a} n > 100 \) for each sample.
\( ^{b} \) Ratios of the emission intensities (535 nm) at ex 495/ex 440 nm.

**Is a Ca Increase Sufficient for Activation?**

As mentioned in the Introduction, the fact that oocytes inseminated in pH 7 seawater fail to activate despite normal Ca²⁺ uptake strongly suggests that the Ca rise is insufficient for activation. However, direct measurements are required to confirm that the Ca rise at pH 7 is indeed the same as that at pH 8. Results are shown in Figs. 1E and 2. Although F/F₀ values tended to be lower in pH 7 inseminated oocytes than in the pH 8 inseminated oocytes, all of the values overlapped (Fig. 2). Plateau phases were also observed in pH 7 inseminated oocytes (see below). None of the oocytes activated, although the formation of fertilization cones showed they were fertilized. Fluorescence did not increase in control oocytes that were kept at pH 7 without insemination. For example, F/F₀ in one oocyte scanned every 5 sec for over 16 min gradually rose to only 1.02. These results show that the Ca increase alone does not activate oocytes. From all of the available data, the conclusion is clear: increases in both pH, and Ca are required for GVBD in Urechis.

**Are There Ca Waves?**

This question was addressed with confocal microscopy. The first series of experiments was performed on a Bio-Rad MRC6000 (see Materials and Methods). Oocytes were focused to obtain optical slices approximately through the center, including the germinal vesicle, and then scanned every 3 or 5 sec. Unfertilized eggs were scanned several times and then sperm were added to the chamber while scanning continued. The first detectable signal was a cortical flash that occurred simultaneously around the entire oocyte; then fluorescence rapidly spread through the cytoplasm into the germinal vesicle (Fig. 4). The timing of fluorescence increases in various regions of the oocyte was quantified by computing the average pixel value within boxes, as shown in Fig. 5. These measurements confirmed that the fluorescence increase began simultaneously in the entire cortex (6/6 oocytes analyzed; Figs. 4 and 5A). These data were obtained with 1-sec scans every 3 or 5 sec, so a faster wave could have been missed. Therefore, additional experiments were performed with a Noran Odyssey confocal microscope and the video recordings were analyzed at 100-msec intervals. The results were the same: in 4/4 oocytes fluorescence rose simultaneously all around the oocyte cortex (Fig. 5B). Polar fertilizations could produce propagated waves arriving simultaneously around the equators of oocytes. However, the poles of the oocytes were usually slightly compressed between two coverslips in the Kiehart chambers (see Materials and Methods) and sperm were observed to arrive at the protruding periphery. Furthermore, the data include optical sections through fertilization sites (e.g., Fig. 5A). Therefore, we conclude that the initial Ca increase is not propagated as a wave from a localized site in the cortex.

**Is Ca Waves?**

Periodic slow Ca oscillations often occurred in fertilized eggs after the plateau phase (Figs. 1B and 7C) and were also encountered in a few spontaneously activating oocytes that had never been exposed to sperm (not shown). In neither case were there propagated waves: Ca rose simultaneously throughout the cortex and center of the oocytes, although it tended to peak slightly later in the center (not shown).

Local Ca increases were sometimes observed in the confocal images. An example from an oocyte inseminated at pH 7 is shown in Fig. 6. Forty seconds later, this oocyte showed a global cortical flash followed by a normal plateau phase. We wondered whether such local Ca increases might be produced by sperm that fail to fertilize the egg. To test this possibility we reinseminated an egg at pH 8, 24 min after the initial fertilization, which had produced a normal Ca response and GVBD. Identical local Ca increases were observed after reinsemination but not before, indicating that nonfertilizing sperm can produce these responses (see Discussion).

**Nuclear Calcium**

Ca began to rise in the nucleoplasm at virtually the same time as in the cortex. When scans were made every 3 or 5 sec, the first detectable rises in both regions were in the same scan in 10/12 oocytes fertilized at pH 7 and 8, and the nuclear increase was delayed by one 5-sec scan in the other 2. In the experiments with video recordings, fluorescence in the centers of the oocytes (probably in the nucleus) also started to rise at the same time as in the cortex (Fig.
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FIG. 4. Pseudo-colored confocal images of the fertilization-induced $\text{Ca}^{2+}$ increase scanned at 5-sec intervals (left to right and top to bottom). (A) Before sperm arrival; the calcium green dextran is spread throughout the cyto- and nucleoplasm but is excluded from yolk granules (dark blue circles). The large dark circle is the oil drop introduced during microinjection (see Materials and Methods) and a bleb of escaped cytoplasm marks the microinjection site at the top of the oocyte. (B) The fertilization response. $\text{Ca}^{2+}$ has increased simultaneously around the entire cortex and has begun to increase in the nucleoplasm. The slight indentation in the lower right half of the oocyte is caused by pressure against an adjacent noninjected oocyte; $\text{Ca}^{2+}$ appears to be less in this region. (C and D) Peak $\text{Ca}^{2+}$ levels are reached later in the nucleoplasm than in the cytoplasm and continue to rise even after cytoplasmic $\text{Ca}^{2+}$ is declining. The color scale is from 0 (dark blue) to 255 (red) pixel intensity.
FIG. 5. Timing of Ca\(^{2+}\) increases in different regions of the oocyte analyzed by confocal microscopy. (A) The initial Ca\(^{2+}\) rise in an oocyte fertilized at pH 8. The rise was detected simultaneously in the nucleoplasm (N) and three regions of the cortex (C: CM, middle, and CB, bottom); however, Ca\(^{2+}\) in the nucleoplasm rose more slowly and peaked later. The data are average pixel values within the boxes indicated, from 2-sec scans (Bio-Rad MRC600) repeated at 5-sec intervals. Region C was a sperm entry site since a penetration cone formed there later. (B) A Ca\(^{2+}\) rise analyzed at 100-msec intervals from the confocal video recording (Noran Odyssey) of an oocyte fertilized at pH 8. Ca\(^{2+}\) began to increase simultaneously throughout the oocyte (a), although it peaked later in the center (b). The letters refer to the boxes: center, top, left, right, bottom. (A) was analyzed with COMOS software (low−high pixel intensities 0−255) and (B) with NIH Image (low−high intensities 255−0) (see Materials and Methods).

However, Ca\(^{2+}\) rose more slowly in the nucleoplasm (Fig. 4); peak values were reached 3–9 sec after the Ca\(^{2+}\) maxima in the cortex (11/12 oocytes; in the exception the peak occurred in the same optical section, Fig. 5A). These data show that Ca\(^{2+}\) spreads rapidly from the entire cortex into the germinal vesicle, but rises more slowly in the nucleoplasm.

However, while F/F\(_0\) ratios in nucleus and cytoplasm were similar during the initial peak, there was a dramatic relative increase in the fluorescence of the nucleoplasm prior to germinal vesicle breakdown in oocytes fertilized at pH 8 (Fig. 7A) that did not occur in oocytes fertilized at pH 7 (Fig. 7B). Results from several experiments are summarized in Table 2. In activating oocytes (pH 8), fluorescence ratios (F/F\(_0\)) in the nucleoplasm rose to an average of about 1.33 times those in the cytoplasm, whereas at pH 7 they tended to remain the same (average 0.96; Table 2).

These data suggest there is a selective accumulation of Ca\(^{2+}\) in the nucleoplasm prior to GVBD. Alternatively, the increased nuclear fluorescence could be an artifact due to dye transport into the germinal vesicle during activation. To distinguish between these possibilities, oocytes were microinjected with both calcium green dextran and tetramethylrhodamine dextran, a Ca\(^{2+}\) and pH-insensitive fluorescent probe. Results are illustrated in Figs. 7C (raw data) and 7D (calcium green/rhodamine F/F\(_0\) ratios from a different oocyte). The dramatic increase in the calcium green signal within the nucleoplasm was not accompanied by a similar increase in rhodamine fluorescence. (The reason for the small increase in nuclear rhodamine fluorescence is not known; one possibility is that it becomes more concentrated as the nucleus shrinks slightly, but this remains to be investigated.) Ca\(_{\text{in}}\) continued to rise in the nucleoplasm after 4 min, when Ca\(_{\text{in}}\) leveled off. On the other hand, the initial Ca\(^{2+}\) increase was the same in both germinal vesicle and cytoplasm when corrected for differences in dye concentration (deltaFR\(_n\)/deltaFR\(_c\) = 1.00, 1.00, and 1.01 in three experiments). The data support the conclusion that Ca\(^{2+}\) diffuses passively from the cortex to the nucleoplasm during the initial transient in oocytes fertilized at both pH 7 and 8 and then selectively accumulates in the nucleoplasm prior to GVBD during activation at pH 8. The biological significance of this accumulation remains to be determined.

**Mechanism of the Ca\(_{\text{in}}\) Increase**

The fact that Ca\(_{\text{in}}\) rises simultaneously around the entire oocyte cortex supports the hypothesis that it is entering through the Ca\(^{2+}\) action potential channels. Experiments with the Ca\(^{2+}\) channel blocking agent Co\(^{2+}\) provide further support. When oocytes are exposed to CoCl\(_2\) + P23 (sperm don't undergo the acrosome reaction in the pres-
oocytes activated (GVBD), consistent with earlier observations that external Ca\textsuperscript{2+} is only required during the first minute after insemination (Gould and Stephano, 1989). Interestingly, Ca oscillations began to occur after 6–12 min in all 4 oocytes perfused with BAPTA as well as in 5/5 oocytes perfused with seawater containing 20 mM CoCl\textsubscript{2} after the Ca peak (not shown), suggesting that they might be a mechanism to compensate for insufficient Ca\textsuperscript{2+} uptake (see Discussion).

Since IP\textsubscript{3}-induced Ca\textsuperscript{2+} release from the endoplasmic reticulum will activate eggs in a variety of species (reviewed by Nuccitelli, 1991; Jaffe, 1996), we investigated whether Urechis oocytes could also be activated by increasing the concentration of IP\textsubscript{3} in the cytoplasm. However, microinjection of IP\textsubscript{3} at estimated internal concentrations of up to 20 \(\mu\)M failed to induce GVBD (9/10 oocytes). We confirmed that IP\textsubscript{3} was indeed causing Ca\textsuperscript{2+} to rise by monitoring fluorescence in oocytes microinjected with caged IP\textsubscript{3} (estimated internal concentration ca. 15 \(\mu\)M) and calcium green. Following release of the caged IP\textsubscript{3} by pulses of UV light (see Materials and Methods), Ca\textsuperscript{2+} transients with \(F / F_0\) ratios of up to 1.6 occurred simultaneously throughout the oocyte, but these decayed rapidly to baseline values within 30 sec. Therefore, IP\textsubscript{3} caused only short Ca\textsuperscript{2+} transients and failed to activate the oocytes. All of the above results show that the Ca\textsuperscript{2+} rise is produced by Ca\textsuperscript{2+} entering through the Ca\textsuperscript{2+} action potential channels, although a supplementary contribution from internal stores is not ruled out (see Discussion).

Can Current Pulses Activate Oocytes?

In previously published work, current pulses of up to 2 min in duration were not followed by oocyte activation (Jaffe et al., 1979; Gould and Stephano, 1987). Since the Ca\textsuperscript{2+} rise during the current pulses was similar in magnitude to the sperm-induced rise, it was of interest to determine if oocytes would be activated by prolonging the current clamp-induced Ca\textsuperscript{2+} rise. We found that oocytes were activated by current clamping them for 3 min (2/2 cases; average plateau potentials +27 and +18 mV, respectively), but not for 1 min (2/2 cases; average plateau potentials +24 mV). An oocyte clamped for 2 min (+25 mV) did activate (polar body formation) and an oocyte that failed to activate during the 15 min following a 1-min clamp activated after receiving a subsequent 2-min clamp (+23 mV). These results show that oocytes can be activated in the absence of sperm by inducing a prolonged Ca\textsuperscript{2+} entry through the Ca\textsuperscript{2+} action potential channels for 3 min and sometimes for 2 min. Since a pH\textsubscript{i} rise as well as the Ca\textsuperscript{2+} increase is required for activation, the proton release mechanism could be activated by the first Ca\textsuperscript{2+} increase, and we are currently investigating this possibility.

Ca\textsuperscript{2+} and the Fertilization Potential

The fact that Ca\textsuperscript{2+} rises simultaneously around the entire oocyte cortex during activation argues against a role in opening the fertilization potential Na\textsuperscript{+} channels, since these channels open locally at the site of sperm–oocyte contact.
FIG. 7.  
Ca$^{2+}$ increases in both cytoplasm and nucleus in oocytes fertilized at pH 8 (A; GVBD began at the arrow) and 7 (B; no GVBD) analyzed from 2-sec scans taken every 5 sec. $F/F_o$ at the initial peak in (A) was 1.49 in nucleoplasm and 1.59 in cytoplasm, but rose to 2.04 in the nucleus just before GVBD began at 4 min, when it was 1.61 in the cytoplasm. By contrast, at pH 7 (B) nuclear and cytoplasmic Ca$^{2+}$ rose in parallel; just before 8 min, $F/F_o$ was 1.8 in nucleoplasm and 2.2 in cytoplasm. (C) Dual confocal recording (see Materials and Methods) of an oocyte injected with both calcium green and tetramethylrhodamine, fertilized at pH 8. Scans were for 1 sec at 3-sec intervals. These data show that the apparent differences in Ca$^{2+}$ levels in the nucleus (CN) and cytoplasm (CC) are not artifacts due to dye compartmentalization, monitored by the Ca$^{2+}$-insensitive probe rhodamine (RN, RC) (see text). GVBD occurred shortly after 6 min (arrow), as indicated by a blurring of the nuclear border. A Ca$^{2+}$ oscillation is seen at 8–9 min. Average pixel intensities were calculated within boxes (not shown) as in (A) and (B). (D) Ratio data from another oocyte injected with calcium green/ 
tetramethylrhodamine, showing the proportionately greater increase in the nucleus. The ratio (C/R) of calcium green/
rhodamine fluorescence in nucleus (N) and cytoplasm (C) was calculated for each scan, then each C/R ratio was divided by the C/R value before the beginning of the Ca$^{2+}$ increase to obtain an $F/F_o$ value. The germinal vesicle border looked blurred at the first arrow and GVBD had definitely occurred by the second arrow.
TABLE 2
Relative Fluorescence Increases of Calcium Green in Nucleus and Cytoplasm of Oocytes Fertilized at pH 7 and 8

<table>
<thead>
<tr>
<th></th>
<th>Cyto</th>
<th>GV</th>
<th>GV/cyto</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Initial peak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8</td>
<td>1.42 ± 0.15</td>
<td>1.38 ± 0.12</td>
<td>0.97 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>1.26 ± 0.09</td>
<td>1.20 ± 0.10</td>
<td>0.95 ± 0.06</td>
</tr>
<tr>
<td>B. GV maximum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8</td>
<td>1.55 ± 0.24</td>
<td>2.01 ± 0.18</td>
<td>1.33 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>1.55 ± 0.48</td>
<td>1.43 ± 0.25</td>
<td>0.96 ± 0.17</td>
</tr>
</tbody>
</table>

*a n = 4 oocytes each at pH 7 and 8. The values shown are F/F₀ ratios (FR) (mean ± SD) for the cytoplasm and nucleus, where F₀ is the average pixel value before insemination and F is the value at the time of either the initial Ca²⁺ peak (A) or when the germinal vesicle fluorescence was maximum during the subsequent plateau phase (B). GV/cyto shows the average of FR₉/FRcyto for each of the four oocytes in the sample. NS, not significant (P > 0.05, Student’s t test); S, significant (P < 0.01).

FIG. 8. The Ca²⁺ increase produced by current clamping unfertilized oocytes to open the voltage-gated Ca²⁺ channels (A) is similar to the Ca²⁺ increase at the initiation of the fertilization potential (B) in the same oocyte. Fluorescence (top trace; cps, counts/sec), membrane potential (middle trace), and current (bottom trace) were recorded simultaneously (see Materials and Methods). Sperm were added about 1.5 min after the current pulse during the period (1.4 min) not included in the figure.

FIG. 9. Effect of perfusion with BAPTA after the initiation of the Ca²⁺ increase in oocytes fertilized at pH 8 (B). Perfusion was begun after the initial peak (P) and by 4 min Ca²⁺ was reduced to levels near those before insemination. Perfusion is inefficient in the microinjection chambers (see Materials and Methods), which probably explains the slow decline. Control oocytes (C) were from different oocyte batches which happened to have slightly lower peak values; in these Ca²⁺ remained high. GVBD occurred in all of the oocytes. Values shown are means ± SD; n = 4 in each group.

FIG. 10. Fertilization potentials in a BAPTA-injected oocyte (A) and in a control oocyte (B) injected with KCl only. The top traces (A, B) show the onset of the potential from the original chart record, and the bottom traces (A', B') are replots with a longer time scale. The fertilization potential Na⁺ channels opened in the BAPTA-injected oocyte but failed to close again (compare A' and B').

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insemination). In the control oocytes (n = 3), membrane potentials returned to negative values between 6 min 8 sec and 7 min 45 sec after the initial positive shift (Fig. 10B). These results suggest that one function of the Ca increase during activation is to activate a mechanism that will eventually close the Na channels. pH does not appear to be involved since fertilization potentials are normal in oocytes fertilized at pH 7 (Gould-Somero et al., 1979). It is interesting to note that prolonged fertilization potentials were also observed in oocytes inseminated in low Ca seawater (1 mM) where Ca uptake is reduced (Jaffe et al., 1979; Gould-Somero et al., 1979). How the Ca increase leads to Na channel closure remains to be determined.

**DISCUSSION**

**Ca Increases**

Our results show that sperm cause a simultaneous Ca increase around the entire cortex of the Urechis oocyte, followed by a sustained elevation (plateau), that declines during GVBD. A Ca increase of the same magnitude is induced by current clamping oocytes to open the Ca action potential channels. No Ca increase occurs when oocytes are exposed to sperm acrosomal peptide (P23) in the presence of the Ca channel blocker, Co, and when oocytes are perfused with CaSW containing BAPTA after the initial peak, Ca levels decrease almost to prefertilization values. These results provide strong evidence that all of the Ca required for activation may be entering through the Ca action potential channels that are open during the fertilization potential.

In fact, the general form of the Ca response, an initial peak followed by a plateau, is similar to the voltage profile during the fertilization potential. In electrical recordings there is an initial peak voltage due to the contribution of the Ca action potential channels superimposed on the Na-dependent fertilization potential, then the potential declines to a plateau as the Ca channels partially self-inactivate (Jaffe et al., 1979; self-inactivation is a common characteristic of voltage-dependent Ca channels, see Hille, 1992). Even the slightly greater Ca increase at pH 8 than at pH 7 (Fig. 2) is correlated with the slightly greater initial peak amplitude of fertilization potentials at pH 8 (+51 mV) than at pH 7 (+41 mV) (Gould-Somero et al., 1979). Both the Ca decrease at the end of the plateau phase and the voltage decrease at the end of the fertilization potential (Jaffe et al., 1979) occur at about the same time as GVBD, although precise correlations would require further study. Ca uptake measurements are also consistent with an extracellular source for the Ca increase. During the first 10 min after fertilization oocytes take up 0.24 pmol 44Ca/oocyte, efflux is low, and there is a net increase of 0.23 pmol Ca/oocyte as measured by atomic absorption spectrophotometry (Johnston and Paul, 1977). During the first 10 sec there is enough influx to raise Ca by 55 μM (0.024 pmol/440 pl estimated cell water volume; Johnston and Paul, 1977; Jaffe et al., 1979) in the absence of buffering and sequestration.

Although the primary source of Ca for activation in Urechis oocytes is extracellular, a supplemental contribution from internal stores is not ruled out. In Urechis the oocytes clearly do possess mechanisms for increasing Ca in addition to Ca action potential channels. In this study, Ca oscillations were observed in about 50% of the fertilized eggs and in all of the eggs that were perfused with BAPTA and CoCl after insemination, yet electrophysiological recordings in the present work and in hundreds of eggs observed previously (Jaffe et al., 1979; Gould-Somero et al., 1979) have never revealed any similar oscillations in membrane potential. Therefore, the oscillations must be due to intracellular Ca release. Since oscillations didn’t always occur following fertilization and always occurred when Ca uptake was reduced by perfusing the oocytes with BAPTA or CoCl, they may be a regulatory mechanism used to increase Ca when influx is insufficient. Our data also show that oocytes possess an IP3-inducible global Ca release mechanism, although we do not yet know when it is used.

In addition to the global Ca increases during the initial peak and plateau phases and subsequent oscillations, transient local Ca increases were sometimes observed in the cortical cytoplasm (Fig. 6). We propose that these are due to interactions with sperm (either unsuccessful sperm in the case of unactivated oocytes or supernumerary sperm in the case of previously activated oocytes) that produce local Ca entry. Supernumerary sperm produce small transient depolarizations in egg membrane potential (Gould-Somero et al., 1979). These could result from Ca entry through a nonspecific leak (e.g., produced by a local membrane distortion) or a transient opening of Na channels with an accompanying small Ca leak. Since similar depolarizations are induced by sperm acrosomal protein (Gould and Stephano, 1989), sperm membrane is not required. In some cases depolarization by supernumerary or unsuccessful sperm (or acrosomal protein) is sufficient to reach the voltage threshold for action potential channels (e.g., Gould and Stephano, 1989). When this happens, transient global Ca increases would be expected and were sometimes observed. Further studies with simultaneous confocal microscopy and electrophysiology will be necessary to clarify the mechanisms of these transient Ca increases.

**Ca, Action Potentials, and Fertilization Potentials in Other Species**

As mentioned in the Introduction, waves of intracellular Ca release have been observed in all animal species where the spatial characteristics of the Ca increases during egg activation have been studied. Thus Urechis is the first clear exception. Is Urechis alone? Although not yet confirmed, indirect evidence suggests other species may use the Urechis strategy to increase Ca during activation. The bi valves Spisula, Barnea, and Mactra all require extracellular
Ca²⁺ to be activated (GVBD) and are activated by raising extracellular K⁺, an effect that requires extracellular Ca²⁺ and is inhibited by the Ca²⁺ channel blocker D600 (reviewed by Jaffe, 1985; Gould and Stephano, 1989; also see Deguchi and Osanai, 1994b). In Urechis oocytes, raising the K⁺ concentration in seawater causes a positive shift in membrane potential that opens the Ca²⁺ action potential channels and activates the oocytes (Gould-Somero et al., 1979; Gould and Stephano, unpublished). It is interesting to note that Urechis, Mactra, Spisula, and Barnea oocytes are all in meiotic prophase at the time of fertilization, whereas fertilization in the other species mentioned occurs at meiotic metaphase I or II or in haploid eggs.

Although propagated waves occur subsequently, there are simultaneous “cortical flashes” at the initiation of the fertilization response in sea urchin (Shen and Buck, 1993), Cerebratulus (Stricker, 1996), and probably Mytilus (Deguchi and Osanai, 1994b); here, a simultaneous Ca²⁺ increase throughout the oocyte was observed by conventional epifluorescence microscopy. In sea urchins these were shown to be due to Ca²⁺ action potential channels by simultaneous electrophysiological recordings and confocal microscopy (McDougall et al., 1993). However, when this Ca²⁺ increase was eliminated (by Ca²⁺ channel blockers or 0CaSW), the subsequent propagated Ca²⁺ wave and egg activation still occurred (McDougall et al., 1993). Therefore, the primary function of the Ca²⁺ action potential appears to be electrical (potentiating a rapid positive shift in egg membrane potential; Jaffe, 1976; Jaffe and Gould, 1985), whereas in Urechis, the Ca²⁺ action potential contributes to both the polyspermy block (Gould-Somero et al., 1979) and oocyte activation.

The initial “flash” in Mytilus and Cerebratulus oocytes may also be produced by Ca²⁺ action potential channels and function primarily in polyspermy prevention. In both Cerebratulus and Mytilus, a flash is generated by high K⁺ seawater, and when eggs are inseminated in the presence of Ca²⁺ channel blocking agents, the flash is eliminated (Deguchi and Osanai, 1994a; Deguchi et al., 1996; Stricker, 1996) and, in the case of Cerebratulus, the eggs are polyspermic (Stricker, 1996); polyspermy was not scored in Mytilus; however, the eggs do possess an electrical polyspermy block with a large initial voltage shift, >+130 mV, that is probably amplified by Ca²⁺ influx (Togo et al., 1995).

Another difference between Urechis and other species in which this has been studied is with respect to the Ca²⁺ dependence of the fertilization potential. In Urechis, sperm open a patch of Na⁺ channels directly. However, in frogs, the fertilization potential Cl⁻ channels are Ca²⁺-dependent (Kline, 1988) and open in a wave around the entire oocyte circumference (Kline and Nuccitelli, 1985; Jaffe et al., 1985). The same is true for the Na⁺ channels that open during the later phase of the fertilization potential in sea urchins (Chambers, 1989; Swann et al., 1992), although the mechanism for the initial phase is still uncertain (Chambers, 1989; Creton and Jaffe, 1995; Jaffe, 1996). The fertilization potential Na⁺ channels are also Ca²⁺-dependent in Cerebratulus (Kline et al., 1986).

**Nuclear Calcium**

During the initial Ca²⁺ rise, Ca²⁺ levels in the germinal vesicle appeared to follow passively the increases in cytoplasm, although the rise time was slower and peak levels were reached 3–9 sec later. Similar kinetics, suggesting passive diffusion into the nucleus often with a slight delay in crossing the nuclear envelope, have been observed in a variety of somatic cells (e.g., Allbritton et al., 1994; O'Malley, 1994), sea urchin zygotes (Gillot and Whitaker, 1994), resting hamster oocytes (Shirakawa and Miyazaki, 1996), and mouse oocytes undergoing Ca²⁺ oscillations during spontaneous maturation in vitro (Carroll et al., 1994).

However, a dramatic difference between cytoplasm and nucleus developed in the Urechis oocytes fertilized at pH 8: there was a substantially greater accumulation of Ca²⁺ in the nucleoplasm that continued after cytoplasmic Ca²⁺ reached a plateau, peaked just before GVBD, and then dissipated as nuceloplasm and cytoplasm mixed following GVBD (Fig. 7). Is there a selective Ca²⁺ increase in the nucleus or is this an artifact?

Possible artifacts that could cause greater fluorescence increases in nucleus than cytoplasm despite equivalent Ca²⁺ activities include the following (see O'Malley, 1994, for an extensive discussion of artifacts in comparing nuclear and cytoplasmic Ca²⁺ levels): (1) Substantial autofluorescence in the cytoplasm: this would increase baseline fluorescence (F₀) so fluorescence increases (F/F₀) would be relatively less in cytoplasm than nucleus. (2) Dye sequestration into cytoplasmic organelles where it would be insensitive to Ca²⁺ increases in the surrounding cytoplasm. This would also raise F₀ with the same effect as in artifact (1). (3) Dye is more concentrated in the nucleus because there is more free water space, preferential binding to nucleoplasmic components, selective uptake, etc. (4) Because of differences in the nuclear “milieu,” the dye has a higher affinity for Ca²⁺ in nucleiplasm, so it produces more fluorescence for a given Ca²⁺ increase. Another possible artifact of whole cell imaging, mistaking fluorescence in perinuclear cytoplasm for nuclear fluorescence, was eliminated by the use of confocal microscopy. [Intrigued by Santella's (1996) claim that germinal vesicles contained no dye when they were manually extruded from Asterina oocytes previously microinjected with calcium green dextran, we tried this maneuver with a Urechis oocyte; however, as expected, the extruded germinal vesicle was fluorescent and then the dye slowly diffused away.]

In our experiments there was no detectable autofluorescence at the laser intensities employed, and we used dextran conjugates to minimize sequestration. Data are expressed as F/F₀ ratios, which corrects for differences in the amount of dye in the two compartments (artifact 3). An additional control for artifact (3) is to include a second probe that is not sensitive to Ca²⁺ and perform ratio measurements, as...
we did with tetramethylrhodamine dextran. As for artifact (4), the observations that nuclear fluorescence increases more or less in parallel with cytoplasmic fluorescence during the initial response at pH 8 and the entire response at pH 7 argue against this possibility. The putative difference in milieu would have to occur selectively in germinal vesicles that are about to disperse, but what could it be? We have some evidence that pH might also be higher in germinal vesicles before GVBD (Gould and Stephano, 1993), but calcium green dextran fluorescence is not increased by increasing pH (e.g., our experiments with NH₄Cl and CaCl₂).

Therefore, we conclude that Ca²⁺ may be selectively increased in the nucleoplasm prior to GVBD. There are many reports of higher Caₐ than Caₐ levels in a variety of somatic cells, but most are questionable because artifacts were not ruled out (see references in O'Malley, 1994; Albritton et al., 1994). We could find no studies that reported different Ca²⁺ levels in nucleus and cytoplasm where confocal microscopy, a ratiometric method, and dextran-conjugated dyes were all used to help avoid artifacts. Mechanisms for selectively increasing Caₐ include (1) release of Ca²⁺ into the nucleoplasm from stores in the nuclear envelope, where Ca²⁺ ATPases, as well as IP₃, IP₄, and CaDP-ribose receptors are known to be present (reviewed by Gerasimenko et al., 1996), (2) decreasing the affinity of nuclear Ca²⁺ buffers, and (3) closing nuclear pores prior to GVBD (also proposed by Al-Mohanna et al., 1994). It will be interesting to explore these possibilities.

Caₐ is required for nuclear envelope breakdown during mitosis of sea urchin and sand dollar embryos (Steinhardt and Alderton, 1988; Twigg et al., 1988; Silver, 1989) and many studies implicate Ca²⁺ binding proteins in regulation of nuclear events (e.g., references in Czubryt et al., 1996). However, it is clear in Urechis that GVBD will not occur unless pH rises as well. The respective roles of these two ions in the series of events leading to GVBD and meiosis reinitiation are currently under investigation.

How Does the Sperm Initiate Activation?

The fertilization potential, the first known response during oocyte activation (Jaffe et al., 1979), is produced by the local opening of Na⁺ channels at the site of sperm interaction (Gould-Somero, 1981). Sperm acrosomal protein and a derived peptide, P23, also open the Na⁺ channels and they rapidly close when the peptide is washed off (Gould et al., 1986; Gould and Stephano, 1991; Stephano and Gould, 1997). In fact, P23 can induce a complete activation including embryo formation under conditions where the maternal centrosomes remain active; Stephano and Gould, 1995, 1997.) Sperm (and lipid-free acrosomal protein) initiate fertilization potentials when fusion is presumably inhibited by clamping the oocyte membrane to positive potentials (Gould and Stephano, 1987). The present results show that Urechis sperm do not activate oocytes by injecting Ca²⁺ (e.g., as suggested by Créton and Jaffe, 1995) or "oscillogenes" (Swann and Lai, 1997) into them. We propose that development is initiated in Urechis when sperm acrosomal protein interacts with receptors on the external surface of the oocyte plasma membrane and that these receptors are the Na⁺ channels or associated molecules. Confirmation of this hypothesis awaits identification of the receptor(s).

ACKNOWLEDGMENTS

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