Cytoplasmic calcium and *Fucus* egg activation

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SUMMARY

Eggs of the marine brown alga, *Fucus serratus*, exhibit small transient elevations of cytosolic Ca²⁺ of variable magnitude, corresponding to the onset of the fertilization potential. Microinjection of Ca²⁺ buffers (BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)) at concentrations sufficient to block any global fertilization-associated Ca²⁺ elevation did not inhibit egg activation (monitored as exocytosis of cell wall) or subsequent development. However, egg activation could be inhibited with higher buffer concentrations. Br₂BAPTA (Kₐ = 1.6 µM) was a more effective inhibitor of egg activation than BAPTA (Kₐ = 0.17 µM). Localized microinjection of Ca²⁺ produced only localized cell wall exocytosis at the injection site. Eggs injected with Br₂BAPTA at intracellular concentrations, which blocked egg activation, exhibited prolonged fertilization potentials. ⁴⁵Ca²⁺ influx across the plasma membrane increased during fertilization. Our results show that a large transient global elevation of Ca²⁺ is not necessary for *Fucus* egg activation but rather a localized elevation to micromolar levels results, at least in part, from increased Ca²⁺ influx across the plasma membrane. This is needed for early fertilization events, including the generation of the fertilization potential and cell wall secretion.

Key words: *Fucus*, egg activation, cytoplasmic calcium, fura-2-dextran, BAPTA

INTRODUCTION

Egg activation represents the switching on of many cellular processes including protein synthesis and cell cycle (Epel, 1990; Whitaker and Patel, 1990) following fertilization of an egg by a sperm. The calcium theory of egg activation (Jaffe, 1983) proposes that an increase in free cytoplasmic Ca²⁺ concentration ([Ca²⁺]cyt) is responsible, at least in part, for signalling the development of the egg. The diverse animal systems so far investigated support this theory. These include eggs of the Medaka fish (Gilkey et al., 1978), sea urchin (Eisen et al., 1984), *Xenopus* (Busa and Nuccitelli, 1985), ascidians (Brownlee and Dale, 1990; Speksnijder et al., 1990), sand dollar (Hamaguchi and Hamaguchi, 1990) and hamster (Miyazaki et al., 1989). All of these eggs, upon fertilization, exhibit large transient increases of Ca²⁺ typically involving (at least in deuterosomes) a wave or waves of propagated Ca²⁺ release from internal stores (Jaffe, 1983; Whitaker and Swann, 1993; Berger, 1993) and a transient depolarization of the membrane potential (fertilization potential). Despite this body of data on animals, little is known of activation mechanisms in plant eggs.

*Fucus serratus* is a dioecious marine brown alga which releases eggs and motile sperm into the surrounding medium. The eggs are arrested as haploid gametes without a cell wall (Callow et al., 1985) following the post-meiotic mitosis of the primary oocyte (Evans et al., 1982; Farmer and Williams, 1898; McCully, 1968). Fertilization is followed by a sequence of activation events including the rapid onset of cell wall production, producing after a few hours, a differentiated polarized zygote with typical plant properties.

Recent work (Brawley, 1991; Taylor and Brownlee, 1993) has shown the occurrence of a fertilization potential in *Fucus* similar in many ways to that found in several animal eggs. A preliminary report (Robinson, 1990), using acquiror-loaded *F. vesiculosus* eggs showed that [Ca²⁺]cyt increased soon after the addition of sperm. However, this was not quantified and measurements were not made on single eggs. Here we investigate further the role of Ca²⁺ in *Fucus* egg activation using Ca²⁺-sensitive dextran-linked dyes to monitor Ca²⁺ in the cytoplasm for at least several hours after injection. We report that while elevated Ca²⁺ is required for the early events of egg activation, a large transient global [Ca²⁺]cyt increase is not observed and the Ca²⁺ elevation can be explained in terms of Ca²⁺ influx across the plasma membrane.

MATERIALS AND METHODS

Gametes

Male and female receptacles from freshly collected *Fucus serratus* plants were washed, blotted dry and stored at 4°C. Eggs were obtained by allowing female plants to spontaneously release oogonia in artificial sea water (ASW) (in mM: 450 NaCl; 10 KCl; 9 CaCl₂; 30 MgCl₂; 16 MgSO₄; 2 NaHCO₃; pH 7.9) at 18°C. Sperm were collected from drying fronds and activated in ASW (Taylor and Brownlee, 1993) unless otherwise stated. Eggs were settled onto coverslips, pre-rinsed with 0.01% poly-L-lysine. All experiments were carried out at 18°C.
After fertilization, zygotes were incubated under unidirectional light (50 μE m⁻² sec⁻¹) at 18°C in ASW.

Membrane potentials and ionophoresis

Microelectrodes were pulled from 1.2 mm filamented borosilicate glass (Clarke, Reading, UK) and dry bevelled (Kaila and Voipio, 1985; Taylor and Brownlee, 1993) to give a resistance of 10-20 MΩ if filled with 3 M KCl. Impalement into eggs was achieved by brief capacity over-compensation while the electrode was resting against the egg membrane (Taylor and Brownlee, 1993). Microelectrodes were back-filled with fura-2-dextran (10×10⁻³ M) or Calcium Green-dextran (10×10⁻⁴ M) (5 mM in 10 mM KCl). Eggs were injected using positive current pulses (1-2 nA, 200 ms, 4.5 Hz) or pressure (see below). All dyes were supplied by Molecular Probes Inc. (Eugene, Oregon, USA). Microelectrodes for dye injection were kept in place where possible to record membrane potential. Intracellular dye concentrations were estimated to be ≤30 μM based on comparative measurements from buffer droplets containing known concentrations of dye and comparisons with dye fluorescence (at the fura-2 isosbestic point) of eggs pressure microinjected with known dye concentrations (see below).

Ratio photometric Ca²⁺ measurements

Ca²⁺-dependent fura-2 fluorescence was monitored using dual wavelength fluorescence microscopy. Fura-2-dextran loaded cells were observed with a Nikon Diaphot microscope, using a ×40 oil immersion objective (1.3 n.a.; Nikon, Japan). Excitation illumination at 350 and 380 nm alternately (8 nm bandwidth, UV line filters, Ealing Electroptics, Watford, UK), was provided from a 150 W xenon lamp via a fibre optic and a rotating filter holder. Fluorescence emission between 520-560 nm was monitored with a photomultiplier tube (PM9924B, EMI, Hayes, UK), the output of which was synchronized with the excitation source by microcomputer. Autofluorescence of an egg was measured at each excitation wavelength before loading with fura-2-dextran. Autofluorescence subtraction and 350/380 nm ratio calculations were performed on-line during the experiment to give one fluorescence ratio per second.

Calibration of fura-2-dextran fluorescence

In vitro calibration of fura-2 fluorescence was obtained using drops of Ca²⁺ buffers containing 50 μM fura-2-dextran (Tsien and Rink, 1980). An in vivo calibration was obtained by pressure microinjecting (see below) Ca²⁺ buffers containing 500 μM fura-2-dextran (pipette concentration) into eggs. Buffer was injected until the egg’s buffering capacity was exceeded and the fluorescence ratio values reached a new steady state. The in vitro and in vivo calibrations differed only by 3-8%.

Pressure microinjection of BAPTA and Br₂BAPTA

Eggs were impaled with bevelled microelectrodes backfilled with ‘intracellular solution’ (in mM: 200 KCl; 10 Hepes; 550 mannitol, pH 7.0) containing 2 mM fura-2-dextran and either 80 mM BAPTA, 80 mM Br₂BAPTA (Calbiochem, Nottingham, UK) or 25 mM BAPTA-dextran (10×10⁻³ M) (Molecular Probes, Eugene, Oregon, USA). A measured pressure pulse was delivered to the back of the electrode using a ‘Pico-injector’ (Medical Systems Corps, NY, USA). Excitation illumination (5 mM in 10 mM KCl). Eggs were injected using positive current pulses (1-2 nA, 200 ms, 4.5 Hz) or pressure (see below). A measured pressure pulse was delivered to the back of the electrode using a ‘Pico-injector’ (Medical Systems Corps, NY, USA). The volume injected was calculated using the fluorescence of fura-2-dextran (isosbestic point: 360 nm excitation (8 nm bandwidth)). A calibration curve relating the fura-2 fluorescence to [fura-2-dextran]₁, and [buffer]₁ was obtained as follows. A known pipette concentration of fura-2-dextran was injected into an egg with a measured pressure pulse and fluorescence at 360 nm excitation recorded. The same pulse protocol was repeated into vegetable oil with the same electrode and the ejected dye droplet was allowed to settle on a silanized coverslip and its volume measured allowing calculation of injected [fura-2-dextran]₂. This procedure was repeated several times with different [fura-2-dextran] to obtain a calibration curve from which [buffer]₁ was calculated. Final cytoplasmic concentrations were corrected for non-cytoplasmic volumes (estimated to be approximately half whole egg volume; cf. Speksnijder et al., 1989). Eggs of similar size (70 μm diameter) were used in all experiments. Injectate volume was ≤3% of total egg volume.

Confocal microscopy

Optical sections of eggs loaded with Calcium Green-dextran (10×10⁻¹ M), obtained with a confocal laser scanning microscope (CLSM; Phoibos 1000, Molecular Dynamics, Inc.), were captured at a rate of 1 every 10-15 seconds. Optical sections from similarly loaded eggs, obtained with a CLSM (Leica Lasertechnik, Heidelberg, FRG), were also captured at a rate of 1 every 1.7 seconds. The fluorescence signal was controlled for time-independent inhomogeneities of dye distribution by dividing each image, pixel by pixel, by a reference image taken at the beginning of the experiment. The resulting ratio images were not calibrated for absolute [Ca²⁺]. Calcium Green-dextran was excited at 488 nm and emission monitored with a 530 nm band pass filter (30 nm bandwidth) and a ×60 or ×40 oil immersion objective (1.4 n.a.; Nikon, Japan).

⁴⁵Ca²⁺ flux experiments

5 cm³ aliquots of unfertilized eggs (approximately 8000 eggs per aliquot) were given a 5 minute pulse of filtered sea water (FSW) (10 mM Ca²⁺) containing approximately 1 μCi cm⁻³ ⁴⁵Ca²⁺, followed by 6 washes with 20 cm³ unlabelled FSW and counted as 2 cm³ aliquots in 10 cm³ of ‘Instagel’ (Packard, Illinois, USA) in a scintillation counter and corrected for quenching. For fertilization, 5 cm³ aliquots of eggs were gently mixed with approximately 10⁶ sperm cm⁻³ and a 5 minute pulse of ⁴⁵Ca²⁺ given 0, 5 or 10 minutes after sperm addition. Aliquots were then washed as above.

Fertilization assay

Cell wall secretion following fertilization was assayed using calcofluor white (CFW) staining (Evans et al., 1982). Eggs were stained with 0.0002% CFW for 10 minutes then washed with ASW. CFW fluorescence (measured at 450 nm with excitation at 360-390 nm) was quantified using a cooled CCD camera (Digital Pixel, Brightdon, UK).

RESULTS

Fertilization potentials and Ca²⁺cyt measurements

Unfertilized eggs had a mean resting potential of −53±8 mV (n=24). Fertilization potentials lasted for a mean of 5.5±1 minute (n=17) and depolarized the membrane to between −20 and −5 mV (Fig. 1). No visible compartmentalization of fura-2-dextran (10×10⁻¹ M) into organelles or vesicles was observed, though dye accumulation was observed in the nuclear region. Resting [Ca²⁺]₁ was 130±17 nM (n=66) (Fig. 1). Addition of sperm to the recording chamber produced significant [Ca²⁺]₁ elevations in 13 out of 43 eggs (30%), coincident with the onset of the fertilization potential (Fig. 1A,B,C). However in 30 out of 43 eggs no significant global [Ca²⁺]₁ elevation was detected during the fertilization potential (Fig. 1D,E) though egg activation had occurred by the time recordings were finished (as assayed by CFW) and zygotes subsequently developed normally. The observed response was not affected by the cytoplasmic dye concentration (see Fig. 1; the level of noise in the Ca²⁺ trace is inversely related to the [dye]₁). The magnitude of the average global [Ca²⁺]₁ rise did not exceed 300 nM and usually corresponded to less than a 100 nM increase. While the duration of the Ca²⁺ elevation varied (Fig. 1), the onset was always coincident with
Fucus egg activation

the onset of the fertilization potential. All eggs exhibiting a fertilization potential produced a cell wall within 20 minutes and were observed to develop normally, growing rhizoids within 20 hours. Unfertilized eggs (no fertilization potential or cell wall production) never showed any $\text{Ca}^{2+}$ elevation ($n=18$). Various treatments to perturb $\text{Ca}^{2+}$ (e.g. ionomycin treatment (100 $\mu$M) or sudden elevation of external $\text{Ca}^{2+}$) showed that the injected dye was monitoring $\text{Ca}^{2+}$ reliably (Fig. 1F).

**Confocal imaging of $\text{Ca}^{2+}_{\text{cyt}}$**

Calcium Green-dextran fluorescence ($10 \times 10^3 M_0$) remained diffuse and presumably cytoplasmic for at least 8 hours after injection, showing apparent accumulation only in the nuclear...
Using the ‘Sarastro’ CLSM, 30/36 (84%) eggs showed no transient increases in dye fluorescence after the addition of sperm (though fertilization had occurred as indicated by CFW fluorescence). Occasionally (6/36) eggs showed a small fluorescence increase throughout the egg lasting 2-4 minutes (data not shown). However, the temporal resolution of these records (1 image acquired per 10-15 seconds) was not sufficient to determine accurately the pattern of the Ca$^{2+}$ increase, though no wave of elevated Ca$^{2+}$ was observed (data not shown). The mean spatially averaged (40%) relative increase in Calcium Green fluorescence represents only a small increase in Ca$^{2+}$ (consistent with fura-2 data (see Fig. 1) since Calcium Green shows a 14-fold increase in fluorescence from calcium-free to calcium-saturated form (Calcium Green is near saturation at 1.4 μM [Ca$^{2+}$]) (Haugland, 1992). To improve temporal resolution, a ‘Leica Lasertechnik’ CLSM was used to scan Calcium Green-loaded eggs with an image capture rate of 1 image per 1.7 seconds (n=5). Records were obtained over 10 minutes (Fig. 2A). As with images obtained at lower temporal resolution, no large whole cell increases in Ca$^{2+}$ were observed during egg activation. Fig. 2 shows small increases in Ca$^{2+}$ occurring in the cortical region of the egg that may represent local increases in Ca$^{2+}$ induced by the sperm. No significant change in fluorescence was observed in the nuclear region. Unfertilized eggs displayed no detectable increase in fluorescence during the 10-12 minutes of data collection (n=22).

Fig. 2. Time sequence of confocal ratiometric images of Ca$^{2+}$ in a single Fucus egg loaded with Calcium Green-dextran. (A) The vertical column plots the ratio signal across an equatorial line through the egg against time which progresses downwards. Corresponding optical sections through the whole egg are shown at various times as indicated by white lines. Optical sections were acquired at 1.7 second intervals for approximately 10 minutes. Sperm was added at time 0. Note the fluorescence ratio increases at the edge of the column corresponding to egg cortical Ca$^{2+}$ increases. (B) Confocal sections of an egg illustrating the cortical calcium increase shown in A. Sections are shown at 3.4 second intervals. The sequence begins at time 1.7 seconds after sperm addition, starting at the top and proceeding from left to right. There is a small increase in Ca$^{2+}$ (ratio increase) in the upper part of the egg 8.5 to 11.9 seconds after insemination that may correspond to a sperm induced Ca$^{2+}$ increase. The slight initial inhomogeneities in the image are the result of dye exclusion by chloroplasts or other organelles and the spatial averaging routine (two passes with a 9 pixel centre-weighted kernel).

Fertilization-induced Ca$^{2+}$ flux

Fertilization induced a significant increase in Ca$^{2+}$ influx (Fig. 3). In the experiment shown, Ca$^{2+}$ influx into unfertilized eggs was 0.02±0.005 pmoles egg$^{-1}$ minute$^{-1}$. This increased to 0.12±0.04 pmoles egg$^{-1}$ minute$^{-1}$ in the first 5 minutes.
following fertilization, falling to prefertilization levels within 10 minutes. In replicate experiments, Ca\(^{2+}\) influx showed a mean 4.35 (±0.74; n=4) -fold increase following fertilization.

**Effects of Ca\(^{2+}\) buffers on egg activation**

Br\(_2\)BAPTA, BAPTA and dextran-BAPTA (10x10\(^3\) M\(_s\)) were used to buffer Ca\(^{2+}\)\(_{cyt}\). The high viscosity of dextran-BAPTA limited its pipette concentration to no more than 25 mM and thus its intracellular concentration to a maximum of 0.75 mM. The free acid form of fura-2 was rapidly removed from the egg cytoplasm (through intracellular compartmentalization and dye loss from the cell) following microinjection (Fig. 4A). This was characterized as loss of fluorescent signal (increasing noise) and an increase in ratio (possibly reflecting higher concentrations of free Ca\(^{2+}\) in other cell compartments). Co-injection with Br\(_2\)BAPTA (1.2 mM cytoplasmic concentration) prevented fura-2 free acid removal from the cytoplasm (Fig. 4B), resulting in prolonged stable Ca\(^{2+}\)\(_{cyt}\) measurements. It follows, therefore, that at the concentrations used in this study, the free acid forms of BAPTA buffers are removed only very slowly from the egg cytoplasm, allowing reliable estimates of [buffer]\(_{cyt}\) during the time course of the experiments.

Cytoplasmic [Br\(_2\)BAPTA] >1 mM inhibited activation in 19 out of 20 eggs, with little or no cell wall production observed (Fig. 5). These eggs either perished within 16-18 hours or remained dormant for at least 48 hours. At [Br\(_2\)BAPTA]\(_{cyt}\) <1 mM, eggs activated and developed normally (including rhizoid growth and cell division) for at least 48 hours (9 out of 10 eggs; Fig. 5). Microinjection of <2 mM [BAPTA]\(_{cyt}\) did not inhibit egg activation. Fourteen out of 18 eggs activated and developed normally and 4 out of 18 eggs did not raise a cell wall immediately but were observed to possess a cell wall 16 hours later and subsequently showed normal zygote development for at least 48 hours. Similar results (i.e. no inhibition of egg activation) were observed using dextran-BAPTA at intracellular concentrations up to 0.5 mM (n=10) (data not shown). Above 2 mM [BAPTA]\(_{cyt}\) 8 out of 12 eggs failed to activate, perishing within 16-18 hours or remaining dormant. Four out of 12 eggs, although initially failing to produce a cell wall were later observed to develop normally. BAPTA (at cytoplasmic concentrations used in this study) clamped average egg [Ca\(^{2+}\)\(_{cyt}\)] to levels significantly below normal resting levels (10-50 nM; though accurate calibration at such low [Ca\(^{2+}\)\(_{cyt}\)] is difficult). Similar concentrations of Br\(_2\)BAPTA only lowered average resting [Ca\(^{2+}\)\(_{cyt}\)] slightly (69±13.8 nM; n=24).

None of the eggs microinjected with BAPTA or Br\(_2\)BAPTA showed any global Ca\(^{2+}\) elevation following addition of sperm (see Fig. 7D), whether or not normal development was subsequently observed. In any one group of eggs, the degree of fertilization-associated cell wall release was inversely related to [Br\(_2\)BAPTA]\(_{cyt}\) (Fig. 6).

Buffering [Ca\(^{2+}\)\(_{cyt}\)] with Br\(_2\)BAPTA did not inhibit the development of the fertilization potential. Eggs microinjected with Br\(_2\)BAPTA\(_{cyt}\) produced prolonged fertilization potentials with membrane potentials remaining depolarized for up to 18 minutes (Fig. 7C) (n=4). Simultaneous measurement of [Ca\(^{2+}\)\(_{cyt}\)] showed no global Ca\(^{2+}\) elevation (Fig. 7D) and cell wall exocytosis was inhibited.

**Other treatments**

High K\(^+\) ASW (in mM): 140 NaCl; 320 KCl; 9 CaCl\(_2\); 30 MgCl\(_2\); 16 MgSO\(_4\); 2 NaHCO\(_3\); pH 7.9) depolarized eggs loaded with fura-2-dextran to between 0 and +10 mV and did not induce wall exocytosis or simultaneous [Ca\(^{2+}\)\(_{cyt}\)] elevations (n=5) (Fig. 8). Ionophoretic injection of Ca\(^{2+}\) caused localized exocytosis of the cell wall, which did not propagate or spread from the site of injection (Fig. 9). Only injections from electrodes impaled near the surface of the egg caused cell wall exocytosis. Control injections using electrodes filled with 100 mM KCl did not elicit cell wall exocytosis.
DISCUSSION

Cytoplasmic Ca\(^{2+}\) transients and Fucus egg activation

In a range of animal species a large transient elevation of [Ca\(^{2+}\)]\(_{cyt}\) signals egg activation. [Ca\(^{2+}\)]\(_{cyt}\) has been reported to increase (from resting levels of 100-200 nM) up to 3 \(\mu\)M in the eggs of the Medaka fish (Gilkey et al., 1978), 10 \(\mu\)M in ascidians (Brownlee and Dale, 1990; Speksnijder et al., 1990), 3 \(\mu\)M or more in sea urchin (Mohri and Hamaguchi, 1991) and 2.2 \(\mu\)M in hamster (Miyazaki et al., 1990). These are examples of the deuterosome egg activation model (e.g. Berger, 1993; Jaffe, 1983; Whitaker and Swann, 1993) in which the increase in [Ca\(^{2+}\)]\(_{cyt}\) propagates as a wave, independent of the presence of external Ca\(^{2+}\) (Schmidt et al., 1982). Injection of Ca\(^{2+}\) buffers (EGTA and BAPTA) inhibit this elevation and prevent further development of the egg (Grandin and Charbonneau, 1992; Swann et al., 1992).

The results presented here demonstrate that while small fertilization-induced elevations in egg Ca\(^{2+}\)\(_{cyt}\) can be observed, a large global increase in [Ca\(^{2+}\)]\(_{cyt}\) is not required for Fucus egg activation or subsequent zygote development. Less than half of the eggs studied displayed any detectable fertilization-associated rise in average [Ca\(^{2+}\)]\(_{cyt}\). Measured increases were small (<300 nM) and unaffected by the cytoplasmic dye concentrations used. Though small whole egg transient Ca\(^{2+}\)\(_{cyt}\) elevations can be observed using photometry and confocal microscopy, it is unlikely that these events are necessary for activation since eggs microinjected with appropriate amounts of BAPTA or Br\(_2\)BAPTA exhibited no detectable fertilization-associated [Ca\(^{2+}\)]\(_{cyt}\) elevations yet could be observed to activate and develop normally.

Cell wall exocytosis is Ca\(^{2+}\) dependent

Although a large global [Ca\(^{2+}\)]\(_{cyt}\) elevation is shown here to be unnecessary for Fucus egg activation, a role for elevated [Ca\(^{2+}\)] is evident, at least in the early stages of activation. Increasing [Br\(_2\)BAPTA]\(_{cyt}\) (>1 mM) and [BAPTA]\(_{cyt}\) (>2 mM) did inhibit cell wall release (an essential step in Fucus egg activation) and further zygote development (Figs 5, 6). Furthermore, Br\(_2\)BAPTA was a more effective buffer than BAPTA (K\(_{d}\), 3.6 \(\mu\)M and 0.7 \(\mu\)M respectively (Pethig et al., 1989)) in preventing egg activation and subsequent development. Assuming a Ca\(^{2+}\)-buffer will be most effective at buffering Ca\(^{2+}\) close to

Fig. 6. (A-C) Calcofluor white (CFW) visualization of cell wall production after fertilization in eggs preinjected with 2 mM, 510 \(\mu\)M and 200 \(\mu\)M Br\(_2\)BAPTA respectively. Plot diagrams quantify CFW fluorescence.
its $K_d$ (Speksnijder et al., 1989) it is unlikely that activation is inhibited simply by the buffering of small global sub-mM fertilization-associated $\text{Ca}^{2+}$ cytosolic episodes as shown in Fig. 1. Furthermore, inhibition of activation at these cytosolic buffer concentrations was not simply a result of lowering resting $\text{Ca}^{2+}$ below physiological levels, since BAPTA, which did significantly lower $\left[\text{Ca}^{2+}\right]_{\text{cyt}}$ was less effective at inhibiting activation than Br$_2$BAPTA, which reduced $\left[\text{Ca}^{2+}\right]_{\text{cyt}}$ only slightly.

Using the facilitated diffusion model to describe the $\text{Ca}^{2+}$-buffers (Speksnijder et al., 1989: equation 7), the concentrations of Br$_2$BAPTA and BAPTA that prevent exocytosis suggest that $\text{Ca}^{2+}$ may increase (during normal activation) to $5-6 \mu$M. This would be reflected in global photometric measurements as a small elevation or possibly be undetected if it occurred very locally. This has been demonstrated in recent studies in sea urchin eggs. Small increases in $\text{Ca}^{2+}$ can be measured corresponding to the latent period, prior to the wave of $\text{Ca}^{2+}$ elevation (McDougall et al., 1993). Using whole cell photometry, this increase is barely measurable above the resting $\left[\text{Ca}^{2+}\right]_{\text{cyt}}$, however confocal imaging clearly shows a significant increase localized to the edge of the egg (McDougall et al., 1993). Small transient localized increases in cortical $\text{Ca}^{2+}$ can be observed in confocal sections of $Fucus$ eggs during fertilization (Fig. 2B) and may reflect the occurrence of sperm-induced $\text{Ca}^{2+}$ elevations to higher levels just beneath the plasma membrane. When summed over the whole egg these are consistent with the pattern of average whole egg $\text{Ca}^{2+}$ elevations observed using ratio photometry. The egg shown in Fig. 2 had produced cell wall by the end of the experiment (10 minutes), though it is not clear whether the temporally separated elevations in cortical $\text{Ca}^{2+}$ represent $\text{Ca}^{2+}$ oscillations or result from several sperm-egg interactions.

The exocytotic mechanism has been shown to be controlled, at least in part, by $\text{Ca}^{2+}$ in both animal (e.g. Baker and Whitaker, 1978; Vogel and Zimmerberg, 1992) and plant (Zorec and Tester, 1992; Gilroy and Jones, 1992) systems. The $\text{Ca}^{2+}$-dependence of fertilization-induced exocytosis in $Fucus$ eggs is further suggested by several observations. Firstly $\text{Ca}^{2+}$ injection causes cell wall exocytosis at the site of injection (Fig. 9); secondly, $\text{Ca}^{2+}$ ionophores have been reported to cause patchy cell wall release in unfertilized $Fucus$ eggs (Brawley and Bell, 1987); and thirdly, the amount of fertilization-induced cell wall exocytosis is inversely related to $\text{Ca}^{2+}$ levels in the egg, as shown in Fig. 2B.
Ca\textsuperscript{2+} influx and Fucus egg activation

In protostome eggs, a sperm-induced Ca\textsuperscript{2+} influx is thought to contribute to an elevation in Ca\textsuperscript{2+}\textsubscript{cyst} and the signalling of egg activation (Jaffe, 1983). For example, in the marine worm, *Urechis caupo*, a large inward Ca\textsuperscript{2+} flux (0.024 pmol s\textsuperscript{-1}·mg protein\textsuperscript{-1}·mg protein\textsuperscript{-1}); Johnson and Paul, 1978) accompanies egg activation but no direct studies have yet been performed to show a rise in [Ca\textsuperscript{2+}]\textsubscript{cyst} to be necessary for protostome egg activation. However, a preliminary report has shown increased quin-2 fluorescence during artificial activation of *Barnea* eggs (Brassard et al., 1988). In *Urechis* Na\textsuperscript{+} influx via sperm-activated channels (Gould-Somero, 1981) is thought to depolarize the membrane and activate a Ca\textsuperscript{2+} influx via putative voltage sensitive Ca\textsuperscript{2+} channels. Protostome eggs can also be activated by simple depolarization of the membrane (Paul, 1975; Dube and Guerrier, 1982), presumably by activation of voltage-sensitive Ca\textsuperscript{2+} channels.

Our data show that a Ca\textsuperscript{2+} influx, comparable to that found in *Urechis*, also occurs in *Fucus* eggs during fertilization. Furthermore, the fertilization potential and egg activation can be prevented by removing external Ca\textsuperscript{2+} (data not shown). This is consistent with the hypothesis (Taylor and Brownlee, 1993; Taylor et al., 1992) that an egg activation mechanism, based primarily on Ca\textsuperscript{2+} influx during the fertilization potential, occurs in *Fucus*. Voltage-dependent Ca\textsuperscript{2+} and K\textsuperscript{+} channels have been identified in the unfertilized *Fucus* egg plasma membrane (Taylor and Brownlee, 1993). In addition, it has been shown that the initial amplitude of the fertilization potential is dependent on external Na\textsuperscript{+} (Brawley, 1991; Roberts and Brownlee, unpublished data), suggesting a role for Na\textsuperscript{+} influx in the initial depolarization. This initial depolarization could activate Ca\textsuperscript{2+} influx via voltage-dependent Ca\textsuperscript{2+} channels, which would also contribute to further depolarization of the membrane during the fertilization potential. This depolarization phase of the fertilization potential in *Fucus* may play a role in preventing polyspermy (Brawley, 1991). Voltage-dependent outward rectifying K\textsuperscript{+} channels (Taylor and Brownlee, 1993) are probably responsible for the repolarization phase of the fertilization potential. The prolonged fertilization potential observed in Br\textsubscript{2}BAPTA-loaded eggs (Fig. 7) could be explained in terms of a prolonged inward Ca\textsuperscript{2+} current resulting from the maintenance of the inward Ca\textsuperscript{2+} gradient across the plasma membrane by preventing localized Ca\textsuperscript{2+}\textsuperscript{+} elevation at the cytoplasmic face of the membrane. An alternative or additional mechanism could involve inhibition of opening of Ca\textsuperscript{2+}\textsuperscript{-dependent voltage-regulated K\textsuperscript{+} channels via Ca\textsuperscript{2+} buffering. However, simple voltage modulation of plasma membrane channels can not fully explain the fertilization potential or the initial events of *Fucus* egg activation. Unlike protostome eggs, *Fucus* eggs fail to activate by simple depolarization with high K\textsuperscript{+} ASW (Fig. 8). This may be explained in terms of spontaneous Ca\textsuperscript{2+} channel inactivation in the absence of some additional factor supplied by the sperm and implies the involvement of an additional signal for transduction of the sperm signal across the egg. Possibilities for this include Ca\textsuperscript{2+} release from Ca\textsuperscript{2+} stores (such as endoplasmic reticulum) localized beneath the plasma membrane (Brawley et al., 1976) which could augment the Ca\textsuperscript{2+} influx, or a membrane-associated phosphorylation cascade. Evidence for donation of sperm of an activating factor during fertilization has been presented for other systems (Dale et al., 1978; Dale et al., 1985; Swann, 1990).

We propose that the fertilization-induced Ca\textsuperscript{2+} influx is responsible, at least in part, for a local elevation of Ca\textsuperscript{2+}\textsubscript{cyst} beneath the plasma membrane, inducing cell wall exocytosis, a vital step in the activation process of *Fucus* eggs. Indeed, localized increases in cortical Ca\textsuperscript{2+} are observed, though not as a uniform simultaneous increase around the cortex which might be expected solely from uniform Ca\textsuperscript{2+} influx. However, irrespective of the precise pattern of Ca\textsuperscript{2+} elevation and involvement of Ca\textsuperscript{2+} in the early stages of *Fucus* egg activation, an early global elevation of Ca\textsuperscript{2+} does not appear to be required to signal the resumption of the arrested cell cycle. The involvement of other messengers in this process remains to be investigated. It also remains to be seen whether the fertilization mechanisms being revealed in *Fucus* are present in other oogamous plant systems.

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