First evidence of a calcium transient in flowering plants at fertilization

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SUMMARY

We report here the first evidence of a transient elevation of free cytosolic Ca\(^{2+}\) following fusion of sperm and egg cell in a flowering plant by the use of an in vitro fertilization system recently developed in maize. Imaging changes in cytosolic Ca\(^{2+}\) at fertilization was undertaken by egg cell loading with the fluorescent Ca\(^{2+}\) indicator dye fluo-3 under controlled physiological conditions. The gamete adhesion step did not induce any cytosolic Ca\(^{2+}\) variation in the egg cell, whereas the fusion step triggered a transient cytosolic Ca\(^{2+}\) rise in the fertilized egg cell, lasting several minutes. This rise occurred after the establishment of gamete cytoplasm continuity. Through these observations, we open the way to the identification of the early signals induced by fertilization in flowering plants that give rise to the calcium transient and to investigations of the role of Ca\(^{2+}\) during egg activation and early zygote development in plants, as has been reported for other better characterized animal and algae systems.

Key words: maize, in vitro fertilization, calcium signaling, fluo-3

INTRODUCTION

The double fertilization characteristic of angiosperms (reviewed by Russell, 1992) occurs internally, deep within maternal tissues and involves two separate fusion events. A sperm-egg cell fusion initiates the development of the embryo. A sperm-central cell fusion leads to the development of the nutritive tissue of the embryo or endosperm. In angiosperms, sperm cells are not released free in the external medium but are delivered close to the female gametes by the pollen tube which enters the embryo sac through a degenerated synergid. An understanding of how non-motile sperm cells recognize and fuse with either the egg or the central cell is thus very difficult to achieve in vivo, due to the inaccessibility of the gametes, the simultaneity and rapidity of the fusion processes and the variability in the time course of double fertilization in maize (Möl et al., 1994).

Separate studies of sperm-egg and sperm-central cell fusions are required to undertake a molecular dissection of the early events and signaling steps associated with the double fertilization in flowering plants. The development of in vitro methods in maize provides this opportunity (Faure et al., 1994b; Dumas and Faure, 1995). Two kinds of in vitro fertilization procedures have been developed (recently reviewed by Kranz and Dresselhaus, 1996). Electroejection of single isolated sperm and egg cells leads to the production of zygotes (Kranz et al., 1991a) which are able to undergo karyogamy (Faure et al., 1993) and regenerate into plants (Kranz and Lörz, 1993). Since this procedure involves forced fusions between sperm cells and non-gametic cells (Kranz et al., 1991b), it is nonspecific and therefore cannot be used to study the mechanisms of the physiological fusion processes between sperm and egg (Kranz and Dresselhaus, 1996). Alternative methods were therefore developed which allow sperm and egg to fuse selectively under conditions that mimic natural fertilization. In vitro fusion of isolated maize gametes has been achieved in the presence of Ca\(^{2+}\) (Faure et al., 1994a) or in combination with high pH (Kranz and Lörz, 1994). These improvements of in vitro fertilization in angiosperms provide the opportunity to study the changes that occur in the fertilized egg cell (Kranz et al., 1995; Tirlapur et al., 1995) as well as the signaling requirements associated with sperm-egg fusion and egg activation (Rougier et al., 1996).

A considerable body of informations is now available on the cascade of signaling events induced by sperm-egg interaction in animals and on the mechanisms of egg activation (for recent reviews see Nucitelli, 1991; Whitaker and Swann, 1993; Jaffe, 1996). Previous studies (Ridgway et al., 1977; Steinhardt et al., 1977; Gilkey et al., 1978) and more recent ones (reviewed by Jaffe, 1991; Whitaker and Swann, 1993; Jaffe, 1996) performed on different models have shown that the sperm triggers a transient rise of intracellular free Ca\(^{2+}\) levels in the fertilized egg. The role of Ca\(^{2+}\) in plant fertilization has been recently investigated in a brown algae, Fucus (Roberts et al., 1994; Roberts and Brownlee, 1995). In this lower plant model, a localized elevation of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_cyt]) to micromolar levels seems to be required for early fertilization events: the generation of the fertilization potential and cell wall secretion. Brownlee (1994) has suggested that, since higher plants reflect a high degree of complexity and specialization in their reproductive strategies, they may have strikingly different mechanisms for signaling gamete fusion and egg activation than animals and lower plants. This is the question that we have addressed.
In this study we were interested in investigating the $\text{Ca}^{2+}$ signals induced by sperm-egg fusion in flowering plants using the in vitro fertilization system developed in maize by Faure et al. (1994a) and a $\text{Ca}^{2+}$ imaging technique well adapted to the delicate and easily burst egg protoplasts. We report here the use of fluo-3 ester loading for the continuous monitoring of cytosolic free $\text{Ca}^{2+}$ in isolated maize egg cells during fertilization. We show that following sperm-egg fusion, a single $\text{Ca}^{2+}$ transient is observed in all eggs investigated, indicating the likely involvement of $\text{Ca}^{2+}$ signaling in early zygote development in flowering plants.

**MATERIALS AND METHODS**

**Plant material**
Maize plants (*Zea mays* L.) of inbred lines A188 used for gamete isolation and HD 99 used for microspore culture were grown in a regulated growth chamber at the École Normale Supérieure, Lyon, France. The temperature ranged from 24°C (day) to 20°C (night) with a 16-hour photoperiod at 70% relative humidity and a light intensity of 450-500 $\text{µE/m}^2$/second provided by high-pressure sodium lamps (400 W, Philips, Belgium). In order to prevent pollination when plants had to be transferred from the incubation medium to the different washing steps were particularly delicate because each isolated egg cell was very fragile when transferred and manipulated.

**Female and male gamete isolation**
Egg cells were isolated from ovules dissected from mature ears using a method modified from Kranz et al. (1991a) and Faure et al. (1994a). Half-ovules were incubated for 6 minutes at room temperature in an enzymatic solution containing: 0.75% pectinase (Sera, Heidelberg, Germany); 0.25% pectolyase (Sigma chimie, St Quentin Fallavier, France), 0.5% Macerozyme Onozuka R10 (Yakult Honsha, Tokyo, Japan); 0.5% Cellulase Onozuka R10 (Yakult Honsha); pH 5.5, adjusted to 560 mOsm with mannitol. Ovule pieces were then washed with 0.57 M mannitol and egg cells were gently removed from the embryo sac by manual microdissection under an inverted microscope. Egg cells were only obtained in limited quantities (10-30 egg cells per ear and per half day) and remained very fragile when transferred and manipulated.

Sperm cells were released from freshly collected tricellular pollen grains (Dupuis et al., 1987) after a pH/osmotic shock in 0.5 M mannitol (Faure et al., 1994a).

**Egg cell loading with fluo-3 AM**
We chose to load the unfertilized egg cell with the acetoxymethyl ester form (AM) of fluo-3 which is highly lipophilic and thus easily crosses the plasma membrane by a non-disruptive route (Tsien, 1981; Kao et al., 1989). The acetoxymethyl ester form of fluo-3 (fluo-3 AM) was obtained from Molecular Probes (Eugene, OR, USA) and made up to a 1 mM stock in anhydrous dimethylsulfoxide (DMSO) (Sigma-Aldrich chimie, St Quentin Fallavier, France).

Isolated egg cells were loaded for 6 minutes at room temperature, in the dark, with 1 $\mu$M final concentration of fluo-3 AM prepared in 0.55 M mannitol. Loaded egg cells were subsequently rinsed 3 times in 0.55 M mannitol and 3 times again in the fusion medium composed of 0.55 M mannitol containing 5 mM $\text{CaCl}_2$. These loading and washing steps were particularly delicate because each isolated egg cell had to be transferred from the incubation medium to the different washing media using a micropipette. All the incubating media were sterile and distributed into microdroplets under mineral oil (Merck, Darmstadt, Germany).

**In vitro fertilization (IVF)**
In vitro fertilization was performed according to Faure et al. (1994a), in the presence of 5 mM $\text{CaCl}_2$, conditions in which almost 80% of sperm-egg pairs fused. Fusion of fluo-3 loaded egg cells with sperm was particularly sensitive to experimental manipulation. To preserve the high percentage of fusion, it was necessary to wash the fluo-3 loaded egg cells six times in order to remove extracellular dye completely.

**Spatial localisation of fluo-3 in egg cells**
In order to control the intracellular distribution of fluo-3 and check the retention of the probe in the cytoplasm without sequestration into organelles (see Read et al., 1992; Callaham and Hepler, 1991), optical sections of fluo-3 AM loaded egg cells were obtained and processed using a confocal laser scanning microscope (Axioplan, Carl Zeiss, Oberkochen, Germany) with a 488 nm excitation from an argon ion laser. A water immersion objective (x16, Plan-NeoFluar, Zeiss) was used for optical sectioning with zoom setting at 100. Z-series images were acquired at spacing steps of 1 $\mu$m in the dual channel splitscreen mode which allows a simultaneous display (side by side) of light and fluorescence confocal images as illustrated in Fig. 1A,B. Observations were made at the Centre Commun de Quantimétrie at Lyon I University.

**Imaging of cytosolic free $\text{Ca}^{2+}$**
For real time dynamics of the $[\text{Ca}^{2+}]_{cyt}$ changes, fluo-3 AM loaded cells were observed with an Olympus IMT2 inverted microscope (Olympus France, Rungis, France), using a Nikon Fluor 40x oil immersion objective (1.3 numerical aperture) (Nikon France, Champigny sur Marne, France) and a non-fluorescent immersion oil (Cargille Laboratories, Cedar Grove, USA). The excitation wavelength was determined using an interference filter (500 nm ±14; Ealing, Les Ulis, France) mounted in a motor-driven excitation filter wheel lambda-10 (Sutter Instruments, Novato, CA) in front of the Biologic 150 W xenon light source (Biologic, Clax, France). The excitation light was delivered to the microscope through an optic fiber. Fluorescence from the dye was monitored during fertilization using an Olympus fluorescent cassette containing a 510 nm dichroic reflector and 520 nm long-pass glass emission filter. The fluorescence emission was measured using a C2400 low light level imaging camera from Hamamatsu and was acquired at the rate of one image every 4 seconds using an ARGUS-50 image analysis system (Hamamatsu Photonics, Massy, France). Images were captured at a resolution of 256x256 pixels and digitized to 256 gray levels. Each image recorded was the mean of eight individual frames. The camera dark signal was recorded prior to each experiment and subtracted on-line. Data were then corrected for photobleaching attenuation using a commercially available program (BioPatch analysis 3.2, Biological). No autofluorescence from the unfertilized or fertilized egg cell was observed at the excitation wavelength of 500 nm.

Since fluo-3 emits fluorescence in response to $\text{Ca}^{2+}$ ions, the increase in the fluorescence intensity is directly related to an increase in free $\text{Ca}^{2+}$ concentration (Minta et al., 1989; Kao et al., 1989). However, accurate calibration of absolute free $\text{Ca}^{2+}$ level is difficult to perform when using single wavelength probes (Stricker et al., 1992; Read et al., 1992) and was unsuccessful when using fluo-3 loaded egg cells. For these reasons, we have chosen to describe the $\text{Ca}^{2+}$ dynamics during fertilization using absolute fluorescence intensity measurements expressed in arbitrary units.

Imaging of fluorescence variation during fertilization was performed with an Olympus IMT2 inverted microscope (Japan) as described for imaging of cytosolic free $\text{Ca}^{2+}$ with fluo-3. After each recording (fluo-3) we checked the occurrence of fertilization by performing DAPI staining of the nuclei. Fusion products were placed in a solution of 14 mM DAPI (4’,6-diamidino-2-9 phenyl-indole, Sigma Chimie) prepared in 0.5 M mannitol-Triton X-100 (0.001% v/v) and observed under an inverted IM 35 Zeiss microscope at 395 nm. Photography was performed using a colour Kodak 400 ASA film.
Zygote division after in vitro fertilization

15 minutes after in vitro fertilization, zygotes were transferred to a drop of liquid culture medium, which consisted of 50% v/v NBM solution (Mol et al., 1993) supplemented with zeatin (1 mg/l), 2,4-D (1 mg/l) and 50% mannitol 0.5 M in 0.75% w/v low melting point agarose (type IX, Sigma Chimie, S Quentin Fallavier, France). This medium was laid over NBM medium containing the same growth regulator combination as above and solidified with 0.8% w/v Sea Plaque agarose (FMC BioProducts, Rockland, ME). Fusion products were kept in the dark at 25°C overnight, then were transferred to a 12 mm diameter Transwell insert (Costar Corp., Cambridge, MA) placed inside a well of a 12-well cluster dish containing 1 ml of a suspension of maize microspores (genotype HD 99, density 2.10^3 microspores per ml) undergoing androgenesis as described by Leduc et al. (1996). Fusion products and microspores were cocultured for at least 48 hours in NBM medium containing zeatin (1 mg/l) and 2,4-D (1 mg/l) and kept in the dark at 25°C.

In order to check whether nuclear or cell division had occurred during culture, fusion products were stained with DAPI, as previously described (Leduc et al., 1996).

RESULTS

Loading of egg cells with fluo-3 AM and subcellular distribution of the Ca^{2+} probe

Our first objective was to be able to image cytosolic Ca^{2+} in maize eggs. Microscopic observations indicated that fluo-3 AM uptake in egg cells started immediately after incubation. This loading time was very short in comparison to those reported for loading other plant protoplasts, generally 1 to 2 hours (Gehring et al., 1990; Irving et al., 1992; Fallon et al., 1993).

Using confocal analysis, we showed that dye-loaded egg cells were morphologically indistinguishable from untreated cells. After isolation, the egg cell appeared round in shape, highly vacuolated and devoid of cell wall, as shown in the bright-field image in Fig. 1A. This protoplast contained a dense cytoplasm surrounding the nucleus from which cytoplasmic strands were directed towards the plasmalemma.

No dye sequestration into the vacuole and the nucleus was found to have occurred when egg cells were loaded with 1 μM fluo-3 AM for 6 minutes. In the fluorescent image (Fig. 1B) of the fluo-3 loaded egg cell shown in Fig. 1A, only the nucleus-associated cytosol and the cytoplasmic strands were fluorescent. This dye distribution remained unchanged for at least 2 hours after transferring the cells into a dye-free medium allowing kinetic studies of Ca^{2+} changes during gametic fusion.

Influence of the loading procedure on the ability of fluo-3-loaded egg cells to fuse in vitro and divide

We checked the ability of isolated egg cells to fuse with sperm after loading with fluo-3. This control revealed the importance of the washing step after the loading procedure in preserving a high percentage of fusion (80%). An additional control was performed to verify that intracellular Ca^{2+} homeostasis (Gilroy et al., 1993) required to maintain normal growth and development was not disturbed by fluo-3 buffering of free Ca^{2+} in the egg. We developed a culture system allowing early steps of zygote development in vitro. In these culture conditions, egg cells loaded in the presence of 1 μM or 5 μM fluo-3 AM proceeded to cleave, following fertilization, in a manner similar to that observed in non-loaded egg cells used as controls. Fig. 2 illustrates one of the multicellular structures obtained from loaded egg cells fertilized in vitro. These observations repeated three times, support the view that the Ca^{2+} dynamics reported here occurred in cells that were capable of subsequent division. Loaded egg cells that failed to fuse with a male gamete (20%) did not cleave when subjected to the same culture procedure.

We concluded from these data that fluo-3 had no cytotoxic effect on the egg cells in our experimental conditions. After loading, these eggs preserved their cellular characteristics, their ability to fuse in vitro with a male gamete and their capacity to initiate development.

Ca^{2+} measurement during adhesion and fusion steps

Since the in vitro fertilization of fluo-3-loaded egg cells with a sperm cell was clearly possible, we were in a position to follow cytosolic Ca^{2+} dynamics during fertilization. Under our experimental conditions, the period between the gamete contact and the beginning of the plasma membrane fusion varied between 30 seconds and 2 minutes. During the first 30-40 seconds of gamete adhesion, we optimized the imaging system to the level of dye fluorescence emission in the egg cell.

Fig. 1. Microscopic characteristics of a single maize egg cell loaded with fluo-3 AM. (A) Bright-field image. The isolated egg cell is a protoplast characterised by an outlying developed vacuoles (V). The most part of the cytoplasm is associated with the nucleus (CS, cytoplasmic strands; NC, nucleus-associated cytoplasm; P, plasmalemma). (B) Confocal image of Ca^{2+}_{cyt} localization in the same egg cell. The dye is mainly restricted to the nucleus-associated cytoplasm and to peripheral cytoplasmic strands (CS). Bar, 10 μm.

Fig. 2. In vitro division of a zygote derived from IVF of a fluo-3 AM-loaded egg cell with a male gamete. (A) Phase contrast micrograph of a 48-hour old divided structure. (B) Fluorescence micrograph of the same structure showing 4 nuclei (arrowheads) after DAPI staining. Bar, 10 μm.
Thus, the first image (0 seconds, s) shown in Fig. 3 was recorded 40 seconds after the establishment of the close contact between the two gametes.

The time course of IVF illustrated in Fig. 3 shows that the adhesion step (0 seconds; s) did not induce any variation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in the maize egg cell. Moreover we observe in the temporal analysis illustrated in Fig. 4A, that adhesion between the two gametes could continue for 30 minutes without further membrane fusion during which time no variation in fluorescence emission signal occurred.

The first sign that fusion had occurred was a sudden influx of fluo-3 into the unloaded male gamete (Fig. 3A,B, 4 s). This is evident as a shoulder on the graph corresponding to the fluorescence emitted along the axis a-b passing through the egg cell at the site of adhesion of the unstained male gamete (first image, 0 s). Arrows and arrowheads shows the position of male gamete incorporation. The egg cell diameter is 60 μm.

**Fig. 3.** Evidence for an intracellular $\text{Ca}^{2+}$ elevation triggered by in vitro fertilization of *Zea mays* egg cells loaded with fluo-3 AM. (A) Time sequence of pseudo-color images illustrating temporal and spatial distribution of the $\text{Ca}^{2+}$ transient triggered by sperm-egg fusion. (B) Magnification of time course of the early events occurring during sperm-egg fusion described in A. The graphs, on the right, report changes in fluorescence intensity measured along an axis (line ab) passing through the egg cell at the site of adhesion of the unstained male gamete (first image, 0 s). Arrows and arrowheads shows the position of male gamete incorporation. The egg cell diameter is 60 μm.

**Fig. 4.** Temporal changes in fluorescence emission monitored during IVF using egg cells loaded with fluo-3 AM. Transient $\text{Ca}^{2+}$ rise in a fluo-3 AM-loaded egg cell. (a) Typical positive response resulting from successful gamete fusion. (b) Recording obtained from egg cell that failed to fuse. Fluorescence emission is the relative changes in fluorescence in arbitrary unit.

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**Ca$^{2+}$ explosion in the fertilized egg cell**

At 12 seconds (Fig. 3A, frame 4), the male gamete was no
longer visible, plasmogamy was achieved and the male nucleus was incorporated in the egg cell. The Ca$^{2+}$ increase triggered by fusion reached its peak amplitude 85 seconds after the beginning of the gametic fusion process. The fluorescence signal remained uniformly elevated for 2 minutes and then decreased to reach the resting [Ca$^{2+}$]_cyt level after 29 minutes of recording (Fig. 4). We have observed this transient increase in the [Ca$^{2+}$]_cyt in all fertilized eggs investigated (n=11). In loaded egg cells maintained in Ca$^{2+}$ enriched fusion medium which failed to fertilize during Ca$^{2+}$ measurement, fluorescence level remained unchanged (Fig. 4).

Analysis showed that at fertilization, the increase in signal amplitude representing the increase in the [Ca$^{2+}$]_cyt is relatively variable from pair to pairs of male and female gametes analyzed (n=11). There was also some variations in the duration of the Ca$^{2+}$ increase (10-30 minutes) (n=11). This may reflect varying degrees of egg cell maturity as reported by Möl et al. (1994) from in vivo studies.

**DISCUSSION**

We report here the first description of a fertilization-associated Ca$^{2+}$ transient in a flowering plant by showing that a Ca$^{2+}$ transient is triggered by sperm-egg fusion in maize. We have been able to investigate these Ca$^{2+}$ dynamics by applying a sensitive Ca$^{2+}$-imaging procedure to a recently developed in vitro fertilization system gaining experimental access to the analysis of sperm-egg fusion at the cellular and molecular levels.

**Successful egg cell loading with fluo-3 as a prelude to Ca$^{2+}$ imaging**

In this study, we show that, contrary to previous reports (Tirlapur et al., 1995), imaging cytosolic Ca$^{2+}$ in egg cells is possible. However, owing to the fact that isolated egg cells are fragile protoplasts available only in low numbers and highly sensitive to osmotic pressure, touch and chemicals, the choice of experimental procedure was critical. Although the use of ratiometric indicators such as fura-2 is recommended to allow accurate calcium measurements in plant cells (Read et al., 1992; Graziana et al., 1993), we were unable to apply such a methodology to egg cells. Several assays using acetoxymethyl esters of fura-2 (fura-2 AM) to load unfertilized egg cells led to their bursting during the loading procedure. Direct loading of plant cells with fura-2 AM has also been previously reported to be unsuccessful due to either extracellular or incomplete internal hydrolysis (Graziana et al., 1993). Another possible method of dye introduction was to directly inject fura-2 into the cytosol. However, microinjection, with its attendant problems, represents a more intrusive protocol, especially when applied to plant protoplasts (Read et al., 1992). Successful microinjections of dyes and reporter genes have been performed in our laboratory (Leduc et al., 1996) using isolated maize zygotes fertilized in planta. However appropriate conditions including immobilization of zygotes in agarose and regeneration of a cell wall were required. Such conditions cannot be applied to unfertilized isolated egg cells which are devoid of cell wall and must be maintained free in the fusion medium to allow in vitro fertilization. Therefore the best adapted technology for detecting qualitative changes in [Ca$^{2+}$]_cyt in isolated maize egg cells during fertilization appeared to be ester loading using the Ca$^{2+}$ indicator fluo-3. As recently outlined by Malho and Trewavas (1996), although the use of such a single-wavelength dye precludes accurate calibration, it does not prevent assessment of differences that result from signaling. In our case, this kind of information was the most crucial.

By demonstrating the ability of fluo-3 to permeate intact egg cells under the form of acetoxymethylster (fluo-3 AM) we confirm and extend earlier data collected from intact plant cells (Gehring et al., 1990) or plant cell protoplasts (Shacklock et al., 1992; Ranjeva et al., 1992; Graziana et al., 1993). However, artefacts may be induced by loading plant cells with AM fluorescent compounds. As previously reported by Gilroy et al. (1991), an unequivocal interpretation of imaging data can only be clearly established if the dye is localized in the cytosol, the cell remains unperturbed by the dye loading and analysis procedures and the cellular response is not altered by the measurement procedure. Our experimental conditions almost fulfilled all these criteria. Within the window of time necessary for imaging Ca$^{2+}$ kinetics, we observed no sequestration of the dye into vacuoles which are the main calcium sequestering organelles in plants (Blumwald and Gelli, 1993; Johannes et al., 1992). Since egg cells are highly vacuolized protoplasts, we consider, as previously outlined by Oparka and Hawes (1992), that the major risk of dye sequestration was in this organelle. We also showed that morphology of egg cells and organelle distribution were not disrupted by dye-loading and that loaded egg cells preserved their ability to fuse and to divide.

**Dynamics of Ca$^{2+}$ rise at fertilization**

Since the Ca$^{2+}$ signal reported here in fertilized maize eggs is single and transient, lasting several minutes, it differs markedly in its form and duration from the transients induced by various stimuli described so far in other plant protoplasts or cells (Bush, 1993, 1995). Among them, mechanical touch (Knight et al., 1991; Trewavas and Knight, 1994) or osmotic shock recently investigated in Fucus rhizoid (Taylor et al., 1996) might have been expected to trigger Ca$^{2+}$ changes within eggs either during manual adhesion of gametes or when incubating egg cells in the loading or fusion media. In addition to the striking differences between the Ca$^{2+}$ signal depicted in maize egg cells and those induced by these two stimuli in plant cells, no [Ca$^{2+}$]_cyt changes could be observed during the adhesion step. They were only measured after completion of sperm-egg fusion. This data also proves that the Ca$^{2+}$ contained in the fusion medium is not responsible of the induction of Ca$^{2+}$ signaling in contrast to data from guard cells showing that exposure to 1 mM CaCl$_2$ can cause large cytoplasmic Ca$^{2+}$ oscillations (McAinsh et al., 1995). Thus we can be certain that modifications in [Ca$^{2+}$]_cyt result from physiological modifications of the egg cell brought about by fertilization and do not originate from any artefactual stimulus. When compared to other calcium signals induced by fertilization in various animals, the single transient signal reported here in a flowering plant is more similar in form, magnitude and duration to the Ca$^{2+}$ transient imaged using fluo-3 in the sea urchin egg (Swann and Whitaker, 1986; Stricker et al., 1992; Gillot and Whitaker, 1993) than to the repetitive transients or oscillations reported in mammals (reviewed by Swann and Ozil, 1994) and...
ascidians (Speksnijder et al., 1989). By contrast, the Ca\(^{2+}\) elevation recorded here in all fertilized maize eggs so far analyzed differs from the smaller, cortical Ca\(^{2+}\) elevation reported in *Fucus* in 30% of the fertilized eggs investigated (Roberts et al., 1994; Roberts and Brownlee, 1995). Thus, although fertilization mechanisms differ markedly between flowering plants and animals, they all involve a calcium signal at the single egg cell level after addition of sperm, as illustrated in the present study, although this signal may show different characteristics.

Imaging such Ca\(^{2+}\) changes in fertilized maize eggs was greatly facilitated by the use of the in vitro fertilization system recently developed in our laboratory (Faure et al., 1994a) allowing direct access to the site of sperm-egg fusion and the precise determination of fusion time. The results illustrated in Fig. 3 indicate that in maize as in sea urchin (Figs 1 and 2 in Swann et al., 1994) and more recently in mouse (Lawrence et al., 1997), diffusion of the dye into the sperm from the egg cytoplasm precedes the Ca\(^{2+}\) increase in the egg. These findings support the view that sperm-egg fusion is the prelude to the egg’s early Ca\(^{2+}\) transient. However, further analyses using confocal microscopy will have to be performed in order to gain spatial data and elucidate the mode of propagation of the Ca\(^{2+}\) transient in fertilized maize eggs. Such analyses would help to indicate whether the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) becomes uniformly elevated throughout the entire fertilized egg or propagates through it in a wave-like fashion as illustrated in sea urchin (Swann and Whitaker, 1986; Stricker et al., 1992; Gilliot and Whitaker, 1993).

**Origin and significance of Ca\(^{2+}\) rise?**

Key questions remain to be addressed regarding the origin and the mechanisms of fertilization-associated Ca\(^{2+}\) signaling in maize. Whether extracellular Ca\(^{2+}\) is solely or partly responsible for the calcium increase or whether there is a role for intracellular stores remains to be determined. To date we know that (1) decreasing extracellular Ca\(^{2+}\) in fusion medium strongly reduces the percentage of fusion in our in vitro fertilization system (unpublished data). This is the period during which \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation occurs. At much later stages of zygote development, other responses include membrane-bound calcium and calmodulin changes (Tirlapuru et al., 1995) or abundant expression of a calreticulin gene (Dresselhaus et al., 1996). The questions remaining to be addressed are: are the early calcium transient reported here and these early and later events correlated and how might they combine to contribute to egg activation and zygote development. The results of numerous experiments mostly performed in animals indicate that the transient rise in intracellular Ca\(^{2+}\) following sperm-egg fusion is essential for the subsequent events that constitute egg activation (Jaffe, 1996). In *Fucus*, fertilization is also followed by a sequence of activation events including the occurrence of a fertilization potential similar in many ways to that found in several animal eggs, and the rapid onset of cell wall production correlated with the localized Ca\(^{2+}\) increase (Roberts et al., 1994). After a few hours, these events lead to the production of a differentiated polarized zygote with typical properties. Investigations using our experimental model are currently in progress to establish whether similar links between the Ca\(^{2+}\) fertilization response and other events presumed to trigger egg activation also occur in flowering plants.

In conclusion, new strategies will have to be developed to elucidate how the transient Ca\(^{2+}\) elevation reported here at fertilization in flowering plants is generated and what the precise function and consequences of this Ca\(^{2+}\) signal on zygote development are. Although it is presently unclear which responses are triggered by this newly observed Ca\(^{2+}\) signal, its discovery contributes significantly to understanding of the cellular and molecular responses triggered by egg-sperm fusion in flowering plants. Moreover, the present study highlights the similarities that do exist between plant and animal fertilization responses and increases our fundamental knowledge of this early and essential event at fertilization both in plants and animals. Finally, this study provides a new contribution to the importance and diversity of Ca\(^{2+}\) functions in plant development (reviewed by Hepler and Wayne, 1985; Kauss, 1987; Bush, 1995).

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