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Intracellular free Ca^{2+} changes during physiological polyspermy in amphibian eggs

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Summary

We have made the first measurements of intracellular free calcium activity ($[Ca^{2+}]_i$) in urodele eggs during physiological polyspermic fertilization. Jellied eggs of the urodele amphibian *Pleurodeles waltlii* were impaled with intracellular Ca^{2+}-selective microelectrodes and inseminated under various conditions of sperm:egg ratio to obtain various degrees of polyspermy. In 17 out of 45 cases the egg $[Ca^{2+}]_i$ level (0.41 μM) showed no variation following fertilization. In 28 other cases, however, the egg displayed a slow increase in $[Ca^{2+}]_i$ of 0.15 μM, starting around 15 minutes after fertilization and reaching a plateau level around 10 minutes later. The amplitude of the fertilization-associated increase in $[Ca^{2+}]_i$ was found to be independent of the number of sperm interacting with the egg surface. Measurements with two Ca^{2+}-microelectrodes impaled in single eggs showed that the increase in $[Ca^{2+}]_i$ did not simultaneously occur at distinct places within the egg cortex and, in some cases, could be detected by only one of the two microelectrodes. This latter observation, as well as the absence of $[Ca^{2+}]_i$ change at fertilization in some experiments, strongly suggested that each sperm interacting with the egg might, at various places, trigger a localized, non-propagating change in $[Ca^{2+}]_i$. Experiments in which eggs were locally inseminated, using a micropipette directed towards the site of impalement of one of the two Ca^{2+}-microelectrodes, clearly established that $[Ca^{2+}]_i$ changes, although incapable of propagating over the entire egg cortex, might nevertheless travel very slowly over short distances, their amplitude vanishing rapidly as they propagate from around the sites of sperm entry. The physiologically polyspermic egg of urodele amphibians appears to represent an exception to the universality of a fertilization-induced Ca^{2+} wave.

Key words: intracellular free calcium, physiological polyspermic fertilization, amphibian eggs, cell cycle reinitiation.

Introduction

Cell activation or reinitiation of the cell cycle in response to various external signals (hormones, growth factors, neurotransmitters, mitogens, sperm, for instance) is in most cases marked by a transient increase in the activity of intracellular free calcium ions ($[Ca^{2+}]_i$) (recently reviewed by Berridge and Irvine, 1989; Meyer, 1991). That calcium response is thought to represent a ubiquitous second messenger. Fertilization certainly represents the best system for studying these intracellular Ca^{2+} transients. Indeed, during fertilization, cell activation is initiated at a single particular point, that of the fusion between the fertilizing sperm and the egg. A transient increase in $[Ca^{2+}]_i$ accompanying egg fertilization (or artificial activation) has been recorded in various species (see references in the Discussion section). In fact, it appears that all deuterostome eggs display a transient Ca^{2+} wave at fertilization (reviewed by Jaffe, 1983; 1985). In most cases, the egg Ca^{2+} waves have been analyzed during monospermic fertilization, because it is the normal way of reproduction in all species studied so far. Groups that exhibit physiological polyspermy have polyploidy-preventing mechanisms operating inside the egg, rather than blocks to polyspermy acting at the egg's outside as in physiologically monospermic species (reviewed by Jaffe and Gould, 1985; Jaffe and Cross, 1986). Amphibians represent a very interesting system since they have both styles of fertilization: anurans (frogs and toads) have monospermic eggs, while urodeles (salamanders and newts) have polyspermic eggs (reviewed by Elinson, 1986). So far, $[Ca^{2+}]_i$ variations associated with amphibian egg fertilization have been measured in some anurans but not in urodeles, with the exception, however, of a report in an abstract form by Kubota et al. (1989) who, using aequorin, observed a calcium release in the egg of *Cynops pyrrhogaster*.

Both the eggs of the anurans *Xenopus laevis* (Busa and Nuccitelli, 1985) and *Discoglossus pictus* (Nuccitelli

et al., 1988) displayed a transient 3-fold increase in $[Ca^{2+}]_i$ at the time of fertilization. In anuran eggs, the period of increased $[Ca^{2+}]_i$ matches the period of cortical granule exocytosis quite well (Busa and Nuccitelli, 1985; Nuccitelli et al., 1988). In addition, the Cl^- conductance increase underlying the depolarizing phase of the fertilization potential in anuran eggs requires an increase in $[Ca^{2+}]_i$ (see references in Nuccitelli et al., 1988). It should be noted that both the fertilization potential (Cross and Elinson, 1980) and cortical granule exocytosis (Wyrick et al., 1974) serve as blocks to polyspermy in anuran eggs. One might postulate, on this basis, that urodele eggs do not need to develop a fertilization-associated Ca^{2+} transient, since they lack both types of block to polyspermy (Wartenberg and Schmidt, 1961; Charbonneau et al., 1983). However, urodele egg activation appears to depend on intracellular Ca^{2+}, since it can be triggered by the calcium ionophore A23187 (Charbonneau et al., 1983), a universal egg activator (Steinhardt et al., 1974). In the present work, we report that polyspermic fertilization of *Pleurodeles* eggs is, in a substantial number of cases (28 out of 45), accompanied by a slow increase in $[Ca^{2+}]_i$, around 0.15 μM from a resting level of 0.41 μM, taking place around 15 minutes after fertilization. Unlike that in anuran eggs, the increase in $[Ca^{2+}]_i$ associated with urodele egg fertilization was permanent or, in some cases, only slowly reversible. In addition, its amplitude was independent of the number of sperm interacting with the egg. These new data are discussed with respect to the corresponding changes in physiologically monospermic eggs and their possible function in regulating cell activation-associated events.

Materials and methods

Animals and solutions

Mature individuals of *Pleurodeles waltlii*, purchased from the Centre d'Elevage des Amphibiens (CNRS, Université de Toulouse, France), were reared in our laboratory and fed daily on Trouw pellets, a complete food for fish (Trouvit, France). Females were induced to ovulate following injection of 600-800 international units of human chorionic gonadotropin (Organon, France). The first eggs were laid around 24 hours after injection, at 25-26°C, while the last eggs that could be used for successful fertilization were laid around 36 hours later. Eggs were either externally expressed by gentle squeezing of the female's flanks or carefully picked up in the water tank immediately after laying. Jellied eggs were allowed to adhere onto the bottom of 4 ml tissue culture plastic dishes (60 × 15 mm) with a center well (Falcon, USA), serving as an experimental chamber for ion activity measurements (see below), for about 5 minutes, and immersed in F1 solution. The physiological solution used throughout the study was F1 solution, composed as follows (mM): 31.2 NaCl, 1.8 KCl, 1.0 $CaCl_2$, 0.1 $MgCl_2$, 2.0 $NaHCO_3$, 1.9 NaOH, buffered at pH 7.4-7.5 with 10.0 mM Hepes. Male spermiducts were surgically removed and stored in 4-fold concentrated F1 solution, at 4°C. Pure sperm fluid was obtained by squeezing, with forceps, a small portion of a spermiduct and was used immediately, either undiluted or diluted in F1 solution.

Intracellular free calcium $[Ca^{2+}]_i$ measurements

Unactivated jellied eggs were each impaled with a Ca^{2+}-selective microelectrode and a membrane potential microelectrode. Ca^{2+}-microelectrodes were made and calibrated as previously described (Grandin and Charbonneau, 1991a). The resin, contained in the microelectrode tip, used to detect intracellular Ca^{2+} activities, was a ready-to-use Ca^{2+} sensor (calcium ionophore I, Cocktail A; Fluka Chemical Corp., Switzerland). It is important to note that in order to measure $[Ca^{2+}]_i$ with selective microelectrodes, it is necessary to impale each of the eggs with two microelectrodes: a potential microlectrode measuring only the membrane potential (Em) and a Ca^{2+}-microelectrode measuring the intracellular activity of Ca^{2+} (pCa, the negative logarithm of intracellular free Ca^{2+} activity) plus the membrane potential. Membrane potential was subtracted from the total signal recorded by the Ca^{2+}-microelectrode at the pen recorder input. Membrane potentials and ion-specific signals were recorded using high input amplifiers (Burr Brown, OPA 104) and connected to the ground via an F1 agarose bridge. Additional details concerning microelectrode impalement and the electrophysiological set-up can be found in Grandin and Charbonneau (1991b). After stabilization of both the electrical (Em traces, in all figures) and ionic parameters (pCa traces, in all figures), jellied eggs were inseminated as described below.

Criteria for egg activation and fertilization

Sperm (pure or diluted in F1 solution) was added around the impaled eggs, in the recording chamber, using a P20 Pipetman. Depending on the concentration of living and motile sperm (assessed with a light microscope prior to insemination) for a given sperm sample, eggs were inseminated with various volumes of sperm suspension. Generally, addition of 20-30 μl of a 5- to 10-fold dilution of the spermiduct-expressed sperm suspension on top of the jelly layers of 5 or 6 eggs was sufficient to produce the appearance of about 10 sperm entry sites on each of these eggs. Addition of undiluted sperm under the same conditions could increase that number of sperm entry sites up to 20-30, or even more when all sperm was added on top of a single egg. Quality of the sperm, its rate of dilution in F1 solution, as well as the distance between the site of sperm addition and the egg, represented the three parameters that were found to influence polyspermy. The degree of polyspermy was evaluated by counting, under a stereomicroscope, the number of sperm entry sites at the egg surface, after mechanically dejellying with forceps. Successful egg activation was assessed by visualizing, in vivo under a stereomicroscope, the disappearance of the maturation spot (concomitant with blurring out of the extremity of the metaphase 2 spindle, a black dot in the center of the maturation spot) and a change of pigmentation from a rough, irregular appearance to a smooth appearance (see references in Elinson, 1986; see also Charbonneau et al., 1983), both events occurring 45-60 minutes after fertilization or artificial activation, at 21-23°C. Successful fertilization was assessed by visualizing, under a stereomicroscope, the sperm entry points appearing at the surface of the eggs. These could be detected around 5 minutes after insemination as large craters of about 15 μm in diameter, progressively expanding their size up to 45 μm and remaining visible for at least 3 hours (Picheral, 1977; Charbonneau et al., 1983). When further embryonic development proceeded normally, first cell division took place around 6 hours after insemination, at 21-23°C, followed by more rapid cell divisions every 90-100 minutes. Subsequent embryo development was assessed by

comparing with previously described events (Gallien and Durocher, 1957).

Results

Conditions of insemination

In all polyspermic urodele species so far studied, 3 to 15 sperm have been found to penetrate each egg following natural insemination, that number sometimes appearing to be somewhat characteristic of each species (see references in Elinson, 1986). In *Pleurodeles waltlii*, 1 to 12 sperm have been seen to reach the egg surface following natural insemination (Picheral, 1977). It is also possible to inseminate eggs artificially under conditions resulting in a normal embryonic development. Amphibian eggs can still be successfully inseminated until around 1 hour following immersion in F1 solution (Hollinger and Corton, 1980), a period of time sufficient for stabilization of electrical and ionic parameters. Under the conditions used in this study, the first sperm entry points (see Materials and methods) were visualized 5-15 minutes after insemination, a 5-10 minute delay corresponding to a situation in which the first sperm reach the egg 2-3 minutes after insemination (see Picheral, 1977; Charbonneau et al., 1983). Various degrees of polyspermy (up to around 60 sperm per egg) could be obtained experimentally (see Materials and methods). In the present study, it was repeatedly observed, in all egg batches, that embryos displaying more than around 30 sperm entry sites failed to cleave subsequently. On the other hand, production of 1 to 30 sperm entry sites per egg resulted in normal embryonic development.

Intracellular free Ca^{2+} during polyspermic fertilization

The $[Ca^{2+}]_i$ level in unactivated jellied eggs of *Pleurodeles* impaled with two microelectrodes was found to be 0.41 ± 0.13 μM (Standard Deviation, $n=89$). Two types of intracellular Ca^{2+} response to fertilization were observed in polyspermic *Pleurodeles* eggs. In 28 out of 45 cases in which fertilization took place (appearance of sperm entry sites at the egg surface and/or occurrence of cell division) $[Ca^{2+}]_i$ began to slowly increase 14.0 ± 8.2 minutes (s.d., $n=28$) after insemination, reaching a plateau level 23.3 ± 9.3 minutes (s.d., $n=28$) after insemination (Fig. 1). $[Ca^{2+}]_i$ did not promptly return back to the original level (Fig. 1). The amplitude of the fertilization-associated increase in $[Ca^{2+}]_i$ was 0.15 ± 0.06 μM (s.d., $n=28$). In the 17 other cases, however, there was no detectable changes in $[Ca^{2+}]_i$ during at least 1.5 hours following insemination (Fig. 2). In control experiments in which eggs were inseminated with dead sperm or did not fertilize following insemination, there was no change in $[Ca^{2+}]_i$ (data not shown). As previously reported (Charbonneau et al., 1983; Iwao, 1985), there was no major change in the egg membrane potential during *Pleurodeles* fertilization (Figs 1, 2), which remained close to the level in the unactivated egg (-12.6 ± 1.6 mV, s.d., $n=89$).

Both the eggs that displayed an increase in $[Ca^{2+}]_i$ and the eggs that did not display such an increase, went on to divide and develop normally with similar efficiency. Thus, among the 17 eggs that did not show changes in $[Ca^{2+}]_i$ at fertilization, all 13 in which embryonic development was followed developed normally (8 were followed during the first two or three cell divisions, 5 until the blastula stage) and displayed less than 30 sperm entry sites (6 eggs with 1-7 sperm entry sites, 2 with 14 and 30, and 9 eggs in which sperm entry sites, detected during recording, were clearly less than 30, but were not precisely counted afterwards). Embryonic development was followed in 16 of the 28 eggs of the other class, those showing an increase in $[Ca^{2+}]_i$ at

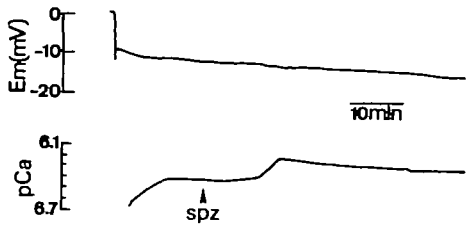

Fig. 1. Representative trace of a polyspermy-induced increase in intracellular free calcium ($[Ca^{2+}]_i$, expressed as pCa, the negative logarithm of intracellular free Ca^{2+} activity) in a *Pleurodeles* egg. Jellied eggs were impaled each with a Ca^{2+}-microelectrode (pCa, bottom trace) and a potential microelectrode (measuring the membrane potential, Em, top trace). After stabilization of the ionic and electrical parameters, the eggs were inseminated (arrowhead "spz") as explained in Materials and methods. In this example, $[Ca^{2+}]_i$ started to increase 10.2 minutes following insemination (fertilization generally follows insemination by 2-3 minutes, see text) and attained a plateau level 8.4 minutes later. In most cases, the fertilization-associated increase in $[Ca^{2+}]_i$ was permanent. In some cases, however, the $[Ca^{2+}]_i$ level very slowly returned back to the original level. It is to be noted that fertilization and particularly two of its key phases, sperm-egg interactions and the increase in $[Ca^{2+}]_i$, did not affect the egg membrane potential at all. Control eggs, which were inseminated with dead sperm or did not show evident signs of fertilization (appearance of sperm entry sites and/or occurrence of cell division), did not display any change in $[Ca^{2+}]_i$. 25 sperm entry sites were counted all over the surface of this egg after its removal from the experimental chamber 1 hour after insemination and dejellying with forceps.

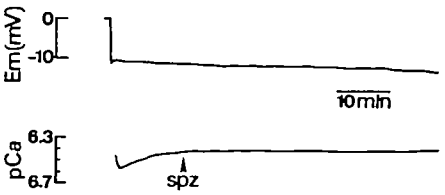

Fig. 2. In some cases, 17 out of 45, polyspermic fertilization of *Pleurodeles* eggs did not induce any change in the $[Ca^{2+}]_i$ level. Same scales as for Fig. 1. The time of insemination is indicated by the arrowhead "spz". Four sperm entry sites were counted at the surface of this egg 1 hour after insemination.

Table 1. *Absence of correlation between the degree of polyspermy and the amplitude of the associated increase in intracellular Ca^{2+} in* Pleurodeles *eggs*

Number of sperm entry sites*	Amplitude of the increase in intracellular Ca^{2+}†
From 3 to 5	0.18±0.00 (n=2)
From 6 to 12	0.12±0.07 (n=3)
15	0.16±0.03 (n=2)
From 25 to 30	0.14±0.09 (n=3)
From 30 to 40	0.08±0.05 (n=2)
From 50 to 60	0.15±0.01 (n=2)

*Counted under a stereomicroscope at the surface of dejellied eggs within 2 hours after insemination.
†Mean value±Standard Deviation (number of eggs).

fertilization. Eight of these 16 eggs developed normally until the blastula stage (all with less than 30 sperm entry sites), while the remaining 8 eggs failed to reach the first cell division (4 had less than 30 sperm entry sites, 4 other 30-60 sperm entry sites). The only noticeable difference between the two classes of eggs was that all the 17 fertilized eggs lacking an increase in $[Ca^{2+}]_i$ had fewer than 30 sperm entry sites, whereas all 4 eggs with more than 30 sperm entry sites (and which did not develop as a consequence) displayed an increase in $[Ca^{2+}]_i$ at fertilization. Therefore, these highly polyspermic eggs represented the only subclass that could be found in the class of eggs showing an increase in $[Ca^{2+}]_i$, but not in the class of eggs showing no change in $[Ca^{2+}]_i$.

To detect any possible relationship between the number of sperm entry sites and the amplitude of the increase in $[Ca^{2+}]_i$, data were arbitrarily classified according to the number of sperm entry sites counted on each egg (only those numbers that were precisely determined were taken into account). Comparison of these various classes clearly shows an absence of correlation between the degree of polyspermy and the amplitude of the fertilization-associated increase in $[Ca^{2+}]_i$ (Table 1).

A Ca^{2+} wave in Pleurodeles *eggs?*

The possible existence of a Ca^{2+} wave in *Pleurodeles* eggs was investigated by impaling single eggs with two Ca^{2+}-microelectrodes (and two potential microelectrodes) and comparing the fertilization-associated $[Ca^{2+}]_i$ changes recorded by each of the two Ca^{2+}-microelectrodes. In one such experiment, there was no increase in $[Ca^{2+}]_i$ at either microelectrode. In three other experiments, $[Ca^{2+}]_i$ increased (by 0.07, 0.09 and 0.15 μM) at only one of the two microelectrodes (Fig. 3A). In the remaining three experiments, $[Ca^{2+}]_i$ increased at both microelectrodes, but, more importantly, each time with a delay between the times of detection at each of the two microelectrodes. In one of these cases, the fertilization-associated increase in $[Ca^{2+}]_i$ had the same amplitude at both microelectrodes (0.06 μM), but began 7.8 minutes after insemination at one microelectrode and 19.2 minutes after insemination at the other microelectrode (Fig. 3B). In two other

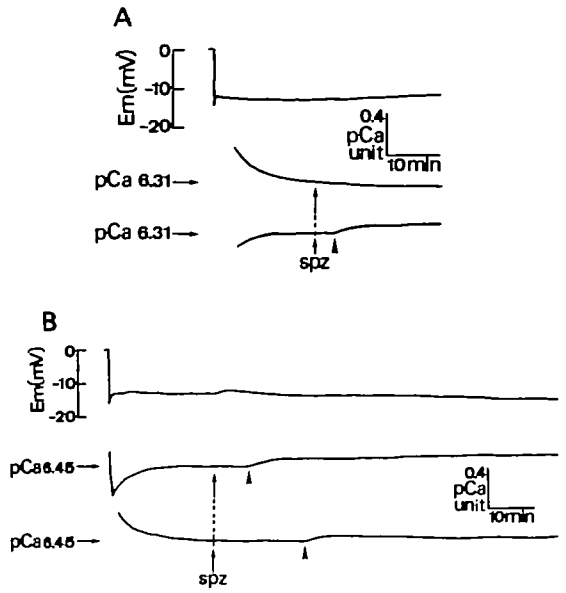

Fig. 3. (A) Single eggs were each impaled with two Ca^{2+}-microelectrodes and two potential microelectrodes. Only one of the two (exactly identical) membrane potential traces is shown (top trace, Em). The two pCa traces (which are not identical) are both shown (middle and bottom traces). One of the two Ca^{2+}-microelectrodes did not record any change in $[Ca^{2+}]_i$ (middle trace) in response to insemination (arrow "spz"), whereas the second Ca^{2+}-microelectrode recorded an increase in $[Ca^{2+}]_i$ 3.6 minutes after insemination (arrowhead, bottom trace). This might be explained either by the fact that the increase in $[Ca^{2+}]_i$ does not propagate all over the egg cortex or by the existence of a Ca^{2+} wave propagating in a non-uniform way which might result in undetectable (because too small) increases in $[Ca^{2+}]_i$ in some regions of the egg (see text). (B) In other experiments, designed exactly as explained in A, the two Ca^{2+}-microelectrodes did record an increase in $[Ca^{2+}]_i$ but at different times following insemination (arrow "spz"). The two arrowheads indicate the beginning of the increase in $[Ca^{2+}]_i$ (7.8 and 19.2 minutes after insemination) at each of the two sites of microelectrode impalement. Interestingly, in this case, the first sperm entry sites that were observed during recording were located near the Ca^{2+}-microelectrode that first detected the increase in $[Ca^{2+}]_i$ (middle trace). Sperm entry sites, counted afterwards (25 to 30), were concentrated around that same Ca^{2+}-microlectrode. This strongly suggests that the increase in $[Ca^{2+}]_i$ starts from around the site(s) of sperm-egg interactions.

experiments, a 0.18 μM increase was detected at one microelectrode 6.0 and 9.0 minutes after insemination, respectively, and 0.25 μM and 0.13 μM increases were detected at the second microelectrode 13.2 and 22.2 minutes after insemination, respectively. However, due to the existence of numerous sperm entry sites, it was not possible to determine temporally and spatially the initiation of the $[Ca^{2+}]_i$ changes, with the exception of one experiment in which the first sperm entry sites, detected during recording, appeared very close to the Ca^{2+}-microelectrode that first recorded the increase in $[Ca^{2+}]_i$ (Fig. 3B).

From the experiments described above, it could not

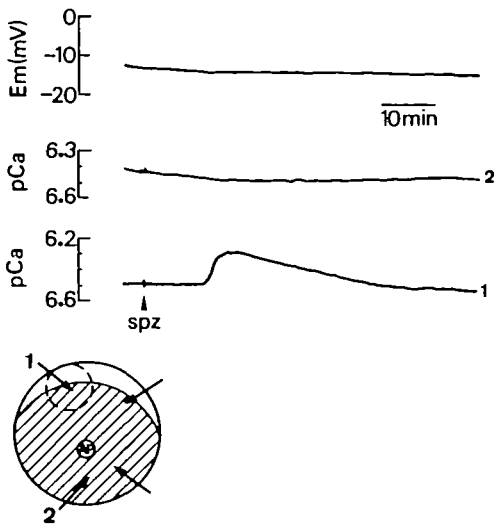

Fig. 4. Changes in $[Ca^{2+}]_i$ in a *Pleurodeles* egg that has been locally inseminated near one of the two Ca^{2+}-microelectrodes, as schematically represented (the external envelopes are not shown). A 0.18 μM increase in $[Ca^{2+}]_i$ (from 0.32 to 0.50 μM) was recorded, around 11 minutes after insemination (arrowhead "Spz"), only by the microelectrode near which sperm had been deposited using a micropipette connected to a 10 ml plastic syringe via a micropipette holder, mounted on a micropositioner, and a flexible plastic tubing. The arrows marked 1 and 2 on the scheme represent the sites of impalement of the Ca^{2+}-microelectrodes (corresponding to the pCa traces marked 1 and 2). The two other arrows represent the position of the two potential microelectrodes (only one of the two identical Em traces is shown). The hatched zone represents the pigmented animal hemisphere of the egg with the animal pole (AP) in its center. The dotted circle around microelectrode 1 represents the approximate location of the dash of sperm following local insemination. A similar local Ca^{2+} response was observed in 8 out of 14 experiments.

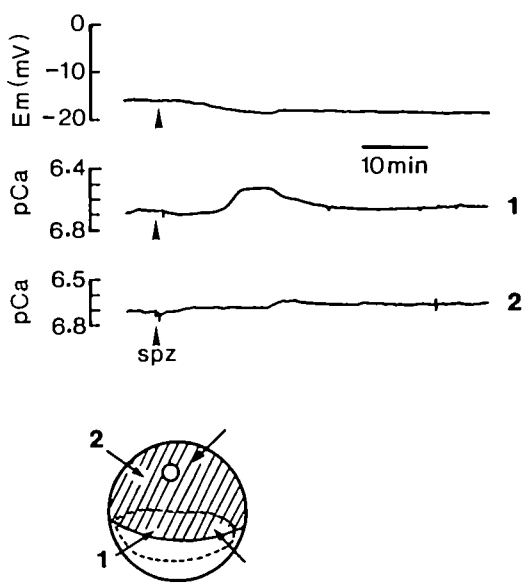

Fig. 5. Other example of changes in $[Ca^{2+}]_i$ in a *Pleurodeles* egg that has been locally inseminated near one of the two Ca^{2+}-microelectrodes, as schematically represented. This pattern was observed in 2 out of 14 experiments (see text). A 0.12 μM increase in $[Ca^{2+}]_i$ (from 0.20 to 0.32 μM) was detected, around 10 minutes after insemination (arrowhead "Spz"), by the microelectrode near which sperm had been locally deposited (same explanations as for Fig. 4 concerning the mode of insemination). A very small increase in $[Ca^{2+}]_i$, 0.03 μM, was detected 8 minutes later by the second microelectrode. The arrows marked 1 and 2 on the scheme represent the sites of impalement of the Ca^{2+}-microelectrodes (corresponding to pCa traces marked 1 and 2). The two other arrows represent the position of the two potential microelectrodes (only one of the two identical Em traces is shown). The hatched zone represents the pigmented animal hemisphere of the egg. The dotted line delimits the zone in which sperm entry sites (around 20) were detected, under a stereomicroscope, around 1 hour after insemination.

be determined whether the increase in $[Ca^{2+}]_i$ measured in the second place represented the same increase as that measured in the first place, spreading as a wave, or rather was triggered by different sperm with a spatially distinct location. To distinguish between the two possibilities, eggs were locally inseminated by positioning a micropipette, containing sperm and mounted on a micropositioner, very near one of the two Ca^{2+}-microelectrodes. In 8 out of 14 such experiments, an increase in $[Ca^{2+}]_i$ (0.10 ± 0.06 μM, s.d., $n=8$, from a basal level of 0.37 ± 0.09 μM, s.d., $n=8$) was detected, 14.3 minutes (±5.6, s.d., $n=8$) after insemination, only in the region that was locally inseminated but not on the opposite side of the egg (Fig. 4), suggesting that the $[Ca^{2+}]_i$ changes cannot propagate, at least far from the site of initiation. In 2 of the 14 experiments, however, an increase in $[Ca^{2+}]_i$ first detected in the locally inseminated region was followed a few minutes later by a much smaller increase at the second microelectrode (Fig. 5). In these 2 experiments, this second increase in $[Ca^{2+}]_i$, recorded 19.2 and 18.0 minutes after insemination, was in fact very small, 0.02 and 0.03 μM, respectively, and followed by 5.1 and 7.8 minutes, respectively, the increase detected in the first place (respectively 0.06 and 0.12 μM). It would be extremely hazardous to speculate that these $[Ca^{2+}]_i$ increases detected in the second place reflect the existence of a propagating Ca^{2+} wave. Indeed, 0.02 μM changes in the $[Ca^{2+}]_i$ level would be barely detectable in normal conditions (see Fig. 5). The fact that these small increases occur at the right time following insemination may open the question concerning their real origin. However, there is nothing more that can be done, using the present technology, that might close the debate. Also, due to technical difficulties in realizing such experiments, one cannot totally rule out the possibility that some sperm do not reach very precisely the region of the egg they were assigned to by the experimentator. It should be noted, too, that in the experiment shown in Fig. 5 the distance between the two Ca^{2+}-microelectrodes was shorter than in the experiment shown in Fig. 4. Finally, in 4 out of 14 locally inseminated eggs which

either displayed sperm entry sites or subsequently cleaved, there was no change at all in $[Ca^{2+}]_i$ at either microelectrode. Data from 6 other eggs, other than those discarded for technical reasons, could not be taken into account, since these eggs did not show any fertilization-associated $[Ca^{2+}]_i$ changes or sperm entry sites, and did not cleave. Among the 14 eggs considered, 4 went at least to the early bud stages (Gallien and Durocher, 1957; they continued to develop but were not individually followed afterwards), 4 arrested at the gastrula or early neurula stages, and 6 did not cleave but displayed a typical $[Ca^{2+}]_i$ increase and sperm entry sites. Injuries around the sites of microelectrode impalement, that healed more or less efficiently according to egg batches, were particularly accentuated in this series in which each egg had 4 microelectrodes in it, and were probably responsible for the failure of these 6 eggs to cleave.

In conclusion, we believe that there is no Ca^{2+} wave in *Pleurodeles* eggs according to the parameters that have been defined by previous authors in other systems (see Discussion). However, our experiments of local insemination of eggs impaled with two Ca^{2+}-microelectrodes do not totally eliminate the possibility of the existence of $[Ca^{2+}]_i$ changes that rapidly vanish in amplitude as they propagate over short distances from around the sites of sperm entry.

Discussion

The present study is the first to report on changes in intracellular free calcium ($[Ca^{2+}]_i$) in eggs during physiological polyspermic fertilization. The relatively late, slow, small and slowly reversible increase in $[Ca^{2+}]_i$ that we have found associated with polyspermic fertilization of the egg of the urodele amphibian *Pleurodeles waltlii* strongly contrasts with the earlier, relatively rapid, larger and transient increase in $[Ca^{2+}]_i$ measured during monospermic fertilization of the egg of the anuran amphibian *Xenopus laevis* (Busa and Nuccitelli, 1985). Relative to the duration of the first mitotic cycle, 1.5 hours in *Xenopus* and 6 hours in *Pleurodeles*, the respective times of occurrence of the increase in $[Ca^{2+}]_i$, around 2 minutes and 15 minutes after fertilization, are comparable although not exactly similar. If, in *Xenopus* eggs, it is clear that the Ca^{2+} wave starts from around the site of entry of the fertilizing sperm (Busa and Nuccitelli, 1985), this is less evident in *Pleurodeles* eggs in which several sperm can enter at any place of the egg surface.

A transient increase in $[Ca^{2+}]_i$ accompanying egg fertilization (or artificial activation) has been recorded in various echinoderms (Steinhardt et al., 1977; Zucker et al., 1978; Eisen and Reynolds, 1984; Eisen et al., 1984; Poenie et al., 1985; Yoshimoto et al., 1985, 1986; Swann and Whitaker, 1986; Tsien and Poenie, 1986; Hafner et al., 1988; Chiba et al., 1990; Whitaker and Patel, 1990), ascidians (Speksnijder et al., 1989, 1990a,b), the medaka fish (Ridgway et al., 1977; Gilkey et al., 1978; Yoshimoto et al., 1985, 1986), anuran amphibians (Busa and Nuccitelli, 1985; Kubota et al., 1987; Nuccitelli et al., 1988) and mammals (Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985; Igusa and Miyazaki, 1986; Miyazaki et al., 1986). All the species studied so far have naturally monospermic eggs, with the exception of the anuran amphibian *Discoglossus* which has recently been reported to present a partial physiological polyspermy (Talevi, 1989). By its characteristics, the *Discoglossus* $[Ca^{2+}]_i$ increase is clearly of the *Xenopus* type, although its duration is longer than that in *Xenopus* (Nuccitelli et al., 1988).

Two observations support the view that *Pleurodeles* egg activation depends on some intracellular release of Ca^{2+} ions. The first one is that *Pleurodeles* eggs can be artificially activated following application of the calcium ionophore A23187 (Charbonneau et al., 1983). The second is the present observation that an increase in $[Ca^{2+}]_i$ associated with egg fertilization can be recorded in a majority of cases. There are two explanations for the observed absence of fertilization-associated $[Ca^{2+}]_i$ changes in some experiments. One is that there are indeed cases in which $[Ca^{2+}]_i$ does not change. The second is that some $[Ca^{2+}]_i$ variations cannot be detected because they are too small. In three experiments, an increase in $[Ca^{2+}]_i$ was recorded only in a given region of the egg but not in the neighbouring region (Fig. 3A). These experiments suggest that the increase in $[Ca^{2+}]_i$ either does not have the same amplitude all over the egg (in which case the increase might be undetectable in some regions because too small) or does not propagate all over the egg (in which case there would be some regions unattained by the $[Ca^{2+}]_i$ increase). Experiments in which local inseminations were performed support the second conclusion. Several characteristics argue against the classification of the *Pleurodeles* $[Ca^{2+}]_i$ increase as a Ca^{2+} wave: it is too small (0.15 μM versus at least 1 μM in most systems); it is permanent or slowly reversible rather than transient; its rate of propagation, if it did propagate, would be extremely low (Fig. 5). In fact, both the absence of a measurable increase in $[Ca^{2+}]_i$ in some fertilized eggs and the observation of localized changes in $[Ca^{2+}]_i$ (Figs 3A, 4) strongly support the existence of several local, non-propagating increases in $[Ca^{2+}]_i$, each possibly triggered by one of the multiple sperm interacting with the egg. Such a possibility has already been evoked, and sometimes demonstrated, in a few other species. Thus, in golden hamster eggs, polyspermy, induced upon reinsemination, was found to be associated with the occurrence of additional Ca^{2+} waves generated at the site of additional sperm entry (Miyazaki et al., 1986). Immature starfish oocytes, lacking polyspermy blocks, displayed a Ca^{2+} transient in which rapid successive peaks might have corresponded to multiple sperm entries (Chiba et al., 1990).

What can be the characteristics of the *Pleurodeles* egg that make it incapable of propagating an intracellular Ca^{2+} signal? In fact, the *Pleurodeles* egg appears to behave, as far as Ca^{2+} propagation is concerned, as an immature *Xenopus* oocyte. Indeed, in *Xenopus*, the cortex of the unactivated egg possesses an extensive

network of cortical endoplasmic reticulum (Campanella and Andreuccetti, 1977; Grey et al., 1974; Charbonneau et al., 1986), absent from the immature oocyte and supposed to be determinant in the propagation of the Ca^{2+} wave (Campanella et al., 1984; Charbonneau and Grey, 1984). In *Pleurodeles*, the cortex of the unactivated egg contains numerous cortical endoplasmic reticulum vesicles, but clearly these vesicles are simple vesicles that lack any connection between them (Charbonneau and Picheral, 1985), unlike the cortical endoplasmic reticulum in *Xenopus* eggs which forms long cisternae interconnecting the cortical granules between them and also makes junctions with the plasma membrane and the subcortical endoplasmic reticulum (Charbonneau et al., 1986). This might account for the absence of increase in $[Ca^{2+}]_i$ in some regions of the *Pleurodeles* egg, for instance when most of the sperm-egg interactions take place far from the region that is being measured.

In anuran eggs, both blocks to polyspermy are highly dependent on an intracellular release of Ca^{2+} (reviewed by Charbonneau and Grandin, 1989). In fact, due to the absence of cortical granule exocytosis and of a fertilization potential, two very rapid Ca^{2+}-dependent reactions, the urodele egg might not need to develop as rapid and massive an increase in $[Ca^{2+}]_i$ as the one present in anuran eggs. There are probably many other Ca^{2+}-dependent reactions associated with egg activation. In particular, the inactivation of MPF, a universal M-phase Promoting Factor (reviewed by Nurse, 1990), which takes place a few minutes after egg activation in *Xenopus* (Gerhart et al., 1984), can be triggered in vitro upon simple addition of Ca^{2+} (Meyerhof and Masui, 1977; Masui, 1982). This suggests that the Ca^{2+} transient in *Xenopus* eggs might serve to trigger MPF inactivation. One of the main roles of the permanent increase in $[Ca^{2+}]_i$ in *Pleurodeles* eggs might also be to trigger MPF inactivation, a reaction that starts around 45 minutes after egg activation (Grandin et al., 1991). If this was the case, *Pleurodeles* eggs might permit a detailed study of MPF inactivation since there is a long delay, around 30 minutes, between the increase in $[Ca^{2+}]_i$ and MPF inactivation.

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