Develop. Growth Differ. (1995) **37**, 703–709

Role of calcium influx during the latent period in sea urchin fertilization

Robbert Créton[1,2] and Lionel F. Jaffe[1]*

[1]*Marine Biological Laboratory, Woods Hole, MA 02543, USA and* [2]*Department of Experimental Zoology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*

After ~7–40 s following gamete fusion, a steadily increasing fraction of a sea urchin's zygotes initiate an activating calcium wave. The fertilization membrane then rises, the cell cycle resumes and development begins. This study focuses on the so-called latent period that occurs between the time that gamete fusion occurs and the initiation of the activating calcium wave. We inhibited calcium influx during this period by adding lanthanum or by reducing external calcium with a buffer at various time points after insemination. Both of these treatments blocked the activation of eggs that had not yet started a wave at the time of treatment. This indicates that an influx of calcium is needed during the latent period to induce egg activation. These results support the sperm conduit model of egg activation in the sea urchin, where calcium flows from the sea through the fused sperms' acrosomal process into a cortical region of the eggs' endoplasmic reticulum.

Key words: calcium, conduit hypothesis, fertilization, latent period, sea urchin.

Introduction

Sperm activate various eggs, including those of sea urchins, by inducing a calcium wave to pass through them (Jaffe 1985; Mohri & Hamaguchi 1991; Nuccitelli 1991; Stricker *et al.* 1992). However, there is no consensus as to how this occurs and so far three hypotheses have been proposed: (i) the sperm contact hypotheses (Foltz & Schilling 1993); (ii) the sperm content hypotheses (Swann 1993); and (iii) the sperm conduit hypothesis (Jaffe 1990; Fig. 1a). In the sea urchin, effective contact is thought to occur between bindin, secreted by the acrosome reacted sperm, and a specific egg receptor (Foltz 1994). According to the current contact hypothesis, the contacted receptor molecules initiate the well known G-protein IP_3 cascade via a protein tyrosine kinase. In the sperm content or oscillogen hypothesis, some proteinaceous, wave-initiating component of the sperm's cytosol is believed to diffuse into the egg after gamete fusion. In the sperm conduit model, extracellular calcium ions are thought to diffuse through channels in the sperm's acrosomal process and then through the interior of this process into the egg, where they are slowly pumped into a local region of the endoplasmic reticulum. At the end of the latent period, the latter overloads and undergoes a luminal mode of calcium induced calcium release, which then induces the first activating calcium or fertilization wave.

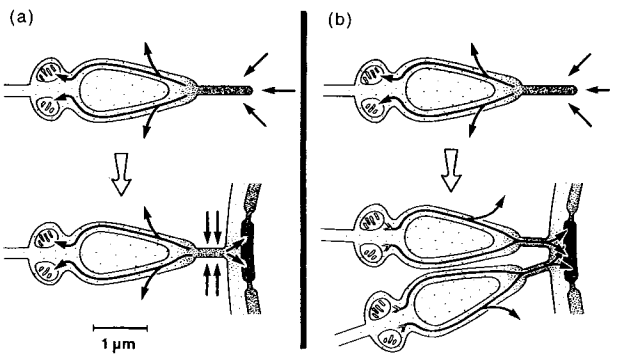

Fig. 1. (a) Calcium conductance and (b) calcium bomb models. (a) Shows the conductance model of sea urchin egg wave initiation in sea water. Top: after the acrosome reaction, but before gamete fusion, calcium leaks into the acrosomal process where some of it diffuses into the sperm's mitochondrion while some is pumped back out. Bottom: after fusion, most of the inflowing calcium is diverted through the fusion pore into the subcisternal space where it is pumped into the endoplasmic reticulum (e.r.). The latter eventually overloads and rapidly releases enough calcium to start a calcium wave. (b) Shows the calcium bomb model invoked to partially explain fertilization in calcium free sea water. After fusion, inflowing Na^+ (not shown) releases Ca^{2+} stored in the *mitochondria*, which then diffuses back into the egg. As these experiments only worked when the concentration of the attached sperm was raised ~50-fold, one cisternum of the e.r. may have received calcium released from several sperm mitochondria. Moreover, normally minor contact and content effects would have been magnified.

*Author to whom correspondence should be addressed.
Received 31 July 1995; accepted 19 August 1995.

If the sperm conduit hypothesis were correct, then natural egg activation would depend upon a steady influx of calcium ions throughout the latent period. We have, therefore, directly tested this proposal in sea urchin eggs.

Materials and Methods

Handling of sea urchin eggs

Eggs and sperm of the sea urchin *Lytechinus variegatus* were obtained by electrical stimulation (12 V) of the urchins. The undiluted sperm was stored at 4°C. The eggs were shed directly into artificial sea water (ASW) containing (mmol/L): NaCl 423; KCl 9; $CaCl_2$ 9; $MgCl_2$ 23; $MgSO_4$ 26; pH 8 1; Tris. The jelly coats of the eggs were removed by 3 min incubation in pH 5 sea water. The dejellied eggs were kept at 20°C and used within 2 h after shedding. To induce an acrosome reaction, the sperm were diluted 1/500 in ASW containing egg jelly. After 30 s incubation, 5 mL of diluted sperm were added to 5 mL of eggs in ASW on a stirrer (120 rpm) at 20°C, resulting in a final sperm dilution of 1/1000.

Inhibition of calcium influx

Calcium influx during fertilization was inhibited using either the calcium channel inhibitor, lanthanum, or the tetrasodium salt of the calcium buffer BAPTA obtained from Molecular Probes, Inc. (Eugene, OR). At various times after insemination, 1 mL of eggs suspended in ASW was added to 1 mL of a well stirred volume of ASW containing 4 mmol/L $LaCl_3$, resulting in a final La^{3+} concentration of 2 mmol/L. In a second series of experiments, fertilized eggs were added to BAPTA-buffered ASW (ASW_B). After addition of the eggs, the ASW_B composition was as follows (mmol/L): BAPTA 10; NaCl 436; KCl 9; $MgCl_2$ 23; $MgSO_4$ 26; Tris 1. In addition, the ASW_B also contained (mmol/L) either: $CaCl_2$ 1 (ASW_{B1}); $CaCl_2$ 10 (ASW_{B10}); or $CaCl_2$ 50 (ASW_{B50}), pH 8. The BAPTA buffer has a K_D of ~1 μmol/L (Pethig *et al.* 1989) that sets the free calcium concentration in ASW_{B1}, ASW_{B10} and ASW_{B50} at 1×10^{-7}, 1×10^{-4} and 4×10^{-2} mol/L, respectively. Four hours after insemination, the eggs were screened for cleavage using a Zeiss light microscope equipped with differential interference contrast optics. For each experiment, the percentage of activation was calculated. Each set of experiments were averaged by calculating the mean and s.e.m. Differences compared with controls were tested for significance using the Student's *t*-test.

The use of aequorin for calcium measurements

Unfertilized eggs were injected with aequorin using a low pressure injection system as described previously (Miller *et al.* 1994). The eggs were immobilized in a Hiramoto/Kiehart chamber (Kiehart 1982) and were injected with 50 pL of semi-synthetic recombinant *f*-aequorin that was kindly supplied by Dr Osamu Shimomura (Shimomura *et al.* 1993). Each injected egg was transferred to a chamber above a photomultiplier tube (Type R464, Hamamatsu Photonic Systems Corporation, Bridgewater, NJ) in order to measure its luminescence before and during fertilization. Sperm was added by the remote-controlled flow of sea water that washed a minute film of dry sperm into the egg chamber.

Results

The latent period

The lag time between the addition of sperm and the onset of the calcium wave can be demonstrated in aequorin loaded eggs (Fig. 2). During the activation wave, the level of luminescence increased by ~1500-fold (from 10 to 15000 counts per second), which corresponds to a 15-fold increase in calcium concentration (Blinks *et al.* 1982). Although it is known that calcium enters the egg during the latent period, this could not be observed in aequorin loaded eggs ($n = 14$). This suggests that the overall rise in calcium during the latent period must be lower than a 10% increase from its resting level, which is the detection limit of our system at low calcium concentrations.

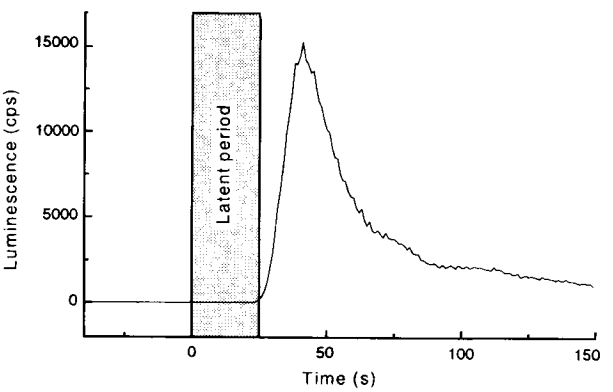

Fig. 2. The latent period as demonstrated in a single aequorin-loaded egg. Eggs were injected with recombinant *f*-aequorin and the resultant luminescence measured with a photomultiplier tube. Luminescence is shown in counts per second (cps). The latent period corresponds to the time difference between insemination at 0 s and the onset of the calcium wave at 25 s. No calcium elevation was observed during the latent period ($n = 14$). Due to the inaccurate method of remote sperm addition used in the present study, refer to Shen and Steinhardt (1984) for more quantitative data on timing of the latent period.

Inhibition of egg activation with lanthanum

Eggs were fertilized in regular ASW to allow efficient binding of sperm to the eggs. The eggs were then transferred to lanthanum containing ASW, at a final concentration of 2 mmol/L lanthanum. The transfer of eggs was performed at set time points after insemination (Fig 3). When the eggs were transferred to the lanthanum bearing medium within ~10 s after insemination, none of the eggs cleaved. These cleavage arrested eggs did not show an elevated fertilization membrane ($n = 50$), suggesting that an influx of calcium is necessary for a very early process in fertilization. This proposal is supported by the fact that the eggs lost their sensitivity to lanthanum at 50 s after insemination. The lanthanum-insensitivity after 50 s, shows that calcium influx is not needed for processes, such as nuclear envelope breakdown, spindle formation and cleavage, which all happen later than the initial 50 s after fertilization. When eggs were transferred to the lanthanum medium 22 s after insemination, half the eggs were activated. The 50% activation time of 22 s after fertilization as well as the long period of increasing activation (10–50 s) corresponds well to the known latent period in sea urchins following fertilization (Shen & Steinhardt 1984). It can therefore be assumed that calcium influx during the latent period is necessary for activation of the sea urchin eggs.

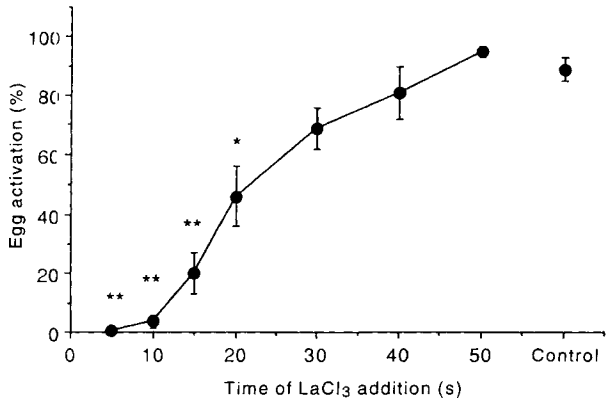

Fig. 3. Sensitivity to lanthanum *vs* time after insemination. Eggs were fertilized in regular ASW to assure sperm-to-egg binding. At different time points after insemination, the eggs were transferred to ASW containing a final concentration of 2 mmol/L lanthanum. Differences compared with control eggs (eggs transferred to ASW without lanthanum) are indicated by an asterisk when they are significant at a 95% confidence limit and by a double asterisk when they are significant at a 99% confidence limit ($n = 5$ experiments; 8 measurements/experiment; 50 eggs/measurement).

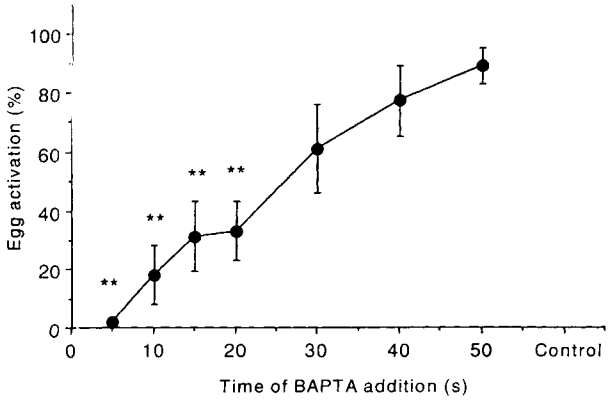

Fig. 4. Sensitivity to the calcium buffer BAPTA *vs* time after insemination. Eggs were fertilized in regular ASW to assure sperm-to-egg binding. At different time points after insemination, the eggs were transferred to ASW containing a final concentration of 1 mmol/L calcium and 10 mmol/L BAPTA. Differences compared with control eggs (eggs transferred to ASW without BAPTA) are indicated by a double asterisk when they are significant at a 99% confidence limit ($n = 4$ experiments; 8 measurements/experiment; 50 eggs/measurement).

Inhibition of egg activation with BAPTA

To assure that the inhibition of activation was caused by an inhibition of the calcium influx and not by some other, non-specific, effect of lanthanum, we repeated the same experiments using the calcium buffer BAPTA (Fig. 4). The eggs were transferred to ASW_{B1} at set time points after insemination. Essentially the same results were obtained in the presence of BAPTA as in the presence of lanthanum. When eggs were transferred to

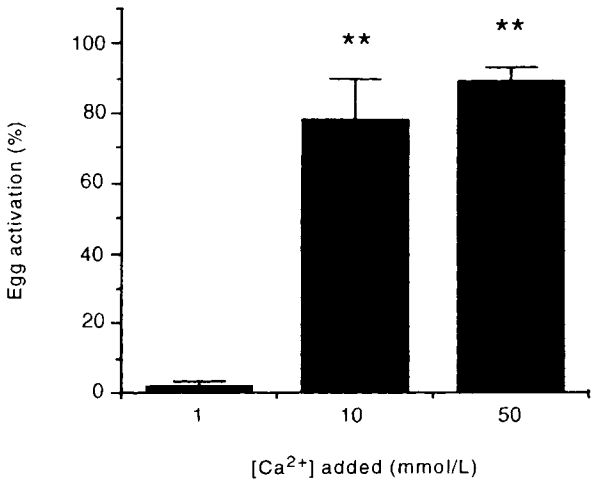

Fig. 5. Rescue of egg activation by the addition of extra calcium to the BAPTA buffer. Eggs were fertilized in regular ASW to assure sperm-to-egg binding. Five seconds after insemination the eggs were transferred to ASW containing 10 mmol/L BAPTA and either 1, 10 or 50 mmol/L calcium. Differences in egg activation compared with control eggs (eggs transferred to BAPTA-buffered ASW with 1 mmol/L calcium) are indicated by a double asterisk when they are significant at a 99% confidence limit ($n = 4$ experiments; 3 measurements/experiment; 50 eggs/measurement).

ASW_{B1} 5 s after insemination, no cleavage occurred; when they were transferred to ASW_{B1} 50 s after insemination, almost all eggs cleaved normally. At intermediate times, the percentage of eggs that cleaved rose steadily, with 50% inhibition occurring ~25 s after insemination. Eggs that were added to a BAPTA-buffer 5 s after insemination could be rescued by the addition of extra calcium to the BAPTA-buffer (Fig. 5). This shows that the inhibitory effect of ASW_B is caused by the lack of calcium and not by a non-specific effect of the BAPTA buffer. Low concentrations of calcium can rescue eggs. In a BAPTA buffer (ASW_{B10}) in which the calcium concentration is set at 100 µmol/L, there is sufficient calcium for the activation of 78% of eggs. This figure is consistent with results of a report in which acrosome reacted *Hemicentrotus* sperm were transferred to various low calcium sea waters before insemination and where 40 µmol/L Ca^{2+} suffices for 50% activation (Sano & Kanatani 1980).

Fifty per cent activation time

To assure rapid fertilization under the present experimental conditions, we have used a high sperm concentration (1/1000). If, at these concentrations, the fusion time between the sperm and egg was the rate limiting step, then one would expect that at lower sperm concentrations fertilization would be delayed. This was examined by measuring the 50% activation time of lanthanum (2 mmol/L) treated eggs at different sperm concentrations (Fig. 6). The results show that sperm concentration did not influence the 50% activation time down to a sperm concentration of 1/3200. Lower concentrations of sperm (1/10000–1/100000) did increase the 50% activation time, suggesting that a sperm concentration of 1/3200 or higher is needed to avoid fusion delay.

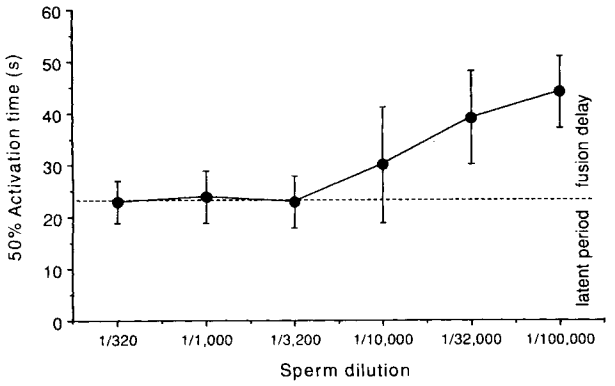

Fig. 6. The 50% activation time *vs* sperm concentration. The 50% activation time was determined at different sperm concentrations by transferring inseminated eggs at different times to ASW containing 2 mmol/L lanthanum ($n = 3$ experiments; 36 measurements/experiment; 50 eggs/measurement).

Discussion

Evidence supporting the conduit model

The results of the simple experiments reported in the present study suggest that an influx of calcium during the latent period is needed to activate sea urchin eggs and thus support the conduit model. The key observations are that the addition of La^{3+} or BAPTA at different times during the latent period is able to prevent activation of eggs and does so with a decreasing probability that parallels the widely variable durations of the latent period. The results of the BAPTA experiments indicate that external calcium is needed in the latent period for activation, while the need of specific calcium influx is suggested by the results of the La^{3+} experiments. These observations are an extension of those made 25 years ago (Baker & Presley 1969; Presley & Baker 1970). These authors observed a similar time course of inhibition after adding the 'spermicides', sodium lauryl sulfate, EDTA and uranyl nitrate. Moreover, the effect of uranyl was later confirmed by Shen & Steinhardt (1984).

However, possible action of the uranyl ion as a calcium channel blocker was not recognized until later (Jaffe 1990). At that time, calculations were published that showed that calcium influx through the fused sperm (perhaps through its fused acrosomal process) is a quantitatively plausible basis for wave initiation and the conduit model was proposed.

These calculations were based upon measurements of the high rates of [$^{45}Ca^{2+}$] entry into sea urchin sperm both during and after acrosome reaction (Schackmann *et al.* 1978; Schackmann & Shapiro 1981). Moreover, the average level of the concentration of cytosolic Ca^{2+} has been clearly seen to rise during this reaction (to 1–2 from 0.1 µmol/L; Trimmer *et al.* 1986; Schackmann 1989).

The proposal of the conduit model was also based upon the plausible assumption that calcium enters through the acrosomal process. Recently attained images of indo-loaded hamster sperm (although not yet of sea urchin sperm) provide some evidence for this assumption (Suarez & Dai 1995), as they suggest that the rise in cytosolic calcium during acrosome reaction is relatively large within the acrosomal region. Also, the suggestion of acrosomal, or at least localized, entry is of interest given the fact that typical calcium channels let in about 1 amol/s of Ca^{2+} when open (Stein 1990). As the role of Ca^{2+} influx after acrosome reaction is ~0.5 amol/s (Jaffe 1990), it may be carried by only one or two new channels and thus be localized.

Another new piece of supporting evidence for the conduit hypothesis lies in the observations made by Shen & Buck (1993). Calcium ions enter through the

whole membrane of the egg following the fertilization potential, but the resultant rise in cortical calcium (the so-called cortical flash) can only be seen during the first few seconds of the latent period and peaks at less than 10% of the fertilization wave level. The application of 125 µmol/L nifedipine eliminates this flash without stopping the calcium wave. However, application of another L-type calcium channel blocker, D-600 (96 µmol/L), does not block calcium entry into acrosome reacted sperm (see Schackmann *et al.* 1978). This indicates that the blockage of egg calcium channels without the concomitant blockage of sperm channels after gamete fusion does not block egg activation. Hence La^{3+} and BAPTA must have blocked egg activation by blocking calcium entry through sperm rather than egg calcium channels.

Objections to the conduit model

How can one correlate the sperm conduit model with the observations of 'fertilization in calcium free media'? When a high enough concentration of acrosome-reacted sperm are mixed with eggs in a cold, sufficiently calcium-free medium the eggs do, indeed, activate (Schmidt *et al.* 1982). However, we propose that they are activated by an unnatural mechanism, specifically by the one shown in Fig. 1b. According to this model, calcium stored in the mitochondrion during acrosome reaction is slowly released by the high extramitochondrial sodium concentration in acrosome-reacted sperm. Therefore, there is not enough calcium mobilized to activate eggs at normal concentrations of attached sperm. However, in experiments performed by Schmidt *et al.* (1982), these surface concentrations became so high that normally weak contact effects enhanced the action of mobilized calcium so as to slowly activate the eggs.

The model Fig. 1b is based upon: (i) the compelling evidence of large increases in mitochondrial calcium during acrosome reaction in sea urchin sperm; (ii) evidence of large increases in extramitochondrial sodium during this same reaction in sea urchins; (iii) evidence that high extramitochondrial sodium acts to release calcium from a variety of other mitochondria (Gunter *et al.* 1994) and (iv) calculations indicating that the concentration of attached sperm in the experiments of Schmidt *et al.* (1982) were ~50 times higher than is normally needed to quickly activate eggs.

Direct, convincing evidence of a large increase in mitochondrial calcium during acrosome reaction has been obtained by energy dispersal X-ray microanalysis of frozen, jelly-activated *S. purpuratus* sperm by Cantino *et al.* (1983). In fact, this increase in mitochondrial calcium probably reached a peak level of ~50 mmol/L, a value that is consistent with earlier observations that ~6 amol of [$^{45}Ca^{2+}$] enter the sperm during this reaction (Schackmann *et al.* 1978). Indeed, so much calcium is normally stored in the mitochondrion of fertilizing sperm that it is easily seen as a brightly fluorescent dot inside living, chlorotetracycline-loaded *Lytechinus* eggs (Hinkly & Newman 1989).

The X-ray microanalysis performed by Cantino *et al.* (1983) also showed large increases in sodium (to at least 100–200 mmol/L) during acrosome reaction of *S. purpuratus* sperm; this value is consistent with the earlier observation that 240 amol of [$^{22}Na^{+}$] enters during the acrosome reaction (Schackmann & Shapiro 1981). Moreover, the calcium-free fertilization medium used by Schmidt *et al.* (1982) contained 450 mmol/L Na^{+} that should have either maintained or even further increased the level of extramitochondrial sodium in sperm during the long latent period by traversing the relatively non-specific ion channels found in acrosome-reacted sperm.

Nevertheless, our own BAPTA experiments (that were performed at normal sperm concentrations) suggest that too little calcium is released from the mitochondria to activate eggs. However, in experiments performed by Schmidt *et al.* (1982), eggs could only be activated at 20 times the normally needed sperm concentration and after 2–3 times the normal latent period. Assuming that the concentration of sperm that were bound to the surface of the eggs was proportional to both the sperm concentration in sea water and to the exposure time of the eggs to these sperm, this means that the concentration of sperm at the egg surface during activation was $\sim 20 \times 2.5$, or 50, times the surface concentration that is normally needed. Indeed, it can be calculated that the surface concentrations of sperm in the experiments of Schmidt *et al.* (1982) probably approached close packing. Schmidt *et al.* (1982) observed an average of 3.3 sperm around each egg's circumference 20 s after adding a 1:10 000 dilution of acrosome-reacted sperm in zero calcium sea water. Assuming that sperm binding is proportional to sperm concentration and exposure time, then $20 \times 4.5 \times 3.3 = 297$ sperm would have been bound at the time of 50% fertilization in calcium free sea water to an egg circumference of $\pi \times 80 = 251$ µm (i.e. 1.2 sperm/µm and a sperm to sperm distance of only 0.8 µm). Considering that the diameter of *S. purpuratus* sperm is ~1 µm, this calculation indicates that the attached sperm may well have approached close packing.

One could imagine two main ways that such potentiation could have occurred. First, there is now substantial evidence that suggests that the attachment of sperm to the sea urchin egg's receptor molecules acts to favor egg activation even though this mechanism is, by itself,

insufficient to bring about significant levels of activation at normal concentrations of bound sperm. Moreover, it has been speculated that this contact activation process is mediated by the G-protein IP_3 cascade (Foltz & Schilling 1993) and would therefore activate eggs via the diffusible IP_3 molecule. A 50-fold increase in the attached sperm concentration could have induced an increase in the concentration of IP_3 within the egg, thereby decreasing the level of calcium needed to start a calcium wave. It is not known whether IP_3 levels are indeed elevated in polyspermic eggs during the latent period; no such increased levels of IP_3 have been found in polyspermic *Xenopus* eggs 5 min after fertilization (Stith *et al.* 1994). However, the crucial experiment of measuring IP_3 levels in polyspermic eggs during the latent period has not yet been performed. Second, close packed sperm may have allowed two or more adjoining sperm to at least transiently fuse with the egg and thus deliver calcium released from several sperm mitochondria as well as the contents from several cytosol sperms'.

Why has the artificial introduction of calcium into sea urchin eggs given such ambiguous results? We would answer that the artificial introduction of calcium in a place, within a medium and at a pace that roughly simulates a sperm's action has only been tried once. In the experiments of Baker *et al.* (1980), 90 µm diameter *Echinus* eggs were electroporated in a medium resembling the ionic composition of the cytosol with the Ca^{2+} concentration set at various levels via 1 mmol/L EGTA. Thirty seconds or more after electroporation in 2–6 µmol/L Ca^{2+}, 60–95% of the cortical granules were gone. This observation does seem to be consistent with the induction of a propagated wave after a lag period.

To our knowledge, in all other published experiments sea urchin eggs have been pricked or electroporated in natural or artificial sea water media containing approximately 10 mmol/L Ca^{2+}, 450 mmol/L Na^+ etc., or they were injected far from the cortex. One of these procedures probably induced a propagated calcium wave but bypassed the normal, initiating lag phase (Moser 1939), while others probably bypassed the whole normal calcium wave process (Millonig 1969; Hamaguchi & Hiramoto 1981; Kinosita *et al.* 1991; Mohri & Hamaguchi, 1991).

Finally, these results raise an interesting question regarding mammalian eggs, namely, would the prevention of calcium influx soon after gamete fusion also block their activation?

Acknowledgements

We are pleased to thank 'de Nederlandse organisatie voor Wetenschappelijk Onderzoek', Universal Imaging Corporation, Surdna Foundation and the National Science Foundation (Grant no. DCB-9103569) for their financial support. We thank Drs O. Shimomura (Marine Biological Laboratory, Woods Hole, MA, USA), Y. Kishi (Harvard University, MA, USA) and S. Inoue (Chisso Corporation) for providing the *f*-aequorin. Much of the work presented in this paper was a part of Dr Créton's PhD thesis (1994) at the University of Utrecht.

References

Baker, P. F., Knight, D. E. & Whitaker, M. J. 1980. The relation between ionized calcium and cortical granule exocytosis in eggs of the sea urchin *Echinus esculentus*. *Proc. R. Soc. Lond.* **B 207**, 149–161.

Baker, P. F. & Presley, R. 1969 Kinetic evidence for an intermediate stage in the fertilization of the sea urchin egg. *Nature* **221**, 488–490.

Blinks, J. R., Wier, W. G., Hess, P. & Prendergast, F. G. 1982. Measurement of Ca^{2+} concentrations in living cells. *Prog. Biophys. Mol. Biol.* **40**, 1–114.

Cantino, M. E., Schackmann, R. W. & Johnson, D. E. 1983. Changes in subcellular elemental distribution accompanying the acrosome reaction in sea urchin sperm. *J. Exp. Zool.* **226**, 255–268.

Foltz, K. R. 1994. The sea urchin egg receptor for sperm. *Semin. Dev. Biol.* **158**, 243–253.

Foltz, K. R. & Schilling, F. M. 1993. Receptor-mediated signal transduction and egg activation. *Zygote* **1**, 276–279.

Gunter, T. E., Gunter, K. K., Sheu, S-S. & Gavin, C. E. 1994. Mitochondrial calcium transport: Physiological and pathological relevance. *Am. J. Physiol.* **267**, C313–339.

Hamaguchi, Y. & Hiramoto, Y. 1981. Activation of sea urchin eggs by microinjection of calcium buffers. *Exp. Cell Res.* **134**, 171–179.

Hinkley, R. E. & Newman, A. N. 1989. Selective identification of the paternal mitochondrion in living sea urchin eggs and embryos by chlorotetracycline. *J. Exp. Zool.* **249**, 111–114.

Jaffe, L. F. 1985. The role of calcium explosions, waves and pulses in activating eggs. In *Biology of Fertilization*, vol. 3 (Eds C. B. Metz & A. Monroy), pp. 127–165. Academic Press, Orlando.

Jaffe, L. F. 1990. The roles of intermembrane calcium in polarizing and activating eggs. In *Mechanisms of Fertilization* (Ed. B. Dale), pp. 389–418. Springer Verlag, Berlin.

Kiehart, D. P. 1982. Microinjection of echinoderm eggs. *Meth. Cell. Biol.* **25**, 13–31.

Kinosita, K., Ito, H., Ishiwata, S., Hirano, K., Nishizaka, T. A & Hayakawa, T. 1991. Dual-view microscopy with a single camera: Real-time imaging of molecular orientations and calcium. *J. Cell Biol.* **115**, 67–73.

Miller, A. L., Karplus, E. & Jaffe, L. F. 1994. Imaging $[Ca^{2+}]_i$ with aequorin using a photon imaging detector. *Meth. Cell. Biol.* **40**, 305–338.

Millonig, G. 1969 Fine structure analysis of the cortical reaction in the sea urchin egg. *J. Submicr. Cytol.* **1**, 69–84.

Mohri, T. & Hamaguchi, Y. 1991. Propagation of transient Ca^{2+} increase in sea urchin eggs upon fertilization and its regulation by microinjecting EGTA solution. *Cell Struct. Funct.* **16**, 157–165.

Moser, F. 1939. Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. *J. Exp. Zool.* **80**, 447–472.

Nuticelli, R. 1991. How do sperm activate eggs? *Curr. Top. Dev. Biol.* **25**, 1–6.

Pethig, R., Kuhn, M., Payne, R., Adler, E., Chen, T-H. & Jaffe, L. F. 1989. On the dissociation constants of BAPTA-type calcium buffers. *Cell Calcium* **10**, 491–498.

Presley, R. & Baker, P. F. 1970. Kinetics of fertilization in the sea urchin. *J. Exp. Biol.* **52**, 455–468.

Sano, K. & Kanatani, H. 1980. External calcum ions are requisite for fertilization of sea urchin eggs by spermatozoa with reacted acrosomes. *Dev. Biol.* **78**, 242–246.

Schackmann, R. W. 1989. Ionic regulation of the sea urchin sperm acrosome reaction and stimulation by egg-derived peptides. In *The Cell Biology of Fertilization* (Eds H. Schatten & G. Schatten), pp. 3–28. Academic Press, San Diego.

Schackmann, R. W., Eddy, E. M. & Shapiro, B. M. 1978. The acrosome reaction of *Strongylocentrotus purpuratus* sperm: Ion requirements and movements. *Dev. Biol.* **65**, 483–495.

Schackmann, R. W. & Shapiro, B. M. 1981. A partial sequence of ionic changes associated with the acrosome reaction of *Strongylocentrotus purpuratus*. *Dev. Biol.* **81**, 145–154.

Schmidt, T., Patton, C. & Epel, D. 1982. Is there a role for the Ca^{2+} influx during fertilization of the sea urchin egg? *Dev. Biol.* **90**, 284–290.

Shen, S. S. & Buck, W. R. 1993. Sources of calcium in sea urchin eggs during the fertilization response. *Dev. Biol.* **157**, 157–169.

Shen, S. S. & Steinhardt, R. A. 1984. Time and voltage windows for reversing the electrical block to fertilization. *Proc. Natl Acad. Sci. USA* **81**, 1436–1439.

Shimomura, O., Musicki, B., Kishi, Y. & Inouye, S. 1993. Light-emitting properties of recombinant semi-synthetic aequorins and recombinant fluorescein-conjugated aequorin for measuring cellular calcium. *Cell Calcium* **14**, 373–378.

Stein, W. D. 1990. *Channels, Carriers and Pumps*. p. 122. Academic Press, San Diego.

Stith, B. J., Espinoza, R., Roberts, D. & Smart, T. 1994. Sperm increase inositol 1,4,5-triphosphate mass in *Xenopus laevis* eggs pre-injected with calcium buffers or heparin. *Dev. Biol.* **165**, 206–215.

Stricker, S. A., Centonze, V. E., Paddock, S. W. & Schatten, G. 1992. Confocal microscopy of fertilization-induced calcium dynamics in sea urchin eggs. *Dev. Biol.* **149**, 370–380.

Suarez, S. S. & Dai, X. 1995. Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. *Mol. Reprod. Dev.* (in press).

Swann, K. 1993. The soluble sperm oscillogen hypothesis. *Zygote* **1**, 273–276.

Trimmer, J. S., Schackmann, R. W. & Vacquier, V. D. 1986. Monoclonal antibodies increase intracellular Ca^{2+} in sea urchin spermatozoa. *Proc. Natl Acad. Sci. USA* **83**, 9055–9059.