An Investigation of the Roles of Calcium Ions in
Mechanisms of Zebrafish Egg Activation

By

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<tr>
<td>AP</td>
<td>Animal Pole</td>
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<tr>
<td>BD</td>
<td>Blastodisc</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C₂H₃NaO₂</td>
<td>Sodium Acetate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium Ion</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>Calcium Nitrate</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CG</td>
<td>Cortical Granule</td>
</tr>
<tr>
<td>CH</td>
<td>Chorion</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N′,N″,N‴-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FITC-dextran</td>
<td>Fluorescein isothiocyanate-dextran</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-hydroxyethyl]piperazine-N″-[2-ethane-sulfonic acid])</td>
</tr>
<tr>
<td>InsP₃</td>
<td>Inositol 1,4,5-Trisphosphate</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilo Pascal</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Magnesium Ion</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium Sulfate</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>Magnesium Sulfate Heptahydrate</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>ml/min</td>
<td>Milliliter per Minute</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>µm/sec</td>
<td>Micrometers per Second</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>MP</td>
<td>Micropyle</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
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<td>NaCl</td>
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<td>Na₂HPO₄</td>
<td>Di-sodium Hydrogen Phosphate</td>
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<td>nl</td>
<td>Nanoliter</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>PD</td>
<td>Parthenogenetic Development</td>
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<tr>
<td>PIM</td>
<td>Photon Imaging Microscope</td>
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<tr>
<td>PM</td>
<td>Plasma Membrane</td>
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<td>PMT</td>
<td>Photo-Multiplier Tube</td>
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<tr>
<td>PSI</td>
<td>Pounds per Square Inch</td>
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<tr>
<td>RA-IPD</td>
<td>Resistive Anode-Imaging Photon Detector</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SOF</td>
<td>Salmon Ovarian Fluid</td>
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<td>VP</td>
<td>Vegetal Pole</td>
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ABSTRACT

The activation process in a variety of deuterostome and protostome eggs is accompanied by cytosolic calcium transients that usually take the form of either a single or multiple propagating waves. Through the injection of aequorin (a calcium-specific luminescent reporter) and the use of a Photon Imaging Microscope (PIM), I report that the eggs of zebrafish (*Danio rerio*) are no exception, in that they generate a single calcium activation wave that traverses the egg at a velocity of around 9 μm/sec. There appears, however, to be no difference between the calcium-mediated activation response of eggs with regards to the presence or absence of sperm in the spawning medium. This suggests that these eggs are normally activated when they come in contact with their spawning medium, and then subsequently fertilized. The aspermic wave is initiated at the animal pole in the region of the micropyle, appears to propagate mainly through the yolk-free egg cortex, then terminates at the vegetal pole. As neither sperm nor external calcium are required for the initiation (or propagation) of the activation wave, this suggests that an alternative wave-trigger must be involved. In zebrafish eggs the propagating calcium wave is not followed by a wave of cortical granule exocytosis as is the case in medaka (*Oryzias latipes*) eggs. What is observed, however, is a near-synchronous exocytosis of cortical granules all over the egg surface some 30-60 seconds after the initiation of the calcium wave. Medaka eggs, however, contain a single layer of cortical granules located just below the plasma membrane, whereas the distribution of cortical granules in zebrafish eggs is more complex. The cortex of the animal hemisphere contains multiple layers of small cortical granules which increase in size (with an accompanying decrease in the number of layers) in an animal to vegetal pole
direction. Thus, the differences in zebrafish and medaka egg morphology might account for the differences in the relationship between the calcium wave and cortical granule exocytosis during activation.
INTRODUCTION

1.1 Calcium Activation Waves

It appears that all vertebrate, invertebrate, and perhaps even some plant eggs are activated by the generation of calcium transients in their cytoplasm (Shen, 1995; Digonnet et al., 1997; Lawrence et al., 1997). In all cases, apart from a recent report from the marine worm *Urechis caupo* (Stephano and Gould, 1997), these transients take the form of propagating calcium waves (Galionne et al., 1993; Jaffe, 1993), which appear to provide a significant component of the activating stimulus required for these eggs (Jaffe, 1985; Epel, 1990; Whitaker and Swann, 1993). It is becoming clear, however, that although an explosive rise in the level of cytosolic Ca\(^{2+}\) is a common feature of this activation response, there are both subtle and significant differences in this response comparing eggs from different species. For example, in fish (Gilkey et al., 1978; Yoshimoto, et al., 1986), echinoderms (Steinhardt et al., 1977; Eisen et al., 1984; Eisen and Reynolds, 1985; Yoshimoto et al., 1986; Striker et al., 1992; Gillot and Whitaker, 1993), and frogs (Busa and Nuccitelli, 1985; Kubota et al., 1987), a single calcium wave is propagated across the activating egg, whereas in annelids (Eckberg and Miller, 1995; Striker, 1996), ascidians (Speksnijder et al., 1990; Albrieux et al., 1997), and mammals (Miyazaki et al., 1986, 1993; Kline and Kline, 1992; Swann, 1994; Miyazaki, 1995) - including humans (Homa and Swann, 1994; Tesarik and Testart, 1994) - activation triggers a series of repetitive calcium waves or oscillations.
1.2 Historical Perspective on the Roles of Ca\textsuperscript{2+} in Fish Egg

Activation

With regards to the role of Ca\textsuperscript{2+} in the activation of fish eggs, most of our information derives from early work on the medaka, *Oryzias latipes* (Gilkey *et al.*, 1978; Yoshimoto *et al.*, 1986; Iwamatsu *et al.*, 1988). The use of this species was an inspired choice, as the eggs of the medaka were particularly suited to these pioneering imaging studies; being large (about 1200 μm in diameter), optically clear, and possessing a peripheral cytoplasm bounded on one side by the plasma membrane, and on the other by the yolk membrane. This morphological feature results in a degree of resolution in the “z-axis”, as the calcium reporter was only loaded into, and thus the resultant signal only recorded from, the outermost 20-40 μm or so of the egg (Gilkey *et al.*, 1978). Hence the clear, unambiguous images and data reported from this system. Another advantage of using medaka was that eggs could be arrested before activation by being placed in a Ringer’s solution (Yamamoto, 1961), thus allowing them to be loaded with a calcium reporter and subsequently activated when required. Furthermore, in medaka a sperm makes contact with the egg surface through a specialized opening in the chorion called the micropyle (MP), which is located at the animal pole (AP; Hart and Donovan, 1983). This makes it easy to relate the calcium wave initiation site to the point of sperm/egg contact, and to the subsequent generation of the body axes of the embryo. The medaka’s chorion is covered by projecting filaments, which are short everywhere except at the vegetal pole (VP), where they are significantly greater in length. These filaments entangle adjacent eggs into a cluster, and serve to attach the cluster to the vent of the female fish (Kirchen and West, 1976). Their presence makes the
identification of the VP, and therefore the AP, easy. Medaka eggs can thus be orientated in a favorable manner in order to aid observation during imaging. Zebrafish eggs do not, unfortunately, possess chorionic hairs. Thus until an egg begins its post-activation segregation, it is very difficult to locate the AP/VP axis except under high magnification that reveals the micropyle apparatus.

1.3 Summary of the Calcium-Related Events during Egg

Activation in Medaka

In medaka, on activation either by sperm or via a parthenogenetic means such as application of the calcium ionophore A23187 (Gilkey et al., 1978), a wave of calcium-induced-calcium-release (CICR) sweeps through the cytoplasm of the egg at a velocity of around 13 μm/sec (Gilkey et al., 1978). This wave originates at the point of sperm entry (or artificial stimulation), and when first visualized by Gilkey et al. (1978) it was considered to represent Yamamoto's invisible "fertilization wave" (Yamamoto, 1944), predicted some 30 years earlier as a key component that re-initiates development in dormant eggs. The calcium wave is followed some 17 seconds later (Yoshimoto et al., 1986) by a wave of cortical granule (CG) fusion, that results in raising the chorion. The latter helps to prevent polyspermy, and provides a protective shell for the developing embryo until hatching (Hart and Yu, 1980). The close correlation between the propagating calcium wave and the wave of CG fusion suggest that the two are linked. Furthermore, supporting data such as the introduction of a calcium buffer argues that the calcium wave directly stimulates CG fusion with the plasma membrane (Gilkey et al., 1978; Iwamatsu et al., 1988).
Calcium activation waves similar to the first one described by Gilkey et al. (1978) have subsequently been found in almost every system examined, and they display a remarkable conservation in wave velocity (see summary reviews by Jaffe, 1991, 1993). This suggests a common mechanism of propagation and indicates that they are perhaps a fundamental feature of egg activation that is conserved from sponges to mammals (Jaffe, 1991).

1.4 Additional Roles of Ca\(^{2+}\) in Egg Activation

Fertilization is a complex process and involves more than just the fusion of gametes and the restoration of the diploid genome. One of the other important components of fertilization involves the activation of the egg from a dormant state and a re-initiation of development. The meiotic division cycle of different species are arrested at different stages before fertilization. In sea urchins and cnidarians, the egg nucleus completes meiosis before it is arrested. On the other hand, the eggs of amphibians, fish and most mammals are arrested at the second meiotic metaphase (Lorca et al., 1993; Gilbert 1997). Thus, as well as stimulating the CG reaction, it has been proposed that the transient rise in cytosolic calcium during activation contributes to the initiation of a series of complex signal transduction cascades that regulate a variety of cellular responses required for the resumption of development (Striker, 1996). A major component of the signal transduction pathway that controls normal cell cycle progression is cyclin. The breakdown of cyclin enables the eggs to return to interphase and resume the cell cycle, an essential step for the generation of metazoan form (Whitaker and Patel, 1990). When cyclin degradation is prevented by cytostatic factor, eggs are arrested (Lorca et al., 1993). It has been shown that the
cytostatic factor can be inactivated *in vitro* by calcium ions at a comparable levels to the calcium transients during egg activation in medaka (Jaffe, 1985).

In addition to returning eggs to interphase, it has also been suggested that calcium transients also function in (1) activating calcium sensitive transcription factors (Clapham, 1995) and protein kinases (Lorca *et al.*, 1993); (2) mediating lipid, protein and DNA synthesis (Epel, 1990; Freeman and Ridgway, 1993; Gilbert, 1997; Tachibana *et al.*, 1997); and (3) initiating amino acid transport synthesis (Ciapa *et al.*, 1995). Once again, these are all key processes in re-initiating development.

### 1.5 Current Hypotheses on the Initiation of Egg Activation

The generation of an autocatalytic propagating wave of calcium release has become an accepted feature of egg activation (Whitaker and Patel, 1990; Jaffe, 1991). However, the mechanism by which this wave is initiated during the egg activation process is a matter of considerable debate (reviewed by Ozil, 1996). There are currently three hypotheses with regards to the initiation of egg activation (Fig. 1). The first hypothesis is the Calcium Conduit Model (Crétot and Jaffe, 1995) derived from work on sea urchins. It proposes the following: After the acrosome reaction, followed by gamete fusion, extracellular Ca\(^{2+}\) diffuse through the interior of the sperm acrosomal process into the egg, where they are pumped slowly into a local region of the endoplasmic reticulum (ER). This eventually becomes overloaded, at which point Ca\(^{2+}\) is rapidly released which initiates a CICR activation wave. The second hypothesis is the Sperm Contact Hypothesis which proposes that the sperm stimulates the production of inositol 1,4,5-trisphosphate (InsP\(_3\)) via a
Fig. 1 Current hypotheses describing the initiation of egg activation.
membrane receptor and GTP-binding proteins. InsP₃ then acts as a second messenger by releasing Ca²⁺ from internal stores (Whitaker and Irvine, 1984; Miyazaki et al., 1993) to generate a CICR wave. The third hypothesis is the Sperm Factor Hypothesis for signaling at fertilization in mammals (Dale et al., 1985; Swann, 1990; 1993). It suggests that after sperm-egg fusion, a protein from the sperm diffuses into the egg cytoplasm and by an unknown, probably enzymatic mechanism, this sperm factor triggers Ca²⁺ release by sensitizing calcium release channels to CICR. This leads to cycles of Ca²⁺ release from the intracellular stores (Swann, 1990, 1994; Parrington et al., 1996). This soluble sperm protein thus exhibits Ca²⁺ oscillation-inducing ("oscillogen") activity in eggs, and has therefore been termed oscillin. It has a size of about 33 kDa and would appear to be a novel mammalian protein. In spite of their differences, a common feature of the above is that sperm play an important role in all three hypotheses.

It is clear, however, that in many cases eggs can be parthenogenetically activated in the absence of sperm by mimicking the fertilization response, i.e., by pricking eggs in calcium-containing media (Kubota et al., 1987) or adding calcium ionophore (Gilkey et al., 1978). This can lead to a variety of degrees of parthenogenetic development (PD) depending on the nature of the stimulation and the species involved.

In natural situations it appears that in most cases sperm provide the activating stimulus, thus activation and fertilization can almost be used as synonymous terms, and often they are. However, it has been reported that some fish eggs such as goldfish (Yamamoto, 1954) and zebrafish (Hart and Yu, 1980; Hart and Fluck, 1995;
Corley-Smith et al., 1996; Sakai et al., 1997) are naturally activated by the spawning medium (i.e., without sperm/egg contact). If sperm are present, they are then subsequently fertilized. Thus, such fish eggs can be naturally activated without fertilization. If sperm are absent, they still undergo an activation reaction, raise a chorion, and proceed through normal ooplasmic segregation. However, after attempting several abortive cleavages they fail to develop further. This represents a natural situation where an activated egg is not necessarily a fertilized egg. Thus the requirement for sperm has been uncoupled from the activation process, and this would suggest that a different mechanism of activation must exist in the eggs of such fish species.

1.6 Zebrafish as a Model System

Zebrafish, Danio rerio, is a fresh-water tropical fish from the upper Ganges River in India (Figs. 2 and 3). Zebrafish are members of the family Cyprinidae that includes many other species of related danios and barbs. The fish are small (about 3 to 4 cm in length) and are easily reared in small aquariums. A 45-liter tank can keep 25 fish that can produce numerous eggs daily (Westerfield, 1994). The eggs are relatively large (Figs. 4 and 5; about 725 and 650 μm in diameter for unactivated and activated eggs respectively) and transparent, which allows for easy manipulation and observation of developmental processes respectively.

1.7 Morphology of Mature Zebrafish Eggs

As mentioned earlier (Section 1.2), most of the information regarding the role of Ca$^{2+}$ in the activation of fish eggs has been derived from medaka. However,
Fig. 2  Zebrafish (*Danio rerio*) are rapidly becoming a popular model for studying vertebrate development. Scale bar is 1 cm.

Fig. 3  Zebrafish pair off after the light comes on. The male fish tend to be more slender than the female fish, however it is not always easy to differentiate between them. Here the upper fish is a male and the lower plumper one is a female.
Fig. 4  Representative bright-field image of an unactivated zebrafish egg in Coho salmon ovarian fluid (SOF). Scale bar is 200 μm.

Fig. 5  Representative bright-field image of an activated zebrafish egg. Scale bar is 200 μm.
teleost fish are known to display a remarkable variety of adaptations with regards to their breeding behavior, the activation/fertilization strategy of their eggs, as well as in their egg morphology (Hart, 1990).

Mature zebrafish eggs differ significantly from those of medaka in that they do not possess a continuous yolk membrane separating a peripheral cytoplasm from a central yolk mass (Gilkey et al., 1978). In zebrafish, individual yolk globules are homogeneously intermingled with ooplasm throughout the mature egg (Beams et al., 1985). Although not structurally separated by a membrane, they do possess a functional ooplasmic cortex some 15-20 μm thick where yolk globules are excluded. This egg cortex contains a network of endoplasmic reticulum (ER), filamentous actin, and an array of CGs (Hart, 1990). The latter are arranged in multiple layers, which is quite unlike the situation found in medaka where they form a single layer just beneath the plasma membrane (Hart and Yu, 1980).

1.8 Ovulation in Zebrafish

Histological studies have shown that ovulation and oviposition (i.e., egg laying) in zebrafish only occur when they mate, i.e., eggs are not present in the ovarian lumen, nor in the oviduct of females even when they are kept together with males. Thus, eggs are found in the oviduct only during ovipositioning. This indicates that the egg is normally retained within the ovarian stroma except when the fish breed (Hisaoka and Firlit, 1962).
When mature zebrafish eggs are discharged from the ovarian stroma and come in contact with the spawning medium, they spontaneously activate. In the absence of sperm, these eggs will undergo a programmed series of developmental steps, i.e., they undergo a CG reaction, raise a chorion, undergo ooplasmic segregation, and attempt abortive cleavages. Medaka eggs on the other hand do not display such activation on contact with their spawning medium, in fact they display the opposite behavior in becoming “less fertilizable” the longer they remain in their spawning medium in the absence of sperm (Yamamoto, 1944, 1961).

During the spontaneous activation process in zebrafish eggs there is an almost instantaneous reduction in egg size (Roosen-Runge, 1938; Hart and Yu, 1980), which results in an separation of the egg surface from the chorion. This initial process is often described as a “raising” of the chorion, a misleading description, as the separation actually results from a contraction of the zebrafish egg (Hart and Fluck, 1995). Some 30 to 60 seconds after spontaneous activation, the CG reaction begins. By this time, the chorion has already separated from the plasma membrane, thus the initial contraction process occurs independently of the onset of cortical granule exocytosis (Hart and Yu, 1980). The CG reaction in zebrafish eggs is initiated and occurs more or less simultaneously over the whole egg surface (Hart and Yu, 1980). This is, therefore, quite unlike the situation found in medaka.

Thus, the focus of my dissertation was to examine the calcium-related activation/fertilization response in the egg of zebrafish, in relation to these significant differences in morphology, activation/fertilization strategy, and cortical granule (CG) reaction when compared to medaka (Hart, 1990). Furthermore, the
resurgent interest in understanding zebrafish embryology as a model system for vertebrate development (Kimmel et al., 1995), made it the most appropriate candidate for this comparative study.

1.9 Calcium Imaging Techniques

There are two general types of calcium reporters used to study calcium dynamics within living cells: luminescent reporters and fluorescent reporters. In this study, two forms of the luminescent reporter aequorin were used.

1.9.1 Aequorin

Aequorin is a bioluminescent calcium sensitive complex composed of a 21 kDa protein (apoaequorin), a lipophilic factor (coelenterazine) and molecular oxygen (Shimomura 1995a). Aequorin was originally isolated from the jellyfish, *Aequorea aequorea* (Fig. 6; Shimomura, 1995a), and emits blue light (~ 475 nm) in the presence of Ca$^{2+}$ through an intramolecular process in which aequorin is transformed into apoaequorin, coelenteramide and CO$_2$ (Fig. 7; Shimomura et al., 1988). Over a physiological range (approximately 0.1 to 100 μM Ca$^{2+}$), the light output of this process is proportional to the square of the free calcium concentration (Shimomura, 1995b) thus providing inherent contrast enhancement. This calcium-sensitive bioluminescent reporter facilitates the reliable and reproducible visualization of calcium dynamics within living cells (Miller et al., 1990; Fluck et al., 1991a; Webb et al., 1997; Webb and Miller, 1999). Since no excitation light is required for the generation of signal, photo-induced cytotoxicity is not a problem in embryos microinjected with aequorins when compared with those loaded with fluorescent
Fig. 6  Jellyfish, *Aequorea aequorea*, from which aequorin was originally extracted and purified (from front cover of *Nature* 358, 1992).
Fig. 7  Schematic illustration of the luminescence and regeneration of aequorin (from Shimomura et al., 1988, *Biochem. J.* **251**: 405-410).
reporters. Most aequorin-loaded embryos continue to develop quite normally after experimentation (Fluck et al., 1991b; Miller et al., 1994; Webb et al., 1997; Gilland et al., 1999). There is, however, a limit to the concentration of aequorin solution that can be used for microinjection as the accompanying increase in viscosity frequently results in a blocked microinjection pipette, a 1% solution is about as concentrated as one can use. This, however, was more than sufficient for the requirements of my project (approximately $10^{-13}$ moles of aequorin were loaded into each unfertilized egg) as signal could still be detected throughout the cleavage period. This indicates that there was still "unspent" aequorin left in the zygote and that the signal recorded during activation was not reporter-limited. Semi-synthetic and recombinant aequorins have been engineered to respond over a wider dynamic range (0.1 μM to 300 μM; Miller et al., 1994) and with an increased sensitivity to calcium ions than natural aequorin (Shimomura, 1991). I used f- and h-aequorin in this project, the relative light intensity generated being 18 and 10 fold brighter than natural aequorin respectively (Shimomura et al., 1989). In addition, apoaequorin (aequorin’s polypeptide component) has been successfully expressed in Dictyostelium cells (Cubitt et al., 1995), yeast cells (Nakajima-Shimada et al., 1991), Nicotiana seedlings (Knight et al., 1991), Chinese hamster ovary cells (Sanchez-Bueno et al., 1996) and the mitochondria of bovine endothelial cells (Rizzuto et al., 1992). However, attempts to produce a transgenic apoaequorin-expressing zebrafish have not as yet been successful, hence the requirement to microinject aequorin in a recombinant form into the unfertilized egg.
1.10 Overall Objectives of the Project

The questions addressed in my thesis are the following: (1) does the activation of zebrafish eggs involve a calcium transient, (2) as the answer to #1 is "yes", what is the nature of this transient, (3) what is the relationship between the transient and the presence or absence of sperm in the spawning medium, (4) what is the relationship between this transient and the observed CG reaction in zebrafish, and finally (5) how does activation in zebrafish eggs compare to medaka eggs.

At the start of my project the answers to all of the above questions were unknown. This was due mainly to the difficulty of collecting unactivated viable eggs and loading them with a calcium reporter without activating them in the process. Using a combination of techniques developed by myself and others (Miller et al., 1994; Westerfield, 1994; Corley-Smith et al., 1996; Sakai et al., 1997), I have begun to address these questions in zebrafish. This is a species chosen by many to be (1) one of the most appropriate models to study vertebrate development (Nusslein-Volhard, 1994; Roush, 1996a; Brown, 1997), (2) a species subjected to the most extensive vertebrate mutagenesis screen (Nusslein-Volhard, 1994; Holder and McMahon, 1996; Roush, 1996b), (3) a species who's genome is currently being mapped as a key component of the human genome project (Gordon, 1997; Roush, 1997), and (4) a species chosen by the National Institutes of Health (NIH) to be a model for non-mammalian biomedical research (Roush, 1997).
In the following chapters, I start to answer these important questions concerning the role of Ca\textsuperscript{2+} signaling in zebrafish activation and thus provide some information on this vital, but poorly understood phase of zebrafish development.
MATERIALS AND METHODS

2.1 Egg Collection

Zebralish (Danio rerio) were maintained on a 14 hr light/10 hr dark cycle to stimulate spawning (Fig. 3; Westerfield, 1994). Unactivated eggs and sperm were squeezed directly from fish by methods modified from Westerfield (1994). Individual fish were anesthetized in tricaine (Sigma Chemical Co., St. Louis, MO, USA; 1.7% in 30% Danieau’s solution: 19.3 mM NaCl; 0.23 mM KCl; 0.13 mM MgSO4.7H2O; 0.2 mM Ca(NO3)2; 1.67 mM HEPES pH 7.2) until gill movement had slowed. After rinsing with egg-water (60 μg/liter ‘Instant Ocean’ (Aquarium Systems, Mentor, OH, USA) in deionized water) and blotting ‘damp-dry’ with a Kimwipe, female fish were transferred to an agarose-coated 60 mm petri-dish, turned ventral side up, then (using damp fingers) squeezed gently along the belly in a head-to-tail motion (Fig. 8). In cases where unactivated eggs were discharged, they were collected using 50 μl micropipettes (Wiretrol; Drummond Scientific Company, Broomall, PA, USA) pre-wetted with Coho salmon (Onchorhynchus kisutch) ovarian fluid (SOF; Corley-Smith et al., 1996; SeaTech Bioproducts, Newton, MA, USA) or 0.5% bovine serum albumin (BSA fraction V; Sigma) in Hank’s buffered saline (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 1.3 mM CaCl2, 1.0 mM MgSO4, 4.2 mM NaHCO3) and placed in a custom-designed injection chamber. Male fish were transferred to a ‘fish-shaped’ groove cut into a sponge, turned ventral side up, then squeezed gently along the belly (Fig. 9). Sperm were collected using a 50 μl micropipette pre-wetted with sperm extender solution (10 mM HEPES, 80 mM KCl, 45 mM NaCl, 45 mM C2H3NaO2, 0.4 mM CaCl2, 0.2 mM MgCl2), and stored in sperm extender solution on ice until required.
Fig. 8  Collection of unfertilized eggs. Female zebrafish are anesthetized, blotted dry on a Kimwipe and then squeezed along the abdomen in a head to tail direction. The unfertilized eggs are collected using a 50 μl Wiretrol micropipette, pre-wetted with either Coho salmon ovarian fluid (SOF) or 0.5% bovine serum albumin (BSA) in Hank's buffered saline, and stored in either of these solutions.

Fig. 9  Collection of sperm. Male zebrafish are anesthetized and blotted dry on a Kimwipe. The fish are then transferred to the “fish-shaped” groove cut into a sponge and squeezed for sperm collection. Sperm are stored on ice in sperm extender solution.
2.2 General Microinjection Procedures

Unactivated zebrafish eggs are quite difficult to microinject, both during the penetration and withdrawal phase. They were therefore placed in a custom-designed injection chamber mounted on a coverglass (22 x 22 mm; No. 1; Chance Propper Ltd., West Midlands, England) attached, using high vacuum silicone grease (Dow Corning Co., Midland, MI, USA), underneath a hole cut in a 35 mm petri-dish lid. These injection chambers consisted of a 800 μm diameter hole cut into a 12 x 10 x 1 mm plexiglass block, with a 200 μm wide slit opening into its side, through which the microinjection pipette could be inserted (See Fig. 10). They were designed to hold unactivated eggs in place during microinjection, and to prevent the eggs from sticking to the tip of the micropipette when withdrawing it following injection. These chambers also provided reasonable inverted viewing optics for imaging purposes, so eggs were also activated and fertilized in them.

Microinjection pipettes were made from borosilicate glass capillaries (1.0 mm diameter, with filling fiber; World Precision Instruments, Inc., Sarasota, FL, USA), using a horizontal puller (Figs. 11 and 12; PN-3; Narishige Scientific Instrument Laboratory, Tokyo, Japan). The tip of each micropipette was broken and then beveled to a 30° angle with a microgrinder (Fig. 13; EG-40; Narishige Scientific Instrument Laboratory, Tokyo, Japan). Sharpened tip diameters were maintained between 5 and 10 μm at the widest part of the bevel.

Unactivated eggs were injected using a pressure-injection system (Fig. 14A; PLI-188; Medical Systems Corp., Greenvale, NY, USA) in combination with a
Fig. 10  Schematic outline of my custom-designed egg injection chamber.
Fig. 11  Narishige PN-3 horizontal puller use for making micropipettes.

Fig. 12  Close-up of the Narishige PN-3 horizontal puller.
Fig. 13 Narishige EG-40 microgrinder used to bevel the micropipettes to aid penetration of unfertilized zebrafish eggs.
Fig. 14  Unfertilized eggs are injected with aequorin using a (A) PLI-188 pressure injection system in combination with (B) a Zeiss MMJ micromanipulator and (C) a Zeiss Stemi SV6 stereomicroscope using bottom illumination.
micromanipulator (Fig. 14B; MMJ; Zeiss, Inc., Jena, Germany) and a bottom illumination stereomicroscope (Fig. 14C; Stemi SV6; Zeiss, Inc., Jena, Germany).

2.3 Microinjection of Aequorins

All glass and plastic-ware associated with the aequorin injections were pre-washed for several minutes with 100 μM EGTA (Fig. 15). Several 50 μl drops 100 μM EGTA solution were placed on the inside of a 35 mm petri-dish lid. The solution was pre-filtered through a 0.2 μm Millipore filter to remove any dust particles that may clog the micropipette. The tip-washing EGTA solution was sucked up and extruded several times to wash both the inside and outside of the micropipette tip. The extent of the internal washing was marked on the micropipette shaft with a marker pen. During this pre-wash, an initial calibration of the injection volume was done by injecting droplets of EGTA solution under EGTA-washed Wesson vegetable oil. The injected volume was controlled by keeping the injection pressure at 15 PSI (105 kPa) and varying the duration of the pressure pulse. The diameter of the droplets produced was measured with an eyepiece reticle (Miller et al., 1994), hence their volumes could be calculated \((\frac{4}{3} \pi r^3)\). Approximately 0.8 μl of aequorin was placed in a pre-washed petri-dish lid with a pre-washed pipette tip. Aequorin was front-loaded into the pre-washed micropipette. The calibration of the micropipette was readjusted by ejecting droplets of aequorin solution under EGTA-washed Wesson vegetable oil. The tip of the aequorin-loaded micropipette was kept in the EGTA-washed oil between injections to prevent evaporation from the tip that might result in clogging.
Fig. 15  Schematic illustration of the procedure used for injecting unfertilized eggs with aequorin, (A) washing, (B) loading and, (C) calibrating the micropipette, and (D) microinjecting aequorin using a high pressure microinjection setup shown in Fig. 14. 1, Micropipette holder; 2, micropipette; 3, 50 µl droplet of a 0.2 µm Millipore-filtered 100 µM EGTA washing solution; 4, EGTA-washed 35 mm petri-dish lid; 5, 0.8 µl droplet of aequorin solution; 6, EGTA-washed Wesson vegetable oil; 7, calibration droplets, diameters measured with eyepiece reticle; 8, unactivated-egg holder; 9, coverglass; 10, unfertilized zebrafish egg; 11, injection chamber.
Approximately 3.0 nl of recombinant f- or h-aequorin (1% in 100 mM KCl, 5 mM MOPS and 50 µM EDTA; Shimomura et al., 1990) was injected into the center of unactivated eggs. Following injection, eggs were transferred to a Photon Imaging Microscope (Figs. 16 and 17; PIM; Science Wares) or a Photo-Multiplier Tube (Figs. 18 and 19; PMT; H 5920-01; Hamamatsu Photonics K. K., Hamamatsu City, Japan) for data acquisition. The aequorin was allowed time to diffuse evenly throughout the egg (a process that took approximately 20 minutes as described in the results; see Section 3.6.1 and Fig. 28) before eggs were activated. During this time a resting level of luminescence was obtained for each experiment.

2.4 Microinjection of a FITC-Dextran

The time required for aequorin to spread throughout the unfertilized zebrafish was estimated by monitoring the spread of a fluorescent dextran of ~21 kDa (i.e. approximately the same molecular weight as aequorin) through the egg. Fluorescein isothiocyanate-dextran (1.2 nl of a 1% solution in 150 mM KCl, 5 mM HEPES, pH 7.2; Sigma) was injected into the center of unfertilized eggs and a time series (every 5 minutes) of the eggs was imaged using the PIM described in section 2.6.1 Images were collected using a Zeiss Fluar 10X/0.5 NA objective.

2.5 Egg Activation/Fertilization

In order to either activate eggs in the absence of sperm, or to activate and fertilize eggs in the presence of sperm, data acquisition was briefly suspended, and most of the 0.5% BSA in Hank's saline, or SOF, was removed from the injection chamber. In the case of activation in the absence of sperm, 100 µl of 0.5% fructose in egg-water
Fig. 16   The Photon Imaging Microscope (PIM) with its controlling and data acquisition computer.

Fig. 17   The Imaging Photon Detector in its custom-built enclosure attached to the bottom "Keller" port of the Zeiss Axiovert 100TV microscope.
Fig. 18  The Photo-Multiplier Tube (PMT) system.

Fig. 19  Inside the dark box of the PMT system showing an injection chamber sitting on top of the mechanical shutter. The photocathode of the PMT is directly below.
was added to the injection chamber. In the case of activation followed by fertilization, 30 μl of the sperm suspension (in sperm extender solution) was placed in the injection chamber. Allowing approximately 30 seconds for the sperm to drop to the level of the egg, 100 μl of 0.5% fructose in egg-water was then added to activate the sperm. Following the above procedures, data acquisition was immediately resumed.

2.6 Aequorin-Derived Data Acquisition and Review

2.6.1 Photon Imaging

2.6.1.1 Outline of the Photon Imaging Microscope

A schematic outline of this system is shown in Fig. 20. A Zeiss Axiovert 100TV microscope is mounted on an air table (TMC; Peabody, MA, USA) covered by a dark box. A hole in the air table allows a custom-built enclosure housing a resistive anode imaging photon detector (RA-IPD; Photek Inc., East Sussex, UK) to be attached to the bottom “Keller” port of the microscope. The enclosure has a dual-overlapping blade mechanical shutter (Uniblitz VS25; Vincent Associates, Rochester, NY, USA) to protect the RA-IPD during bright-field illumination, and a recirculating water-cooled thermoelectric system that chills the RA-IPD to -12 ± 1°C. The cooling water is recirculated with AC piston pumps through a small refrigerator bath that maintains the water at 8 ± 2°C. The enclosure is attached to the microscope via a c-mount to Zeiss-port adapter containing a 0.5X reducing lens. This adapter both reduces the size of the image projected onto the photocathode of the RA-IPD and seals off a chamber over the photocathode into which dry air with a -100°F dew point is flowed at a rate of 200 ml/min (Balston 75-20 Instrument Air Drier; Haverhill, MA, USA). This prevents condensation from forming and freezing on the cooled photocathode surface.
Fig. 20 Schematic outline of the Photon Imaging Microscope (PIM) system.
The Zeiss Axiovert 100TV microscope has a sliding 100% mirror located directly below the tube lens in line with the objective lens. When placed in the optical path, the mirror reflects light through the trinocular headpiece to either the eyepieces or a CCD camera (TK 1281; JVC, Yokohama, Japan). When the mirror is removed from the optical path, light from the objective passes directly through the 0.5X reducing lens and (if the shutter is open) onto the photocathode of the RA-IPD. The mirror motion is automated by a reversible permanent magnet DC motor attached to the mirror slider handle. The actuator system allows the mirror to lock into place to ensure the correct optical alignment of the image projected to the eyepieces or the CCD camera.

The microscope stage and focus are automated with a custom-built microstepping motor system (Science Wares, Inc.; East Falmouth, MA, USA) that enables remote control of the sample position and microscope focus while the dark box remains closed. The epi-fluorescence filter slider is automated with a custom-built optical limit switch system (Science Wares, Inc., East Falmouth, MA, USA) that enables remote control of the filter position while the dark box remains closed. Bright-field and epi-fluorescence illumination of the sample is accomplished by mounting the standard Zeiss 100W tungsten filament bulb housing and Zeiss HBO 100W mercury arc lamp housing with variable intensity atto arc lamp control outside the dark box and coupling the light from the light source through a G. W. Ellis fiber-optic light scrambler with appropriate fittings (Technical Video, Ltd., Woods Hole, MA, USA) to the illuminator carrier of the microscope respectively. A second computer-driven shutter is located between the lamp housing and collecting lens housing for the fiber optic scrambler in order to prevent light (via the fiber optic) from entering the dark box during photon collection. Both bright-field, fluorescent and photon images were
collected using either a Zeiss Fluar 10X/0.5 NA, Zeiss Plan Neofluar 10X/0.3 NA or 5X/0.15 NA objectives.

2.6.1.2 Data Acquisition

Microscope automation and data acquisition are controlled by a custom-built application developed in Microsoft Visual C++ (Science Wares, East Falmouth, MA, USA) for a Windows95 platform (subsequently referred to as IpdWin95). This manages the data acquisition and records all experimental operations in a central file that references photon image data files to bright-field image data files. The photon image data for an entire experiment consists of only two files; a list of photon coordinate pairs (8-bit x, 8-bit y) and a list that pairs time tags with indices into the photon coordinate pair list. Time tags are generated by a periodic software interrupt (at approximately 500 ms intervals), which stores the total number of milliseconds elapsed since the start of the experiment. The time-tag index file is used to determine which photon coordinate pairs to read from the photon coordinate pair list file when reconstructing a photon image for a given time interval. The CCD camera PAL video signal output is digitized by a frame-grabber (Matrox Meteor; Matrox Image Products Group, Quebec, Canada) into a 768 X 768 pixel 8-bit grayscale image and stored as a .TIFF 6.0 format file.

After data acquisition has been completed, the IpdWin95 software is then used to review the acquired photon, fluorescent and bright-field image data simultaneously with any chosen integration periods and time steps. The software can also be used to superimpose photon data on captured bright-field or fluorescent images, thus allowing the correlation of photon events with morphological features. For subsequent detailed
quantitative data analysis, a series of photon and corresponding bright-field or fluorescent images are exported in .TIFF file format to Metamorph 3.0 (Universal Imaging, Inc., West Chester, PA, USA) for image processing. For figure preparation and presentation files are downloaded into Corel PHOTO PAINT 7 and Corel DRAW 7 (Corel Corp., Ontario, Canada).

2.6.2 Photon Counting

Quantitative aequorin-generated data was also acquired using a PMT system, which is outlined in Fig. 21. The system was designed so as to place the aequorin-loaded egg (within the injection chamber, see Fig. 10) as close to the bi-alkali photocathode as possible. The whole system is mounted within a dark box and a 25 mm diameter dual-overlapping blade mechanical shutter (Uniblitz VS25; Vincent Associates, Rochester, NY, USA) is positioned directly above the photocathode in order to protect it from ambient light during sample manipulation. During data collection, the entire injection chamber is covered with a concave reflective mirror to direct light from the egg onto the photocathode surface. The quantum efficiency of the photocathode is approximately 11% at 470 nm (data supplied by Hamamatsu), and the dark counts (i.e., the system noise) are in the order of 10-15 photons/sec. The whole system is mounted within a dark box designed to block ambient light from the photocathode when it is exposed to light from the sample. The output pulses from the PMT are recorded by a computerized data acquisition system, called PMT for Windows 95 (Science Wares), via an electronic interface unit. This software stores the count rate values in a sequential file, and allows data acquisition parameters and text comments from an experimental log to be recorded on a separate file. The program provides real-time and
Fig. 21  Schematic outline of the Photo-Multiplier Tube (PMT) system.
post-experimental data displays as well as a variety of analysis functions. PMT-generated data are also downloaded into Microsoft Excel 97 for graph plotting, and finally into Corel DRAW 7 for figure preparation.

2.7 Microinjection of Fluorescent-Microspheres

Any rotational movements of eggs during the activation process (i.e., once the plasma membrane and chorion separate) could potentially lead to a misinterpretation of the imaging data. At activation, however the homogenous zebrafish egg has no distinctive features that would make the observation of rotational movements obvious. Thus, an experimental protocol had to be developed to identify any rotational movements of the eggs during activation. Approximately 2.3 nl of 0.1% nile red-labeled microspheres (2 μm; carboxylate-modified; dissolved in 150 mM KCl, 5 mM HEPES, pH 7.0; Molecular Probes, Eugene, OR, USA) were injected into the periphery of eggs. Injected eggs were then observed via a Zeiss Plan Neofluar 10X/0.3 NA objective using a Bio-Rad MRC-600 laser scanning confocal microscope (Fig. 22; Bio-Rad Labs., Richmond, CA, USA) equipped with a krypton/argon laser, mounted on an upright microscope (Axioskop, Zeiss Inc., Jena, Germany). The most appropriate optical plane for viewing the fluorescent microspheres was located. Eggs were then parthenogenetically activated using 0.5% fructose in egg-water (as described in Section 2.5) and a confocal time-series (every 15 seconds) was recorded. From this I could determine any rotational movements of the egg during activation.

The corresponding fluorescent and bright-field images were reviewed using Confocal Assistant 4.02 (down-loaded from the Bio-Rad FTP-server: ftp://ftp.genetics.biorad.com/public/confocal in .PIC file format), and Metamorph 3.0.
Fig. 22  The Bio-Rad MRC 600 Confocal Laser Scanning Microscope.
Presentation files for figure preparation were downloaded into Corel PHOTO PAINT 7 and Corel DRAW 7.

2.8 Experiments with No Added Calcium

In a series of experiments, precautions were taken to minimize levels of free Ca\(^{2+}\) external to unactivated eggs, before and during the activation process. 0.5% BSA in Hank's buffered saline solution with no added calcium was prepared in ultra-pure H\(_2\)O by omitting CaCl\(_2\), and adding 0.3 mM EGTA to chelate trace levels of contaminating calcium arising from the other components. All glassware and plasticware used in handling eggs was also pre-washed for several minutes with 100 µM EGTA solution. Unactivated eggs were collected (see Section 2.1) in the modified medium described above.

2.9 Real-Time Video Recording of Egg Activation

Real-time video recordings of the activation process both in the presence and absence of sperm were made using a CCD camera (TK-C1381; JVC, Yokohama, Japan) mounted on a Zeiss stereomicroscope (Stemi SV6; Zeiss Inc., Jena, Germany), with images recorded on a VCR (AG-6750; Panasonic, Matsushita Electrical Industrial Co. Ltd., Japan). Images were then exported to Metamorph 3.0 for data analysis. Three aspects of the activation process were analyzed both in the presence and absence of sperm in the activation medium. (1) The timing of activation events (i.e., the onset of the cortical granule reaction, followed by the raising of the chorion) after the addition of the activation medium. (2) The nature of the cortical granule reaction during the activation process. (3) An estimation of size changes of eggs following activation.
RESULTS

3.1 Collection of Unactivated Zebrafish Eggs

A major impediment to the study of the activation/fertilization process in zebrafish lay in the difficulties in collecting and maintaining unactivated eggs. This is due to the fact that they spontaneously activate when they come into contact with the very medium the fish live in, i.e., water. This reproductive strategy appears to be linked to the breeding behavior of these fish, and is discussed in Section 4.1.

At 29°C, female zebrafish exhibit an ovarian cycle of approximately 2 days in optimal, healthy conditions (Hisaoka and Firlit, 1962). However, one cannot tell from the external appearance of a female fish where she is in her ovarian cycle, the only way being to anesthetize the fish then attempting to squeeze eggs out of her as described in Section 2.1. The process of ovipositioning is stimulated by a combination of the photoperiod (i.e., the lights coming on), and the physical presence of a male fish. The latter is thought to release some kind of pheromone into the water in close proximity to a particular female when they pair-off during their mating behavior (Hoar, 1955).

If a ripe female is not stimulated by a male fish to start laying her eggs, she will begin to re-absorb them back into her body in order to recycle components for her next ovulation (Hisaoka and Firlit, 1962). This strategy is not surprising considering the ratio of egg to fish size in zebrafish (i.e., this small 3 to 4 cm fish lays between 300-500, 725 μm diameter eggs at each ovulation). Thus, the quality of the eggs in terms of their eventual fertilizibility deteriorates with time. This
means that after the light comes on, the longer it takes you to find a ripe female who is in a condition to lay, the poorer the quality the eggs will be. In practical terms, it means you have about 40 minutes to find a ripe fish.

Thus, the logistics of just obtaining good biological material to begin experimentation with is extremely complex. The outcome of a day’s experiment often relies on luck. Even when one is successful in finding and squeezing a ripe fish and she discharges good eggs, this is where a new set of experimental challenges begins. How do you prevent these eggs from activating?

3.2 Holding Media for Unactivated Eggs

The first successful strategy used to hold zebrafish eggs in an unactivated state, was to mimic conditions within the zebrafish ovary. This was done using ovarian fluid collected from Coho salmon (SOF). The rational for using SOF was that it could be collected in sufficient quantities to make its use practical. It was reported that SOF could hold zebrafish eggs in an unactivated state for up to 90 minutes (Corley-Smith et al., 1996), and its eventual commercial availability (from SeaTech Bioproducts) opened a new chapter in studying the activation/fertilization mechanisms in zebrafish. This was one of the major factors that inspired my choice of research project.

However, the supply of SOF was erratic, and the effectiveness of each individual batch obtained varied. This made it very frustrating, as SOF was a key component of my research methodology. Suspecting that the effectiveness of SOF
resulted from osmotic factors rather than a specific component(s), Sakai et al. (1997) reported that concentrations of bovine serum albumin (BSA) in Hank’s buffered saline proved to be an effective and reliable substitute for SOF. Concentrations below 0.1% had no effect on preventing egg activation, while above 0.1% an effect was observed, which reached a maximum efficiency at 0.5%. This concentration dependency also suggests that the activation trigger might be osmotic in nature. Thus, midway through my project I switched from using SOF to 0.5% BSA in Hank’s buffered saline to maintain eggs in an unactivated state.

In both SOF and BSA, there is a significant decrease in the fertilization percentage of eggs the longer they are held in these solutions. This raises additional problems with regards to a project based on Ca$^{2+}$ imaging, as I had to load an egg with a reporter, then wait until this had diffused evenly throughout the egg (i.e., approximately 20 minutes; see Section 3.6.1 and Fig. 28) before inducing activation. This means in reality that out of each batch of good eggs collected and loaded with aequorin (discussed in the following section) perhaps only one egg could actually be successfully activated and provide data during each experimental session. The data reported in my thesis resulted from over 400 individual experiments all of which began with the collection, then maintenance of eggs in an unactivated state.

3.3 Microinjection of Unactivated Zebrafish Eggs

The tough chorion of an unactivated egg adheres to the plasma membrane. This makes these eggs very difficult to microinject. Even with a sharpened micropipette tip, injection is like attempting to push a finger into a balloon full of
water. Thus, if the egg is not supported through 360°, it will just roll in any direction when force is applied to effect micropipette penetration. Furthermore, once the egg has been penetrated, it is extremely “sticky”, and it is very hard to remove the micropipette without bringing the egg with it. This is why I had to design a custom-made injection and viewing chamber (see Fig. 10). This chamber provided the necessary movement-restricting support to allow unfertilized eggs to be injected, and was also able to hold them in place during the withdrawal of the micropipette. Eggs could be activated and fertilized within these chambers while being viewed with inverted optics through their coverglass base.

3.4 Calcium Wave Velocities

Fig. 23 shows a representative example (n = 4) of a calcium wave traversing a zebrafish egg following spontaneous activation in the absence of sperm. For imaging purposes, this particular egg was in a perfect side-on orientation, with the animal pole (AP) located at the left hand side. This made analyzing the velocity of the wave easy. This was done using the “Linescan” function in Metamorph 3.0. A plot of wavefronts at three separate times is illustrated in Fig. 24, indicating a velocity along the AP to vegetal pole (VP) axis of approximately 6 μm/sec. When comparing bright-field images before and after activation (i.e., Figs. 23A with 23K respectively), the chorion has clearly elevated by the time the calcium wave has traversed the egg. Eggs activated in this manner segregate normally to form a blastodisc, then attempt abortive cleavages. They do not, however, develop any further.
Fig. 23 Representative sequence of images from an aequorin-loaded egg illustrating changes in intracellular free Ca$^{2+}$ during activation in the absence of sperm. Panel A is a bright-field image of the unactivated egg just prior to the initiation of the signal, and shows no separation between the chorion and the egg plasma membrane, whereas Panel K shows the egg after the passage of the calcium wave, clearly indicating (see arrowhead) a raised chorion. Each photon image (Panels B to J) represents 60 seconds of accumulated light, with a 20 second gap separating each successive image. Panel L shows a schematic illustration of the location of the animal pole (AP) after activation. This egg developed only as far as a few abortive cleavages. Color scale indicates luminescent flux in photons per pixel. Scale bar is 200 μm.
Fig. 24  Analysis of the wave illustrated in Fig. 23 for egg activation in the absence of sperm. Profiles of the intensity of photon emission, taken from Panels D, F, and H in Fig. 23, were measured using the Linescan function of Metamorph 3.0. A line length of 700 μm was used, as this was the diameter of the egg. Each profile shows the relative intensity of photons collected for 60 seconds. The schematic diagram in the top corner shows subsequent blastodisc (BD) formation, and thus the location of the AP during wave propagation. The dashed lines indicate the half-heights used as an arbitrary indication of the position of the wave fronts at each time point. The Linescan analysis assumes that the wave passes directly through the egg along the AP to VP axis, thus I refer to this value as the “axial velocity”. This is converted to a “cortical velocity” which assumes that the wave travels the hemi-circumference distance between the AP and the VP (i.e., this equals approximately 1100 μm for this egg). AP and VP are the animal and vegetal poles respectively.
Fig. 25 illustrates a representative example (n = 3) of a calcium wave in an egg activated in the presence of sperm, which was then subsequently seen to have been fertilized - as it went on to divide and develop normally (see Fig. 25L). In this example, the egg is not in a perfect side-on orientation for imaging, as its AP is tilted away from the collecting lens as indicated in the schematic outlines illustrated in Fig. 26. There is no clear morphological indicator of the AP or VP in unfertilized zebrafish eggs. The most reliable polarity marker is the eventual location of the forming blastodisc, assuming that the egg does not move or rotate during activation and early segregation. Unfertilized eggs were therefore loaded into the injection chambers in a random orientation, and as a result, I never imaged an egg activated and fertilized in the presence of sperm, in a perfect side-on orientation as illustrated in Fig. 23. Thus, the imperfect orientation of the egg shown in Fig. 25 led me to use only the left-hand-side of the egg to generate the wave-front plots indicated in Fig. 26, and so the right-hand-side of each plot is represented by a broken line. This analysis once again reveals a wave velocity of around 6 μm/sec. In both Figs. 24 and 26 I used the half-height of the advancing wave front in my calculation. Whether activated in the presence or absence of sperm, all waves were initiated at the AP, the site of the micropyle.

3.5 Characteristics of the Calcium Transients

Fig. 27 compares the profile, duration, and extent of the aequorin-generated luminescence in two representative examples of eggs activated in the absence (Figs. 27 A and C) or presence of sperm (Figs. 27 B and D). These data were collected using the PMT system (see Figs. 18, 19 and 21), and are included in the data
Fig. 25  Representative sequence of images from an aequorin-loaded egg illustrating changes in intracellular free Ca$^{2+}$ during activation in the presence of sperm. Panel A is a bright-field image of the unactivated egg just prior to the initiation of the signal, and once again shows no separation between the chorion and the egg plasma membrane, whereas Panel K shows the egg after the passage of the calcium wave, clearly indicating (see arrowhead) a raised chorion. Panel L shows the egg at the 4-cell stage, indicating that it has been fertilized and is developing normally. The photon images (Panels B to J) represent 60 seconds of accumulated light, with a 20 second gap separating each successive image. The dividing blastodisc in Panel L indicates the location of the AP. Color scale indicates luminescent flux in photons per pixel. Scale bar is 200 $\mu$m.
Fig. 26    Analysis of the wave illustrated in Fig. 25 for egg activation in the presence of sperm. Profiles of the intensity of photon emission from Panels C, D, and E in Fig. 25, were measured using the Linescan function of Metamorph 3.0. A line length of 750 μm was used, as this was the diameter of the egg. Each profile shows the relative intensity of photons collected for 60 seconds. The schematic diagram shows subsequent formation of the blastodisc (BD), and thus the location of the AP during wave propagation. The broken profile lines indicate that only the left-hand-side of this plot was used to calculate a wave velocity (see Section 3.4). The dashed lines indicate the half-heights used as an arbitrary indication of the position of the wave fronts at each time point. The Linescan analysis assumes that the wave passes directly through the egg along the AP to VP axis, thus I refer to this value as the "axial velocity". This is converted to a "cortical velocity" which assumes the wave travels the semi-circumference distance between the AP and the VP (i.e., this equals approximately 1178 μm for this egg). AP and VP are the animal and vegetal poles respectively.
Fig. 27 Representative profiles of luminescence from aequorin-loaded eggs activated either in the absence (A and C), or presence (B and D) of sperm. These data were gathered using the PMT system. Figs. A and B illustrate plots (every 5 seconds) of the total luminescent output for the entire activation process. Panels C and D show luminescent output at a higher temporal resolution (every 1 second) indicating an initial period (around 3-5 seconds) of lower level emission prior to the explosive rise. Red arrows in A and B indicate the addition to the injection chamber of 0.5% fructose in egg-water (A), or 0.5% fructose in egg-water 30 seconds after the addition of the sperm suspension (B). Panel C illustrates the first 10 seconds of the activation response of the egg shown in Fig. 23, indicating the initial low intensity localized response around the micropyle (see arrowhead) at the animal pole. Color scale indicates luminescent flux in photons per pixel. Scale bar is 200 μm.
summary presented in Table 1 (eggs activated in the absence of sperm, n = 12; in the presence of sperm, n = 3). Like the wave propagation velocities described in the previous section, there were no significant differences between the profiles of the egg responses under the two activation conditions (see Appendices IV to VI). The higher time resolution plots of the initiation of the transients shown in Figs. 27 C and D, indicate that under both conditions there is an initial period (of a few seconds) where there is a slow increase in luminescent output, followed by a main explosive rise, which again is very similar under the two experimental conditions. Fig. 27C' illustrates that the initial slow luminescent increase is localized to a region surrounding the micropyle at the AP.

3.6 Wave Propagation Pathway

3.6.1 Spreading Time of a FITC-Dextran

Fig. 28 illustrates a representative example (n = 5) of the spreading time of a 21 kDa FITC-dextran injected into the center of an unfertilized egg. The time series of images indicates that it takes approximately 20 minutes for the FITC-dextran to spread to the periphery of the egg. It is reasonable therefore, to assume that the 21 kDa aequorin molecule would do the same in a similar time period. This led me to introduce a “20 minute wait” in my experimental protocol between injecting aequorin into an unfertilized egg, and activating it.

It is also clear that a considerable level of fluorescent signal remains in the center of the egg after this time. Again assuming that aequorin behaves in the same way, it indicates that if the calcium activation wave were to pass through the center
Fig. 28 A representative example of a zebrafish egg which was injected with a 21 kDa FITC-dextran (molecular weight approximately the same as aequorin) into the center of the egg. Panel A is bright-field image and Panel B the corresponding fluorescent image taken immediately after injection. Panels C to F are fluorescent images taken at 5 minute intervals after the injection. In the fluorescent images (Panels B to F), the outline of the egg is indicated. Scale bar is 200 μm.
of the egg, there would be sufficient reporter present to visualize its passage. This supports our aequorin-based observations discussed in the next section, in that it indicates that the limited aequorin-generated signal from the center of the egg is not due to a lack of reporter.

3.6.2 Aequorin-Generated VP View of the Activation Wave

The VP view of a representative activation wave \( n = 4 \) illustrated in Fig. 29 indicates that the calcium wave appears to propagate mainly through the periphery of the egg in the yolk free cortex. It also suggests that the level of free \( \text{Ca}^{2+} \) in the deep interior of the egg also seems to rise during the propagation of the wave, but not to the same extent as in the cortex.

3.7 Corrected Cortical Velocities

The method of analyzing activation wave velocities using the Linescan function of Metamorph results in “axial velocities”, i.e., it assumes that the wave passes directly through the egg along the AP to VP axis. As indicated above, my imaging data (Fig. 29) suggests that the propagating calcium wave passes through the cortex of the egg, i.e., a distance more like the hemi-circumference rather than the diameter of the egg. Thus, I corrected my Linescan-derived axial velocity values to reflect this, calling them “cortical velocities” (see Appendix III, Figs. 24 and 26, and Table 1). This correction results in activation wave velocities of around 9 \( \mu \text{m/sec} \) for eggs activated in either presence or absence of sperm
Fig. 29  Representative series of images of a vegetal pole (VP) view of an activation wave indicating that the wave propagates mainly in the periphery of the egg. Panels A and F are bright-field images of the egg before and after activation respectively. The arrowhead in Panel F indicates the raised chorion. Panel B illustrates the resting level of luminescence prior to activation. Panel C indicates the activation wave at the equator of the egg, and Panels D and E demonstrate the subsequent progression of the wave to the VP. The outline of the egg is indicated. Panels B to E show 60 seconds of accumulated light. Color scale indicates luminescence flux in photons per pixel. Scale bar is 200 μm.
<table>
<thead>
<tr>
<th></th>
<th>Activation without sperm</th>
<th>Activation with sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>From PIM Experiments</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cortical Ca(^{2+}) Wave Velocity</strong></td>
<td>8.9 ± 0.8 μm/sec (n = 4)</td>
<td>9.0 ± 0.2 μm/sec (n = 3)</td>
</tr>
<tr>
<td>Wave Initiation Site</td>
<td>Animal Pole</td>
<td>Animal Pole</td>
</tr>
<tr>
<td><strong>Cortical Granule Reaction</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Chorion Elevation</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Ooplasmic Segregation</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Cleavage</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Normal Development</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>From PMT Experiments</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Duration of Luminescence Pulse</strong></td>
<td>7.1 ± 2.0 min (n = 12)</td>
<td>7.4 ± 1.2 min (n = 3)</td>
</tr>
<tr>
<td><strong>Rise Time to Luminescence Peak</strong></td>
<td>1.0 ± 0.5 min (n = 12)</td>
<td>0.7 ± 0.1 min (n = 3)</td>
</tr>
<tr>
<td><strong>Ratio of Ca(^{2+}) Rise above Resting Level (/Luminescence)</strong></td>
<td>138.00 ± 21.8 fold (n = 3)</td>
<td>116.4 ± 11.0 fold (n = 3)</td>
</tr>
</tbody>
</table>

**Table 1** Data summary from the PIM and PMT experiments. Values represent mean ± SD.
3.8 Rotational Movements during Activation

My data indicate that zebrafish eggs do not rotate once the chorion has separated from the plasma membrane during the activation process. Fig. 30 (a representative example, n = 3) clearly indicates that nile red-labeled microspheres injected into the periphery of an unactivated egg maintain their general position throughout the activation process.

3.9 Activation in Medium with No Added Calcium

When eggs were collected in 0.5% BSA in Hank’s saline with no added calcium, they remained unactivated for five minutes, then spontaneously activated (n = 30). These experiments indicated two factors. Firstly, that external calcium was required in the 0.5% BSA Hank’s solution to maintain the eggs in an unactivated condition, and secondly, that the calcium both initiating and generating the activation signal could be provided mainly by internal stores.

3.10 Real-time Video Analysis of Egg Activation

A close analysis of real-time recordings, using Metamorph 3.0, indicated that during activation in either the absence or presence of sperm, there were no qualitative or quantitative differences in the timing of the activation events. The onset of the cortical granule reaction followed by the raising of the chorion was the same, approximately 30 to 60 seconds after the addition of the activating media.

I also confirmed the observation of Hart and Yu (1980), that the cortical granule reaction in zebrafish eggs occurs all over the surface of the egg at
**Fig. 30** A representative example of a zebrafish egg loaded with fluorescent microspheres into its periphery. Panels A and F are bright-field images taken before and after activation respectively. Panel A shows no separation between the chorion and the egg plasma membrane, whereas in Panel F, the chorion is clearly raised (see arrowhead). Panels B to D indicate fluorescent confocal images taken at an optical plane containing the fluorescent microspheres. The image in Panel B was taken just before the addition of 0.5% fructose in egg-water, whilst those in Panels C and D were taken 2.5 and 5 minutes after activation was initiated. Panel E shows the fluorescent images from Panels B, C, and D superimposed on one another. This demonstrates that the egg does not rotate within its expanding chorion during the activation process. Scale bar is 200 μm.
approximately the same time, i.e., there is not a distinct wave of cortical granule fusion and exocytosis as displayed by medaka eggs (Gilkey et al., 1978). This near-simultaneous exocytosis was observed under both activation regimes (n = 4 in the absence of sperm; Fig. 31 and n = 2 in the presence of sperm; Fig. 32). Finally, changes in the diameter of eggs initiated by the activation process were the same (i.e., an approximate reduction in diameter of 15%) under both activation conditions.
Fig. 31 Representative bright-field images of a zebrafish egg activated in the absence of sperm. The image in Panel A was taken before activation whilst those in panels B to H were taken every 15 seconds after the addition of 100 µl 0.5% fructose in egg-water. Scale bar is 200 µm.
Fig. 32 Representative bright-field images of a zebrafish egg activated in the presence of sperm. The image in Panel A was taken before activation whilst those in Panels B to H were taken every 15 seconds after the addition of 30 μl sperm suspension followed by 100 μl 0.5% fructose in egg-water. Scale bar is 200 μm.
DISCUSSION

4.1 Activation followed by Fertilization

I observed no striking differences between the calcium waves generated by eggs activated by the spawning medium in the absence of sperm, i.e., which undergo a cortical granule reaction, raise a chorion, segregate normally, attempt abortive cleavages, then fail to develop - and those subsequently shown (in the presence of sperm) to be fertilized, i.e., which undergo a cortical granule reaction, raise a chorion, segregate, then divide and develop normally. This leads me to suggest that sperm do not compete with the spawning medium to activate zebrafish eggs, rather that the eggs are normally first activated by the spawning medium, then fertilized. Here I define activation to be the initiation of an explosive rise in intracellular calcium that propagates across the egg in a wave-like manner and results in the resumption of the dormant egg’s development, while fertilization is defined as the fusion of gametes followed by the subsequent steps that lead to the restoration of the diploid genome.

In the natural situation where a male and female fish simultaneously discharge eggs and milt into the spawning medium, their breeding behavior brings the gametes together in a matter of seconds. Examination of a slow motion video (AIMS Multimedia Video; “Meiosis and Mitosis: Fertilization and Sexual Reproduction”-ISBN 0-8068-8878-4) of a pair of breeding zebrafish indicates clearly that following an intimate sequence of paired swimming movements, the discharge of eggs and milt is accompanied by a simultaneous twisting flick of their
tails. This creates an upwardly directed vortex in the water column that brings the eggs and sperm together very quickly. Indeed, in vitro studies have reported zebrafish sperm to be attached to the microvilli of the sperm entry site at the egg micropyle within 5 to 10 seconds after insemination (Wolenski and Hart, 1987). Thus, although I am making a distinction between activation and fertilization, it is clear that when sperm are present these two events are normally separated by perhaps only a few to tens of seconds. Once the cortical granule reaction is initiated and the chorion begins to rise, some 30 seconds after the first morphological signs of activation in zebrafish (Hart and Yu, 1980), sperm are no longer able to reach the plasma membrane through the micropyle and a short, but lethal program of parthenogenetic development is initiated. In summary, I suggest that eggs are initially activated by the spawning medium, there then follows a restricted window of opportunity (presumably less than 30 seconds - but perhaps only between 5 and 10 seconds) for sperm to fertilize the egg. This strategy helps to ensure that only sperm from the successful male paired with the spawning female fertilizes her eggs. Presumably the behavioral-mediated selection of breeding partners followed by this fertilization strategy, confers some selective advantage to the progeny.

This is, however, an activation/fertilization strategy somewhat different than that described for medaka (Gilkey et al., 1978; Yoshimoto et al., 1986; Iwamatsu et al., 1988). Thus, as predicted more than 40 years ago by Lord Rothschild (1958), this study of a different teleost species is revealing several other interesting variations that may help us to understand fertilization in vertebrates.
Zebrafish do not represent an isolated case, as a somewhat similar strategy has been reported with regards to the activation of eggs of other fish species such as goldfish (*Carassius auratus*) and the pond smelt (*Hypomesus olidus*) (Yamamoto, 1954, 1961). Furthermore, both the marine shrimp *Sicyonia ingentis* (Lindsay *et al.*, 1992) and the prawn *Palaemon serratus* (Goudeau and Goudeau, 1996) also display this strategy, and in both cases, the initiation of a calcium activation wave once again does not require sperm. Instead, these eggs are activated when they are exposed to seawater Mg$^{2+}$ during spawning. In both cases, if eggs remain unfertilized, they will still resume meiosis, form a hatching envelope and in the case of *Sicyonia ingentis* eventually undergo abnormal cleavage (Pillai and Clark, 1987; Goudeau *et al.*, 1991). This maternally-derived developmental program is very much like the situation observed in zebrafish. It has also been determined that for both crustacean species, external Ca$^{2+}$ is not required for the Mg$^{2+}$-induced activation wave. Once again, this is similar to zebrafish where I report that external calcium is likewise not required to generate an activation wave. The presence of external calcium is, however, required (in 0.5% BSA in Hank’s buffered saline) to maintain eggs in an unactivated state for any period of time. I would suggest that this might be due to the fact that bathing unactivated eggs in a calcium-free form of this medium stimulates a release of calcium from internal stores, such as the corticul endoplasmic reticulum (ER), which reaches a level after a few minutes where a calcium-induced-calcium-release (CICR) activation wave is induced.
4.2 Wave Initiation Point

In situations where sperm are thought to normally activate mature fish eggs, such as in the case of medaka, it is from the point of sperm contact (i.e., at the micropyle) that the calcium wave is initiated (Gilkey et al., 1978); the wave of cortical granule (CG) extrusion begins (Yoshimoto et al., 1986); and the chorion progressively lifts away from the egg surface (Yamamoto, 1961). In the case of the activation in the absence of sperm in zebrafish egg, I report that the calcium wave is still initiated from the micropyle region at the animal pole (AP; Fig. 27, Panel C’). This might be due to the fact that the spawning medium (and/or any activating factor in it) makes contact with the egg plasma membrane more rapidly via the micropyle than through the chorion (Hart, 1990). Furthermore, it has been reported that there is a “tuft” of plasma membrane-derived microvilli at the micropyle to which a sperm head eventually binds (Hart and Yu, 1980). This tuft might also serve to increase the surface area of the plasma membrane coming in contact with the spawning medium, resulting in an amplification of a triggering signal, thus ensuring that the calcium wave is initiated from this location. Another morphological feature that may favor initiation of the calcium wave at the micropyle, is the presence of an extensive array of ER beneath the plasma membrane at this location (Hart, 1990). It has been suggested that the ER is the calcium store required both to initiate and propagate calcium activation waves (Jaffe, 1991).

4.3 Path of Wave Propagation through the Egg

In medaka the calcium wave is only propagated through the peripheral cytoplasm of the egg (Gilkey et al., 1978). In this species, the interior of the egg is
filled with a single, large membrane-bound yolk compartment. In zebrafish, the morphology of yolk encapsulation is somewhat different due to the presence of many membrane-bound yolk globules (Beams et al., 1985). These, however, are excluded from the outermost 15 to 20 μm of the egg, resulting in a functional yolk-free cortex (Hart, 1990). The aequorin-generated data from the PIM system suggests that in zebrafish the activation wave also propagates mainly through the cortex (see Fig. 29) rather than through the whole egg. This suggestion is supported by observations regarding egg morphology. In addition to the array beneath the micropyle, the ER in the zebrafish cortex forms an extensive peripheral network and has been reported to form intimate contacts between both the plasma membrane and the membranes of the CGs (Beams and Kessel, 1973). Thus, the egg cortex possesses the structural element (i.e., an extensive ER Ca\textsuperscript{2+} store) suggested necessary to propagate a CICR wave (Jaffe, 1991).

On the other hand, it is not clear if such a wave-propagating network of ER extends through the deep interior of the zebrafish egg, thus connecting the restricted and convoluted inter-globule space in the same way as it does in the cortex (Hart and Fluck, 1995). One has also to consider other elements of the wave propagation mechanism, such as the distribution of the required calcium-release channels, when comparing peripheral domains of ER to deep ones (Gillot and Whitaker, 1993). These considerations, along with my new data suggest that the zebrafish egg might resemble that of the medaka, in that its fast calcium activation wave is also propagated mainly through the egg cortex. The lack of signal from deep within the egg could arise from a lack of reporter from this domain. However, my injectate
distribution experiments with a 21 kDa FITC-dextran (see Fig. 28) suggests that there should be plenty 21 kDa aequorin deep within the egg.

4.4 Activation Wave Velocity

My method of analyzing activation wave velocities using the Linescan function of Metamorph (see Section 3.7) result in “axial velocities”, i.e., it assumes that the wave passes directly through the egg along the AP to VP axis. As discussed in the previous section, my imaging data suggests that the propagating calcium wave passes through the cortex of the egg, i.e., a distance more like the hemi-circumference rather than the diameter of the egg. Thus, I corrected my Linescan-derived axial velocity values to reflect this, calling them “cortical velocities” (see Appendix III, Figs. 24 and 26, and Table 1). This correction results in activation wave velocities of around 9 μm/sec for eggs activated in either the presence or absence of sperm, which is in the same range as the ionomycin-induced activation waves reported previously in zebrafish (Lee et al., 1996). These velocities clearly fall into the category of “fast” calcium activation waves proposed by Jaffe (1993). Thus, zebrafish are another species that display a highly conserved calcium activation wave velocity, supporting the suggestion that a common mechanism of wave propagation is found in the eggs of both deuterostomes (Jaffe, 1991; Jaffe and Créton, 1998), and protostomes (Eckberg and Miller, 1995; Striker, 1996).

4.5 Estimation of Peak Calcium Levels during Wave Propagation

Aequorin-generated data from the PMT system indicates that there is an approximate 10,000-fold difference in luminescence at the peak of the activation
response, compared to the resting level before activation (see Fig. 27 and Table 1). If I assume that the luminescence of \( f \) and \( h \)-aequorin varies with the calcium concentration \textit{in vivo} as it does \textit{in vitro}, i.e., to the second power (Shimomura, 1995b), this represents a 100-fold difference in the levels of cytosolic free calcium. If I use a value of around 60 nM reported by Créton \textit{et al.} (1998) for the resting level of cytosolic free calcium in fertilized zebrafish eggs, then I get a peak calcium rise of around 6 \( \mu \text{M} \). Thus, my estimation of the peak calcium rise during the activation wave in zebrafish is approximately one fifth that estimated for medaka, i.e., around 30 \( \mu \text{M} \) (Gilkey \textit{et al.}, 1978). However, it is very much in line with the peak levels reported for a variety of sea urchin species (see Table III in Browne \textit{et al.}, 1996).

The difference between the means of the ratios of the \( \text{Ca}^{2+} \) rise above the resting levels in the presence and absence of sperm was not significant \( (t_{\alpha=0.05,n_1+n_2=6} = 1.53; \) see Appendix IV).

4.6 No Fast-Block to Polyspermy

One essential element in my proposal that zebrafish eggs are activated by their spawning medium, then subsequently fertilized, is that there has to be no electrically-mediated fast-block to polyspermy associated with the activation response. From voltage-clamping experiments, Nuccitelli (1980) showed that the membrane potential of the medaka egg only shifts slightly on fertilization for a period of about 20 seconds. Furthermore, he reported that voltage clamping an egg's membrane potential between -80 and +48 mV did not prevent fertilization. This observation is contrary to the fast electrically mediated block to polyspermy that has been reported in a number of invertebrate and vertebrate eggs (Miyazaki and Hirai,
1979; Jaffe and Gould, 1985). Hence, fertilization in medaka eggs is not inhibited by a rapid, positive membrane potential at the plasma membrane. If a similar situation exists in zebrafish, this would help to support the proposal that after eggs have been activated by the spawning medium, they can still be fertilized by sperm when the latter are present. Monospermy in zebrafish is guaranteed by a morphological strategy (rather than an electrical one) where the spherical head of the fertilizing sperm is around 2.5-2.8 μm in diameter, while the inner aperture of the micropylar canal is only slightly larger (Hart and Donovan, 1983). Thus, since two spermatozoa cannot pass simultaneously through the inner micropylar aperture, the block to polyspermy in zebrafish would appear to be relatively fast, mechanical, and mediated by the first sperm to reach the egg surface (Hart, 1990).

4.7 Relationship between the Calcium Wave and CG Breakdown

There appear to be at least two distinctive patterns of CG breakdown in teleost eggs. For example, in medaka (Gilkey et al., 1978), and Fundulus (Brummett and Dumont, 1981), the exocytosis of CGs begins at the AP, at or close to the micropyle. It then propagates itself in a wave-like fashion over the egg surface to the antipode at the VP. In the case of medaka, the wave of cortical CG fusion follows approximately 17 seconds after the propagating calcium wave (Yoshimoto et al., 1986). However, a second pattern occurs in eggs of zebrafish where CG exocytosis is not initiated at the AP, and does not propagate from there in a wave-like fashion. In this case, after a delay of about 30 seconds following activation, the reaction is initiated randomly and more or less simultaneously over the egg surface (Hart and Yu, 1980; Schalkoff and Hart, 1986).
The organization, arrangement, and size of CGs, however, also appear to be variable in the eggs of teleosts. In medaka they are approximately 10-40 μm in diameter, and form a tightly packed monolayer in the cortical cytoplasm (Gilkey et al., 1978). By contrast, the CGs of zebrafish tend to be smaller (between 3-22 μm in diameter), and are arranged in several irregular layers throughout most of the cortex. Furthermore, the granules in these layers are organized in a gradient of progressively increasing size from the AP to the VP along with a corresponding reduction in the number of layers (Hart and Donovan, 1983). Hart (1990) has suggested that in zebrafish, adjacent small CGs may first fuse together to form secretory complexes of larger CGs, and that such intergranule fusion may precede CG exocytosis (i.e., fusion of CGs to the plasma membrane). Such CG to CG fusion has been reported from other systems such as sea urchins (Vacquier, 1975), and mammals (Rousseau et al., 1977). It was proposed that this intergranule fusion may provide a more efficient exit for the liberation of the contents of deeper lying granules (Hart, 1990).

Thus in medaka, where there are no additional deep layers of CGs, the only fusion required is between the monolayer of large CGs and the egg plasma membrane. It has been proposed that this fusion is stimulated by the propagating calcium wave, and thus explains its associated wave-like progression (Gilkey et al., 1978; Yoshimoto et al., 1986). In zebrafish, where there is a delay of some 30 to 60 seconds before the near-synchronous fusion of CGs all over the egg surface (Hart and Yu, 1980), I would like to propose that in this case the calcium wave might stimulate a wave of CG to CG fusion. This forms and primes a secretory complex of larger CGs which subsequently fuse with the plasma membrane in a synchronous fashion. This would explain the observed delay in CG exocytosis, the lack of
correlation between the calcium activation wave and a wave of CG exocytosis, and finally the near simultaneous nature of the exocytotic reaction. Furthermore, the duration of the cytosolic calcium elevation resulting from the activation wave (some 7 minutes, see Table 1) correlates well with the time required to complete the CG reaction and elevate the chorion (around 6 minutes; Hart and Yu, 1980).

4.8 Possible Mechanism for Triggering the Activation Wave

At the moment, the exact mechanism through which the spawning medium triggers the activating calcium wave in zebrafish eggs remains unknown. However, it clearly does not involve any sperm-derived factors as suggested for other systems (Swann, 1993). Thus, the situation in zebrafish is quite unlike that found for example in the mouse, where sperm-egg fusion must occur before the calcium transient is initiated, and where intracellular transients are never observed under any conditions in the absence of sperm-egg fusion (Lawrence et al., 1997). In mouse, however, activation involves multiple calcium oscillations (Swann, 1994), whereas in zebrafish it requires only a single propagating calcium transient. Perhaps some sperm-derived factor is required for maintaining multiple oscillations (Parrington et al., 1996; Swann and Lai, 1997).

My data suggest (see Figs. 27 C, C' and D) that there is a slow localized build-up of intracellular Ca\(^{2+}\) for a few seconds prior to the explosive propagating calcium wave. I have already mentioned the extensive ER network located at the micropyle (Hart, 1990), which I would suggest is the store responsible for this initial Ca\(^{2+}\) release which eventually triggers the wave. The fact that eggs can be activated
when bathed in calcium-free medium would appear to eliminate Ca^{2+} itself as the
diffusible messenger entering the egg via the micropyle and stimulating the
intracellular Ca^{2+} release (Créton and Jaffe, 1995). This leaves the possibility of
some transmembrane receptor generating a calcium release agent as a reasonable
suggestion for a triggering mechanism. In the case of the Mg^{2+}-activated shrimp
egg, Lindsay et al. (1992) suggest that a Mg^{2+}-sensitive receptor linked to a G-
protein-based signal transduction pathway generates inositol 1,4,5-trisphosphate
(InsP\textsubscript{3}), which in turn stimulates the release of Ca^{2+} and thus the initiation and
propagation of the calcium wave. There is some additional evidence to support this
idea, in that another external divalent cation (i.e., cadmium) has been shown to
increase intracellular InsP\textsubscript{3} and Ca^{2+} levels in human skin fibroblasts (Smith et al.,
1989).

4.9 Hypothetical Model for the Activation/Fertilization Process in
Zebrafish Eggs

To summarize my new data, I propose a simple model to explain the
activation and fertilization strategy in zebrafish eggs (Fig. 33). Although the whole
chorion is permeable to the spawning medium, the micropyle guarantees faster entry
at the AP (Hart, 1990). Through mechanisms as yet unknown, this results in a
localized increase in intracellular Ca^{2+}, presumably released from the ER. When this
reaches a threshold level (after only a few seconds) it triggers a CICR activation
wave that begins to propagate across the egg at a velocity of around 9 \mu m/sec.
Following the initiation of this wave, sperm (if present) have a chance to bind to the
egg via the micropyle before chorion-raising forms a mechanical block to
fertilization. This "fertilization window" might last for up to 30 seconds (i.e., till when the chorion begins to rise). It is clear that the propagating calcium wave in zebrafish eggs does not induce a following wave of exocytotic CG to plasma membrane fusion as is seen in the medaka egg. What one does see in zebrafish eggs, however, is a near-synchronous fusion of CGs to the plasma membrane some 30 to 60 seconds after the initiation of the calcium wave. As there is some evidence (Hart and Yu, 1980) that there may be a pre-fusion of CG to each other before fusion to the plasma membrane, I have suggested that the calcium wave in zebrafish might induce a wave of CG to CG fusion, followed by a near synchronous fusion of these pre-fused CGs to the plasma membrane. This is schematically shown in Fig. 33, Panels II to VI.

If sperm are absent, the egg will undergo a short, maternally programmed pattern of development that terminates in abortive cleavages. When sperm are present eggs undergo a post-activation fertilization, and continue to develop beyond the initial maternally determined program.

My new data pose several interesting questions concerning the initiation of zebrafish egg activation. What, for example, are the signaling pathways involved in triggering a calcium wave during natural sperm-free activation? Does this mean adding a 4th egg activation hypothesis to the three discussed previously in Section 1.5 and reviewed by Ozil (1996)? The work presented in this dissertation represents the first few steps along the road to addressing these challenging questions.
Fig. 33  Hypothetical model illustrating the activation/fertilization strategy of zebrafish eggs, relating the calcium activation wave to morphological events. Features not drawn to scale, and the timing of events are approximations.
V.

Ca²⁺ Wave Reaching VP and CG to CG Fusion Wave Complete
\[ t = 50 \text{ sec} \]

VI.

Ca²⁺ Wave Complete
\[ t = 60 \text{ sec} \]

VII.

Chorion Fully Elevated
\[ t = 6 \text{ min} \]

VIII.

First Cell Division
\[ t = 45 \text{ min} \]

Fig. 33 - Continued
APPENDIX I

Abstract of work presented at:

The 13th International Congress and 56th SDB Annual Meeting of
Developmental Biology
Snowbird, Utah, USA.

July 5-10, 1997

FAST CALCIUM WAVES ACCOMPANY FERTILIZATION OF ZEBRAFISH EGGS.

Karen W. Lee, Sarah E. Webb, and Andrew L. Miller

Department of Biology, HKUST, Clear Water Bay, Kowloon, Hong Kong, PRC.

The fertilization process in a variety of deuterostomes and protostomes is accompanied by the propagation of fast Ca\(^{2+}\) waves. We have previously reported that such waves can be induced by the application of the calcium ionophore ionomycin, and result in the activation of unfertilized zebrafish eggs. We have now extended this study in zebrafish to include sperm activation, as well as other means of parthenogenetic activation. Unactivated eggs were collected from anesthetized (1.7% tricine in 30% Danieau's solution) female zebrafish, and held in Coho salmon (Oncorhynchus kisutch) ovarian fluid (SOF). Eggs were injected with f-aequorin, a calcium-sensitive bioluminescent reporter, and left for 20 minutes to allow for diffusion of the reporter. Sperm was collected from anesthetized male fish, and held in a sperm-extender solution until required. Unactivated, aequorin-loaded eggs were imaged via an Imaging Photon Detector-based system, and after a pre-fertilization resting level of aequorin-generated light was obtained, a sperm suspension, followed by a sperm-activation solution (0.5% fructose in egg-water) was added. Following a short delay (required for the sperm to be activated and swim to the egg), the first sign of activation was a localized rise in intracellular calcium, from which a calcium wave propagates across the egg, traveling at approximately 10 μm/sec. Similar Ca\(^{2+}\) waves (both in velocity and intensity) could also be induced by dilution of the SOF. (Supported by RGC Grant HKUST650/96M). The f-aequorin was a kind gift from O. Shimomura.
APPENDIX II

Figure 1A shows the current clamp response of an oocyte to a 25-s light flash. After a 6-s delay from light onset, the membrane depolarized to a level of $-12$ mV, and it returned to pre-stimulus baseline about 1 min after light offset. Responses of all other oocytes were similar to this, depolarizing to $-12$ to $-23$mV, with latencies that ranged from 6 to 24 s. Figure 1B shows the response of another oocyte to light while voltage clamped at various potentials. Light flashes evoked sustained inward currents at clamp potentials more negative than $-25$mV, and outward currents at potentials more positive than $-20$mV. The cause of the reduction in inward current before light offset at clamp potentials of $-30$ and $-40$mV is not known and was not observed in other oocytes. The reversal potential for the current for this oocyte was $-23$mV. Light-evoked currents from other oocytes reversed direction at holding potentials between $-12$ and $-23$mV, suggesting that this response is mediated by the endogenous calcium-activated chloride conductance (7, 8). Many other expressed receptor proteins also are known to couple into this pathway, and the long response latency we observed is typical of the activation of this chloride conductance (9). No light responses were detected in mRNA-injected oocytes before the application of 11-cis retinal. Other studies using the same expression system detected no responses in non-injected oocytes after incubation with 11-cis retinal (5).

Figure 1C shows that repetitive flashes of light can evoke repetitive responses from oocytes without the need for additional incubation with 11-cis retinal. This sustained light sensitivity supports an earlier finding that Limulus metarhodopsin is a relatively stable and photoreversible photoprodut of Limulus rhodopsin (10). This is not the case for bovine rhodopsin expressed in Xenopus oocytes (5), which requires additional incubation with 11-cis retinal to maintain light sensitivity.

Note that the light-evoked currents in Figure 1C increased in amplitude in response to repeated flashes of a constant intensity, while the response latencies decreased from 10 s for the first response to about 4 s for subsequent responses. Decreasing the intensity of the light flashes decreased both the response amplitude and the steady state level. Flashes of low intensity often failed to evoke any response to the first test flash, but not to subsequent ones (data not shown). Assuming that repetitive flashes of equal intensity generate equal levels of activated rhodopsin, the increasing response amplitude in Figure 1C may reflect the accumulation of an internal transmitter involved in the transduction cascade that yields the light-dependent currents we record. The occasional failure of the first test flash in a series to evoke a response points to the existence of a threshold for action for one or more internal constituents of this transduction pathway. We have not explored this facility response to subsequent light flashes in sufficient detail to determine either the exact threshold for action for the internal transmitter or the time course of its delay.

In conclusion, we have demonstrated that Xenopus oocytes efficiently translate Limulus retinal mRNA and provide a suitable system for studying characteristics of light transduction. We will combine this technology with molecular biological techniques to study how the properties of Limulus rhodopsin influence photoreceptor sensitivity and noise.

Supported by grants from the National Institutes of Health, and the National Science Foundation.

**Literature Cited**


**Ionophore-Induced Calcium Waves Activate Unfertilized Zebrafish (Danio rerio) Eggs**

*Karen W. Lee* (The Hong Kong University of Science and Technology), Robert Baker1, Antony Galione2, Edwin H. Gilland3, Roger T. Hanlon1, and Andrew L. Miller4

Egg activation is accompanied by propagating Ca\(^{2+}\) waves that can be induced by factors such as sperm, prickling, calcium ionophores, and injected IP3 or cADPR (1). Such waves have been reported from a variety of deuterostomes and protostomes (2, 3). One notable omission from this ever-expanding list was the zebrafish (*Danio rerio*). In light of the increasing popularity of this fish as a model for study of vertebrate development, we present data showing that zebrafish eggs are likewise activated by a regenererative Ca\(^{2+}\) wave.

In the past, the major obstacle to such an investigation lay in the inability to delay *in vitro* fertilization long enough to load an unactivated egg with a calcium reporter. This difficulty has recently been overcome by the discovery that unactivated zebrafish eggs can be held in Coho salmon (*Onchorhynchus kisutch*) ovarian fluid (Sea Tech Bioproducts) for as long as 1.5 hours and still be successfully activated (4).

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1New York University Medical Center.
2Oxford University.
3Marine Biological Laboratory.
4The Hong Kong University of Science and Technology.
Figure 1. An example of an ionophore-induced Ca\(^{2+}\) wave crossing a zebrafish egg. Panel A illustrates a transmitted light image of an unactivated egg. Panels B through H are successive (microchannel-plate-generated) fluorescent images showing a Ca\(^{2+}\) wave crossing the egg at a velocity of about 10 \(\mu\)m/s (ionomycin solution added at Panel B: inj. indicates the injection site). Panel I is a second transmitted light image collected after the passage of the wave, clearly illustrating that the egg had been activated (arrowhead indicates the rising chorion). Panels J through L show that activated (but unfertilized) eggs loaded with Fluo-3 undergo normal ooplasmic segregation, forming a regular blastodisc (L is an overlay of J and K). It is clear that indicator dye injected into the center of an unactivated egg is carried via ooplasmic streaming (indicated by arrow in Panel J) into the forming blastodisc. Panel J (60 min post-activation) illustrates a fully elevated chorion. Scale bars in Panels A and J equal 100 \(\mu\)m.
We stripped unactivated eggs from ripe, anesthetized females and immediately immersed them in 100 μl of salmon ovarian fluid (SOF) in agarose-coated viewing chambers. Eggs were injected with 3 to 5 nl of the calcium reporter Fluo-3 (pipette concentration: 300 μM) and left for 20 to 30 min to allow the reporter to diffuse throughout the egg. Eggs were then checked for dye distribution by using a multichannel plate detector (Motion Analysis Inc.) mounted on a Zeiss IM35 platform. During each experiment, several eggs were simultaneously imaged through a Zeiss Plan 6.3x objective while under the appropriate excitation illumination for Fluo-3. Eggs were checked for signs of activation under brightfield illumination. Both brightfield and fluorescent images were stored on videotape for subsequent analysis.

Eggs were activated by adding 10 μl of 50 μM ionomycin. Three distinct observations were made. (1) In cases where there were no signs of activation (46% of the eggs observed), no increases in intracellular Ca^{2+} were detected. (2) Where activation was complete, judged by a comprehensive raising of the chorion (23% of the eggs observed), an accompanying wave of elevated Ca^{2+} traversed the egg. (3) When we recorded a localized, non-propagating rise in intracellular Ca^{2+} (31% of the eggs observed), it was coincident with localized egg activation, judged by localized raising of the chorion.

Figure 1 shows a representative example of an ionophore-induced Ca^{2+} wave. Such waves took around 60 s to cross the 625-μm-diameter egg (at 24°C), indicating a velocity of around 10 μm/s. Thus for zebrafish eggs, our results are consistent with the general observation that an explosive rise in free calcium provides most of the activating stimulus.

This work was supported by NIH R24 RR101291-01A1 awarded to ALM and RTH, and Research Grants Council (Hong Kong) HKUST650/96M awarded to ALM.

**Literature Cited**

Axial Distance = X \mu m
Time = t \text{ sec}
Axial Velocity = X/t \mu m/\text{sec}

Cortical Distance = X(\pi)/2 \mu m
Time = t \text{ sec}
Cortical Velocity = X(\pi)/2t \mu m/\text{sec}

III Calculation of the cortical velocity from axial velocity.
## APPENDIX IV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Activation</th>
<th>Ratio of Ca\textsuperscript{2+} Rise above Resting Level (\sqrt{Luminescence})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>without sperm</td>
<td>161.95</td>
</tr>
<tr>
<td>2</td>
<td>without sperm</td>
<td>119.23</td>
</tr>
<tr>
<td>3</td>
<td>without sperm</td>
<td>132.83</td>
</tr>
<tr>
<td>mean ± SD</td>
<td></td>
<td>138.00 ± 21.8 fold</td>
</tr>
<tr>
<td>4</td>
<td>with sperm</td>
<td>129.00</td>
</tr>
<tr>
<td>5</td>
<td>with sperm</td>
<td>111.46</td>
</tr>
<tr>
<td>6</td>
<td>with sperm</td>
<td>108.63</td>
</tr>
<tr>
<td>mean ± SD</td>
<td></td>
<td>116.4 ± 11.0 fold</td>
</tr>
</tbody>
</table>

IV PMT Experiments: Data summary of the ratio of Ca\textsuperscript{2+} rise above resting level. The difference between the means was not significant ($t_{0.05,n_1+n_2=6} = 1.53$) comparing activation in the absence or presence of sperm. $t$-value calculated as follows:-
Calculation of $t$-value:

**Data Set #1: Ratios of Ca\(^{2+}\) Rise above Resting in the Absence of Sperm**

<table>
<thead>
<tr>
<th>Expt #</th>
<th>Value</th>
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<tbody>
<tr>
<td>1</td>
<td>161.95</td>
</tr>
<tr>
<td>2</td>
<td>119.23</td>
</tr>
<tr>
<td>3</td>
<td>132.83</td>
</tr>
</tbody>
</table>

mean $\pm$ SD = 138.00 $\pm$ 21.80 fold

**Data Set #2: Ratios of Ca\(^{2+}\) Rise above Resting in the Presence of Sperm**

<table>
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<th>Value</th>
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</thead>
<tbody>
<tr>
<td>4</td>
<td>129.00</td>
</tr>
<tr>
<td>5</td>
<td>111.46</td>
</tr>
<tr>
<td>6</td>
<td>108.63</td>
</tr>
</tbody>
</table>

mean $\pm$ SD = 116.40 $\pm$ 11.80 fold

**Analysis used: 2-tailed $t$-test**

$H_0$: there is no significant difference between the two sample means

$H_1$: there is a significant difference between the two means

Sample size for set #1 = $n_1 = 3$
Sample size for set #2 = $n_2 = 3$

Selected $\alpha = 0.05$, i.e., there is a 95% chance of making a correct decision about $H_0$. 
Formula used:-

\[ t = \frac{\bar{x}_1 - \bar{x}_2}{SE(\bar{x}_1 - \bar{x}_2)} \],

where

\[ SE(\bar{x}_1 - \bar{x}_2) = \sqrt{\frac{S_p^2}{n_1} + \frac{S_p^2}{n_2}} \]

and

\[ S_p^2 = \frac{SS_1 + SS_2}{d.f.1 + d.f.2} = \frac{\sum (x_1 - \bar{x}_1)^2 + \sum (x_2 - \bar{x}_2)^2}{(n_1 - 1) + (n_2 - 1)} \],

therefore

\[ S_p^2 = \frac{\left[ \frac{\sum x_1^2 - (\frac{\sum x_1}{n_1})^2}{n_1} \right] + \left[ \frac{\sum x_2^2 - (\frac{\sum x_2}{n_2})^2}{n_2} \right]}{(n_1 + n_2 - 2)} \]

To calculate \( S_p^2 \):

\( \sum x_1^2 \) is the sum of the squared values of each measurement in set #1

\[ 161.95^2 + 119.23^2 + 132.83^2 = 58087.40 \]

\( \frac{\left( \sum x_1 \right)^2}{n_1} \) is the sum of all the values in set 1, squared, then divided by the number of observations in set #1

\[ \frac{(161.95 + 119.23 + 132.83)^2}{3} = 57134.76 \]
This was repeated for set #2

\[ \sum x_2^2 \] is the sum of the squared values of each measurement in set #2

\[ 129.00^2 + 111.46^2 + 108.63^2 = 40864.81 \]

\[ \left( \frac{\sum x_2}{n_2} \right)^2 \] is the sum of all the values in set #2, squared, then divided by the number of observations in set #2

\[ \frac{(129.00 + 111.46 + 108.63)^2}{3} = 40621.27 \]

Therefore:

\[ S_{p^2} = \frac{[58087.40 - 57134.76] + [40864.81 - 40621.27]}{(3 + 3 - 2)} = \frac{952.64 + 243.54}{4} = 299.05 \]

and

\[ SE(x_1 - x_2) = \sqrt{\frac{S_{p^2}}{n_1} + \frac{S_{p^2}}{n_2}} \]

therefore

\[ SE(x_1 - x_2) = \sqrt{\frac{299.05}{3} + \frac{299.05}{3}} = \sqrt{199.37} = 14.12 \]

And, as \( t = \frac{x_1 - x_2}{SE(x_1 - x_2)} \), and the means are 138.00 and 116.4 for set #1 and #2 respectively.
Therefore,

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{SE(\bar{x}_1 - \bar{x}_2)} = \frac{138.00 - 116.40}{14.12} = \frac{21.60}{14.12} = 1.53
\]

From my calculated \( t \) value of 1.53, the selection of \( \alpha \) as 0.05, and the degrees of freedom (i.e., the measure of the number of statistical parameters that had to be estimated from the sample rather than known as facts) equal to \( n_1 + n_2 - 2 = 4 \), consultation with a standard set of \( t \)-tables reveals a corresponding \( t \) value for these parameters of 2.776.

As my calculated \( t \) value (1.53) is less than this, I conclude that \( H_0 \) is correct and there is no significant difference between data set #1 and #2.

A similar calculation was used to compare other characteristics of the Ca\(^{2+}\) waves generated in the absence and presence of sperm (see Appendices V and VI).
**APPENDIX V**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Activation</th>
<th>Duration of the transients (min)</th>
<th>Rise time of the transients (min)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>without sperm</td>
<td>11.8</td>
<td>1.73</td>
</tr>
<tr>
<td>2</td>
<td>without sperm</td>
<td>6.5</td>
<td>1.05</td>
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<tr>
<td>3</td>
<td>without sperm</td>
<td>4.9</td>
<td>0.35</td>
</tr>
<tr>
<td>4</td>
<td>without sperm</td>
<td>5.1</td>
<td>0.78</td>
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<tr>
<td>5</td>
<td>without sperm</td>
<td>6.8</td>
<td>1.22</td>
</tr>
<tr>
<td>6</td>
<td>without sperm</td>
<td>7.3</td>
<td>0.65</td>
</tr>
<tr>
<td>7</td>
<td>without sperm</td>
<td>5.1</td>
<td>0.75</td>
</tr>
<tr>
<td>8</td>
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<td>8.5</td>
<td>0.75</td>
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<td>9</td>
<td>without sperm</td>
<td>8.9</td>
<td>2.17</td>
</tr>
<tr>
<td>10</td>
<td>without sperm</td>
<td>6.9</td>
<td>0.83</td>
</tr>
<tr>
<td>11</td>
<td>without sperm</td>
<td>5.5</td>
<td>0.88</td>
</tr>
<tr>
<td>12</td>
<td>without sperm</td>
<td>7.3</td>
<td>0.65</td>
</tr>
</tbody>
</table>

mean ± SD  
7.1 ± 2.0  
1.0 ± 0.5

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Activation</th>
<th>Duration of the transients (min)</th>
<th>Rise time of the transients (min)</th>
</tr>
</thead>
<tbody>
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<td>13</td>
<td>with sperm</td>
<td>8.7</td>
<td>0.62</td>
</tr>
<tr>
<td>14</td>
<td>with sperm</td>
<td>6.4</td>
<td>0.60</td>
</tr>
<tr>
<td>15</td>
<td>with sperm</td>
<td>7.0</td>
<td>0.77</td>
</tr>
</tbody>
</table>

mean ± SD  
7.4 ± 1.20  
0.7 ± 0.1

V PMT Experiments: Data summary of the duration and rise time of the transients from eggs. The difference between the means was not significant for either duration ($t_{a=0.05,n_1+n_2=15} = 0.25$) or rise time ($t_{a=0.05,n_1+n_2=15} = 1.00$) comparing activation in the absence or presence of sperm. For calculation of t-values, see Appendix IV.
### APPENDIX VI

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Activation</th>
<th>Cortical Ca$^{2+}$ Wave Velocity (μm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>without sperm</td>
<td>8.3</td>
</tr>
<tr>
<td>2</td>
<td>without sperm</td>
<td>9.8</td>
</tr>
<tr>
<td>3</td>
<td>without sperm</td>
<td>9.3</td>
</tr>
<tr>
<td>4</td>
<td>without sperm</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>mean ± SD</strong></td>
<td></td>
<td><strong>8.9 ± 0.8</strong></td>
</tr>
<tr>
<td>5</td>
<td>with sperm</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>with sperm</td>
<td>8.8</td>
</tr>
<tr>
<td>7</td>
<td>with sperm</td>
<td>9.1</td>
</tr>
<tr>
<td><strong>mean ± SD</strong></td>
<td></td>
<td><strong>9.0 ± 0.2</strong></td>
</tr>
</tbody>
</table>

**VI**  PIM Experiments: Data summary of the cortical Ca$^{2+}$ wave velocity. The difference between the means was not significant ($t_{0.05,n_1+n_2=7} = 0.22$) comparing activation in the absence or presence of sperm. For calculation of $t$-values, see Appendix IV.
REFERENCES

Albrieux, M., Sardet, C., and Villaz, M. (1997). The two intracellular Ca\textsuperscript{2+} release channels, ryanodine receptor and inositol 1,4,5-trisphosphate receptor, play different roles during fertilization in ascidians. *Dev. Biol.* **189**: 174-185.


Miyazaki, S., Shirakawa, H., Nakada, K., and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/Ca\textsuperscript{2+} release channel in Ca\textsuperscript{2+} waves and Ca\textsuperscript{2+} oscillations at fertilization of mammalian eggs. Dev. Biol. 158: 62-78.


Nuccitelli, R. (1980). The fertilization potential is not necessary for the block to polyspermy or the activation of development in the medaka egg. Dev. Biol. 76: 499-504.


