Calcium signalling in early embryos

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The onset of development in most species studied is triggered by one of the largest and longest calcium transients known to us. It is the most studied and best understood aspect of the calcium signals that accompany and control development. Its properties and mechanisms demonstrate what embryos are capable of and thus how the less-understood calcium signals later in development may be generated. The downstream targets of the fertilization calcium signal have also been identified, providing some pointers to the probable targets of calcium signals further on in the process of development.

In one species or another, the fertilization calcium signal involves all the known calcium-releasing second messengers and many of the known calcium-signalling mechanisms. These calcium signals also usually take the form of a propagating calcium wave or waves.

Fertilization causes the cell cycle to resume, and therefore fertilization signals are cell-cycle signals. In some early embryonic cell cycles, calcium signals also control the progress through each cell cycle, controlling mitosis.

Studies of these early embryonic calcium-signalling mechanisms provide a background to the calcium-signalling events discussed in the articles in this issue.

Keywords: calcium; fertilization; cell cycle; signalling

1. CALCIUM AS A MESSENGER AT FERTILIZATION

Although the idea that calcium might cause egg activation is an old one (Heilbrunn 1928), our detailed understanding of calcium as a signalling ion began in nerves and muscles. Using the squid giant axon, a 1 mm wide centimetres long nerve cell, the basic features of cellular calcium handling were established (Baker 1972). The main points were that the free calcium concentration in the cytoplasm is approximately 10^{-7} M, between 10 000 and 100 000 times less than the concentrations in extracellular fluids; this was so because calcium ions were tightly bound to cytoplasmic proteins and active, energy-requiring mechanisms maintained intracellular stores of calcium that were sequestered from the cytoplasm. In the muscle, the action potential that activates contraction stimulates the release from this internal store, the sarcoplasmic reticulum (Endo *et al.* 1970). Cytoplasmic calcium concentration could also quickly rise when voltage-activated calcium channels open, the calcium flux driven by the very large calcium gradient across the plasmalemma, as at the synapse to cause neurotransmitter release (Katz & Miledi 1971). In a very few years, the calcium-signalling paradigm was established: a transient increase in cytoplasmic calcium caused by either calcium influx or internal calcium release and terminated by the resequestration or extrusion of calcium ions by ATP-driven calcium pumps (Ebashi & Lipmann 1962;

Martonosi & Feretos 1964; Maclellan 1970; Schatzmann 1973; Hasselbach & Oetliker 1983).

(a) *Calcium as the fertilization activator*

A calcium-transporting antibiotic, an ionophore A23187 (Reed & Lardy 1972), was the new tool that led to the demonstration that an increase in calcium itself was sufficient rapidly to induce most of the hallmarks of fertilization, including release from cell-cycle arrest (Chambers *et al.* 1974; Lallier 1974; Schroeder & Strickland 1974; Steinhardt & Epel 1974; Steinhardt *et al.* 1974). In sea urchin eggs, it was shown that fertilization membrane elevation, membrane conductance changes, the respiratory burst and increases in protein and DNA synthesis were all induced by ionophore treatment (Steinhardt & Epel 1974). These fertilization-like changes did not require calcium in the surrounding seawater, therefore demonstrating that the ionophore was releasing calcium from internal stores. However, very few eggs divided. This was later shown to be due to the absence of centrioles, without which mitotic spindles cannot form in the sea urchin egg (Schatten 1994). Using the method of enhancing parthenogenesis that Loeb (1913) had developed, Brandriff and his colleagues were able to create adult female sea urchins after parthenogenetic activation with A23187 (Brandriff *et al.* 1975). Thus, calcium, an ionic signal, is the egg activator (Whitaker & Steinhardt 1982).

(b) *Fertilization calcium waves*

It had been inferred from the 20-fold increase in ^{45}Ca efflux after ionophore treatment (Steinhardt & Epel 1974) and at fertilization (Azarnia & Chambers 1969,

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1976) that cytoplasmic calcium increases substantially during egg activation. A direct demonstration of the calcium increase was first made using a calcium-sensitive photoprotein from the jellyfish *Aequorea victoria*, the same species that was the source of the original green fluorescent protein, now widely used as a fluorescent tracer in developmental and cell biology. The photoprotein, aequorin, emits very low levels of blue light that can be detected using a photomultiplier or image intensifier. A calcium increase comparable with that seen during a twitch in skeletal muscle, but of a much longer duration (10 min rather than 10 ms), was observed in sea urchin eggs using a photomultiplier (Steinhardt *et al.* 1977). Using image intensification in the much larger fish egg, the calcium increase was observed to start at the point of the sperm entry and to progress slowly around the Medaka egg's rim of the cytoplasm to the antipode where it extinguished (Ridgway *et al.* 1977; Gilkey *et al.* 1978). The magnitude and duration of fertilization calcium waves led to them being described as calcium explosions (Jaffe 1980). For more details on the calcium waves, see Jaffe (2008).

Aequorin was also used eventually to measure a large free calcium increase in the mouse oocyte (Cuthbertson *et al.* 1981) and image intensification was used to record calcium waves in the smaller (relative to Medaka) sea urchin and starfish eggs (Eisen & Reynolds 1984; Eisen *et al.* 1984; Swann & Whitaker 1986). A calcium wave was also measured in frog eggs using calcium-sensitive microelectrodes (Busa & Nuccitelli 1985). Once Roger Tsien had invented calcium-sensitive fluorescent dyes (Tsien 1984; Grynkiewicz *et al.* 1985), they became the calcium indicator of choice (Busa & Nuccitelli 1985): calcium fertilization waves were measured using confocal microscopy with enhanced spatial and temporal resolution (Hafner *et al.* 1988; Fujiwara *et al.* 1993; Galione *et al.* 1993; Gillot & Whitaker 1993; Deguchi & Osanai 1994; Stricker *et al.* 1994; Eckberg & Miller 1995; McDougall & Sardet 1995; Stricker 1996; Fontanilla & Nuccitelli 1998).

(c) *Calcium chelators*

A formal proof that an increase in intracellular free calcium is the activating messenger at fertilization requires that activation is blocked if the calcium signal is prevented. This was first shown in the sea urchin egg (Zucker & Steinhardt 1978) and later confirmed (Swann & Whitaker 1986; Swann *et al.* 1992) by microinjection of the tetracarboxylate calcium chelator EGTA. Microinjection of EGTA was also shown to prevent the activation of mouse (Miyazaki & Igusa 1982) and ascidian oocytes (Tosti & Dale 1994). Using the higher-affinity chelator BAPTA developed by Tsien (Lew *et al.* 1982; Tsien 1984), very similar results were obtained in frog (Kline 1988), mouse (Kline & Kline 1992a) and molluscan oocytes (Guerrier *et al.* 1993) and in sea urchin eggs (Swann *et al.* 1992).

(d) *The fertilization calcium signal*

Based on the experiments described so far, it is readily concluded that the sperm gives rise to a large calcium signal that rises from a resting free calcium concentration of approximately 200 nM to a peak of several

micromoles over a period of tens of seconds or less. The initial calcium signal has the form of a calcium wave travelling across the egg at a velocity of approximately $5\text{--}10\ \mu\text{M s}^{-1}$.

Once the wave has propagated across the egg, free calcium concentration remains elevated for several minutes, even tens of minutes in the case of the large frog egg where the calcium wave takes up to 5 min to traverse the egg. This very large calcium signal arises, it appears, because the endoplasmic reticulum (ER) calcium stores are sensitized to calcium release during oocyte maturation, probably through enhanced expression of calcium-releasing receptors (Mehlmann *et al.* 1996; Parrington *et al.* 1998); this is what likely leads to the development of the positive feedback mechanisms necessary for the explosive propagation of the calcium wave (Fujiwara *et al.* 1993; Carroll *et al.* 1994). The reorganization of the ER into stacked lamellar clusters during oocyte maturation also contributes to the wave mechanism (Kline *et al.* 1999). The clusters may also play some part in the downregulation of the wave mechanism, as they disappear in tandem with the ability of the oocyte to generate calcium waves (Kline 2000; Terasaki *et al.* 2001; FitzHarris *et al.* 2003; reviewed in Whitaker 2006). The resequestration of calcium into the ER at the end of the fertilization calcium transient is less well studied, but it is known that the SERCA pump and calreticulin/calsequestrin play the major role, as they do in somatic cells (Henson *et al.* 1989; Galione *et al.* 1991; Roderick *et al.* 2000).

A graphic image of the fertilization calcium wave can be had by imagining that during oocyte maturation, calcium release mechanisms are primed to generate a calcium explosion (Jaffe 1980) and that the sperm lights the fuse at fertilization (Whitaker & Swann 1993); in this way, the sperm, tiny when compared with the egg, can make its presence felt and can activate the entire egg cytoplasm.

2. PHOSPHOINOSITIDE SIGNALLING AT FERTILIZATION

Phosphoinositide lipids were unique in showing rapid turnover during secretory activity in the pancreas (Hokin & Hokin 1958). A large body of evidence was accumulated to support the idea that phosphoinositide turnover was involved in the mobilization of intracellular calcium during calcium signalling (Michell *et al.* 1981). The extent of calcium mobilization was directly related to the levels of phosphoinositide lipids (Berridge 1981); the most rapidly cycling were polyphosphoinositides (Creba *et al.* 1983; Michell 1983). The bisphosphoinositide lipid head group, inositol trisphosphate (InsP_3), was found to cause the release of calcium from calcium-sequestering vesicles prepared from pancreatic acinar cells (Streb *et al.* 1983); the complementary product of the hydrolysis of phosphatidylinositol(1,4)-bisphosphate, diacylglycerol, was discovered to be a very potent stimulant of a protein kinase C (PKC; Castagna *et al.* 1982; Kikkawa *et al.* 1983). These discoveries defined the phosphoinositide signalling pathway in which receptor stimulation by its ligand led to the activation

of a phospholipase C that in turn generated InsP_3 and diacylglycerol, InsP_3 causing calcium release and diacylglycerol activation of PKC.

It was found that polyphosphoinositide turnover increases markedly at fertilization in sea urchin eggs (Turner *et al.* 1984) and that the microinjection of InsP_3 into unfertilized sea urchin eggs caused their activation (Whitaker & Irvine 1984). These two sets of observations established the idea that InsP_3 was a key second messenger at fertilization.

3. G-PROTEIN SIGNALLING; THE INSP_3 RECEPTOR

Trimeric GTP-binding proteins had been shown to couple plasma membrane hormone receptors to adenylcyclase to produce the messenger cAMP (Rodbell 1980). cAMP is perhaps the only second messenger that has not been found to play at least a putative role at fertilization, but the connection is that a then novel subset of these G-proteins appeared to couple hormone receptors to the phospholipase that generated the two messengers of the phosphoinositide pathway (Bokoch & Gilman 1984; Cockcroft 1987; Casey & Gilman 1988).

InsP_3 was found to release calcium into the cytoplasm from the ER through its receptor/channel (Furuichi *et al.* 1989) located on the ER membrane. There are many structural similarities between the InsP_3 receptor and the ryanodine receptor (RyR) responsible for calcium release from the sarcoplasmic reticulum (Mikoshiba *et al.* 1994). The receptor has three isoforms that are differentially regulated by Ca^{2+} ; the receptor is regulated by tyrosine kinases and PKC and can be dephosphorylated by calcineurin, a calcium-activated signalling molecule (Mikoshiba 1997).

4. FERTILIZATION SECOND MESSENGERS

As the calcium wave is central to egg activation at fertilization, so does InsP_3 seem to be central to the calcium wave. The microinjection of InsP_3 at sub-micromolar concentrations will activate frog (Nuccitelli *et al.* 1993), sea urchin (Whitaker & Irvine 1984; Swann & Whitaker 1986; Turner *et al.* 1986), starfish (Picard *et al.* 1985; Santella *et al.* 2000), ascidian (McDougall & Sardet 1995; Roegiers *et al.* 1995) and mouse eggs (Swann *et al.* 1989). These commonly studied species are all deuterostomes. Stricker's excellent survey of calcium signalling across the animal kingdom (Stricker 1999) demonstrates that all eggs from protostomes tested so far (cnidarians, nemerteans, molluscs and echiurans) respond to InsP_3 , as do algal gametes (Roberts & Brownlee 1995). Stricker's summary table contains the important caveat that this response demonstrates only that all oocytes are *capable* of responding to InsP_3 , not that InsP_3 is a fertilization messenger in all species. To demonstrate the latter, it must be shown at least that blocking the effects of InsP_3 prevents egg activation and preferably that InsP_3 increases with the appropriate timing at fertilization. InsP_3 antagonists have been shown to prevent egg activation in frogs (Nuccitelli *et al.* 1993), sea urchins (Crossley *et al.* 1991; Galione *et al.* 1993; Lee *et al.* 1993; Lee & Shen 1998; Mohri *et al.* 1995),

starfish (Stricker 1995; Santella *et al.* 1999a), ascidians (McDougall & Sardet 1995) and mice (Kline & Kline 1994). Only in frog (Stith *et al.* 1993, 1994) and sea urchin (Ciapa & Whitaker 1986; Kuroda *et al.* 2001) eggs has InsP_3 been shown to increase at fertilization; measurements have not been reported in other species.

Sea urchin eggs were the material with which two new second messengers were discovered. The story began with the demonstration that pyridine nucleotide derivatives could release calcium from the egg's stores independently of InsP_3 (Clapper *et al.* 1987). The first of these derivatives to be purified was cyclic ADP-ribose (cADPr; Lee *et al.* 1989) and the second was nicotinic acid adenine dinucleotide phosphate (NAADP; Lee & Aarhus 1995). cADPr was found to mobilize an internal calcium store that was functionally distinct from the InsP_3 -mobilized calcium store and neither was a mitochondrial calcium store (Clapper & Lee 1985). NAADP was found to cause calcium release in both ascidian (Albrieux *et al.* 1998) and sea urchin eggs (Albrieux *et al.* 1998; Galione *et al.* 2000). The calcium store mobilized by NAADP is an acidic compartment, not the ER, and probably represents lysosomes (Churchill *et al.* 2002), while cADPr targets the RyR on the ER (Lee *et al.* 1994). Both messengers have been found to contribute to the calcium-signalling repertoire of somatic cells (Galione 1994; Thorn *et al.* 1994; Iino *et al.* 1997; Galione & Churchill 2000, 2002; Lee 2001).

As with InsP_3 , so with cADPr and NAADP: a *capacity* for a response to these agonists does not itself demonstrate a role at fertilization. cADPr increases during fertilization in sea urchin eggs (Kuroda *et al.* 2001; Leckie *et al.* 2003) and NAADP is found in sea urchin spermatozoa (Billington *et al.* 2002; Churchill *et al.* 2003), but the antagonists of neither NAADP (Churchill & Galione 2000) nor cADPr (Galione *et al.* 1993; Lee *et al.* 1993, 1996; Leckie *et al.* 2003) prevent the calcium wave in sea urchin eggs, nor do they in frog (Galione *et al.* 1993), starfish (Santella *et al.* 1999b; Moccia *et al.* 2004), ascidian (Albrieux & Villaz 2000) or mouse eggs (Ayabe *et al.* 1995).

5. INITIATION AND PROPAGATION OF THE FERTILIZATION CALCIUM WAVE

Now that we have all the actors on stage, our play can begin. Its plot sets out how the fertilizing sperm, tiny compared with the egg, triggers the fertilization calcium explosion and how in turn the calcium wave propagates across the egg.

(a) *Act I: calcium wave initiation*

This act comprises a number of subplots that will converge in Act II, the point being that the wave initiation mechanism appears to vary significantly between species. However, there are two broad themes. The first suggests that sperm-egg interaction is at the outset analogous to a hormone-receptor interaction, and the second one suggests that sperm and egg must fuse to enable calcium wave initiation.

Mutual recognition by gametes is an important mechanism of speciation (Swanson & Vacquier 1998; Vacquier 1998) and involves egg and sperm receptors.

Receptor interactions are required to trigger the acrosome reaction in the sperm (Darszon *et al.* 1999); the acrosome reaction releases enzymes that aid the travel of the sperm towards the egg surface and expose the plasma membrane patch on the sperm that will ultimately fuse with the egg plasma membrane. In mammalian oocytes, the cell–cell recognition process between gametes involves an integrin/disintegrin interaction mediated by the CD9 tetraspannin protein, ADAM proteases and the izumi CD9 receptor on the sperm (Evans *et al.* 1997; Cho *et al.* 1998; Fenichel & Durand-Clement 1998; Eto *et al.* 2000; Kaji *et al.* 2000; Miller *et al.* 2000; Zhu & Evans Janice 2002; Inoue *et al.* 2007; Vjugina & Evans Janice *in press*). In the sea urchin, the acrosomal protein, bindin, interacts with an EGF family receptor on the egg (Glabe 1985; Kamei *et al.* 2000).

The receptor transduction hypothesis originated in an observation that trimeric G-proteins were involved at fertilization in the sea urchin: the G-protein antagonist GDP β S prevented cortical granule exocytosis at fertilization, one of the signs of egg activation (Turner *et al.* 1986). However, it turned out that this antagonist did not prevent the fertilization calcium wave itself (Crossley *et al.* 1991). Integrin-derived RGD peptides can trigger the activation of both frog (Iwao & Fujimura 1996) and mammalian oocytes (Campbell *et al.* 2000; Viets *et al.* 2001) and integrins have a well-known signal transduction function (Akiyama 1996; Woods & Shimizu 2001). In frog oocytes, RGD peptides have been shown to prevent sperm binding and fusion (Iwao & Fujimura 1996). It is difficult, though, to unravel the effects of cell–cell adhesion from the putative signal transduction event. It is perhaps informative that the EGF receptor-like bindin in the sea urchin receptor has no signal transduction motif, perhaps implying that the sperm–egg receptor interaction has evolved for binding, not for signal transduction (C. G. Glabe 2005, personal communication).

Despite the absence of signal transduction motifs in the bindin receptor, Src-family tyrosine kinases have been shown to play a part in the initiation of the calcium wave in sea urchin and starfish eggs. Dominant-interfering SH2 domains from Src kinase delay or prevent the fertilization wave and an anti-Src antibody triggers a calcium wave (Giusti *et al.* 1999b, 2000a, 2003). The Src kinase stimulates PLC γ to produce InsP $_3$ (Giusti *et al.* 1999a, 2000b; Shearer *et al.* 1999; Jaffe *et al.* 2001; Runft Linda *et al.* 2004). SH2 domains inhibitory to PLC γ and Src-family kinases will block fertilization in sea urchins, starfish, zebrafish and ascidians (Carroll *et al.* 1997, 1999; Runft *et al.* 1999, 2002; Shearer *et al.* 1999; Rongish & Kinsey 2000; Kinsey William *et al.* 2003), but not in mammals or frogs (Mehlmann *et al.* 1998; Mehlmann & Jaffe 2005). Nonetheless, in frogs, Src-related tyrosine kinase co-immunoprecipitates with PLC γ after fertilization and complex formation is blocked by the protein tyrosine kinase inhibitor PP1 (Sato *et al.* 2000). Depleting cholesterol from lipid-signalling rafts with methyl- β -cyclodextrin caused a reduction in tyrosine kinase activity and blocked the fertilization calcium transient (Sato *et al.* 2002). The tyrosine kinase inhibitors lavendustin A and tyrphostin B46 prevented

the fertilization calcium wave, as did a 20 amino acid truncation of the Src SH2 domain (Glahn *et al.* 1999).

The second broad hypothesis imagines that sperm–egg receptor interactions induce the fusion of egg and sperm plasma membranes by an as yet unknown mechanism: sea urchin bindin will induce the fusion of membrane vesicles (Glabe 1985) and CD9 is known to be involved in the myocyte fusion (Kaji *et al.* 2000). The egg is then activated once the sperm and the egg fuse via the transfer of an activating factor from the egg to the sperm. To me, this is a more plausible idea given the evidence, although the activating factor most definitely varies from species to species.

The clearest evidence is found in the mouse. It is known that the fertilization calcium transient occurs some minutes after sperm–egg fusion (Lawrence *et al.* 1997). Mammalian sperm extracts produce the characteristic repetitive fertilization calcium waves of mouse, human and hamster eggs (Swann 1990, 1996; Homa & Swann 1994). After a false start (Parrington *et al.* 1996; Shevchenko *et al.* 1998), the active component was identified as a novel phospholipase C, PLC ζ , that was present only in the testis and the sperm (Rice *et al.* 2000; Saunders *et al.* 2002). This entirely specific tissue distribution argues very strongly that PLC ζ is the activating agent in mammalian fertilization; there is other indirect evidence: PLC ζ is sequestered into the zygote nucleus as it reforms after fertilization just as the fertilization calcium transients are dying away; if sequestration is prevented, the transients persist (Kono *et al.* 1995; Larman *et al.* 2004; Saunders *et al.* 2007). It has to be admitted though that the definitive proof by gene knockout is lacking.

The experiment of grinding up sperm and micro-injecting the resultant extract into its egg has been performed in quite a number of species (Dale *et al.* 1978; Swann 1990; Santella & Kyozuka 1994; Wilding & Dale 1997, 1998; Runft & Jaffe 2000; Li *et al.* 2001; Wu *et al.* 2001; Yamamoto *et al.* 2001; Runft *et al.* 2002; Coward *et al.* 2003; Howell *et al.* 2003; Knott *et al.* 2003; Santella *et al.* 2004), but the molecular identity of the activating factor is still known only in mammals, perhaps because the signature of the calcium transient is so distinctive and so sensitive to dose. Eggs that produce only a single calcium transient at fertilization, for example amphibians (Yamamoto *et al.* 2001) and sea urchins (Dale *et al.* 1978), are too promiscuous in their response to a variety of activating agents to permit ready biochemical enrichment of the putative activating factor. On the other hand, ascidian oocytes resemble mammalian oocytes in having a distinctive calcium signature evoked by sperm extract (Kyozuka *et al.* 1998; Wilding & Dale 1998; McDougall *et al.* 2000; Runft & Jaffe 2000), and therefore offer better chance of identifying the factor, which, in ascidians, acts through the Src-PLC γ pathway (Runft *et al.* 1999) that has been shown to be the activating mechanism at fertilization.

In echinoderms, both nitric oxide (NO) and cGMP have been suggested to activate messengers at fertilization, based on the fact that both NO and cGMP increase at fertilization and can trigger a calcium transient. Measurements of cGMP show it to rise by 20 s after insemination, the earliest time point measured (Ciapa & Epel 1996; Kuroda *et al.* 2001), though this is late

relative to the timing of the calcium transient (Whitaker 2005). A concentration of 5–10 μM cGMP in the cytoplasm is required to activate a sea urchin egg (Whalley *et al.* 1992; Kuroda *et al.* 2001), while peak concentrations of cGMP at fertilization have been measured to be 20–100 nM (Ciapa & Epel 1996; Kuroda *et al.* 2001), which seems too little. Compare InsP_3 whose peak concentration reaches 0.2–0.3 μM at 20 s after insemination (Kuroda *et al.* 2001) while eggs that have been shown to activate at cytoplasmic concentrations of 2 nM (Whitaker & Irvine 1984). The G-kinase inhibitor RcAMPS completely blocks cGMP-induced calcium release, but cannot prevent fertilization wave initiation (Galione *et al.* 1993; Lee *et al.* 1993, 1996).

NO may (Kuo *et al.* 2000) or may not (Leckie *et al.* 2003) increase very soon after fertilization and there is a similar disagreement over whether the NO scavenger, oxyhaemoglobin, can block the fertilization calcium transient (Kuo *et al.* 2000; Leckie *et al.* 2003). NO activates eggs via the cGMP/cADPr pathway (Willmott *et al.* 1996), and it is known that blocking the downstream elements of this pathway does not prevent the fertilization calcium transient from occurring (Galione *et al.* 1993; Lee *et al.* 1993, 1996; Leckie *et al.* 2003). The NO pathway is absent in ascidian and mouse eggs (Hyslop *et al.* 2001). Since NO-induced egg activation can be blocked by antagonists of the cGMP pathway, it is unlikely that a different mechanism, such as nitrosylation (Kuo *et al.* 2000), occurs at fertilization.

NAADP is also a candidate as an activation messenger in echinoderms. It triggers calcium release in unfertilized eggs (Lee 2001; Churchill *et al.* 2003; Santella *et al.* 2004) and is present at activating concentrations in sperm (Billington *et al.* 2002; Churchill *et al.* 2003). But the inactivation of the NAADP pathway inhibits membrane currents (Churchill *et al.* 2003; Moccia *et al.* 2004), not the fertilization calcium wave (Churchill *et al.* 2003). In starfish, the fertilization wave is prevented by an InsP_3 chelator (Iwasaki *et al.* 2002) and by SH2 domains directed against Src and $\text{PLC}\gamma$ (Carroll *et al.* 1997; Giusti *et al.* 1999b), but equally NAADP can mobilize calcium even when the InsP_3 pathway is downregulated (Lim *et al.* 2001).

The most elegant mechanism proposed to explain egg activation after sperm–egg fusion is the conduit hypothesis (Jaffe 1997; Xu & Sternberg Paul 2003). The idea is that calcium channels in the sperm provide a source of calcium that diffuses into the egg once the sperm and the egg fuse. Once inside the egg, it provokes calcium-induced calcium release (CICR), a mechanism known to be important in stimulus contraction coupling in the heart (Cheng *et al.* 1993). In the heart, though, the mechanism is, as would be expected, completely dependent on the presence of extracellular calcium. In contrast, the fertilization calcium wave is unaffected by the removal of external calcium (Chambers 1980; Chambers & Angeloni 1981; Epel *et al.* 1982).

(b) The interval

While waiting for Act II, we can sum up the available data on calcium wave initiation at fertilization. Signalling pathways thought to be implicated in the

Figure 1. Messengers implicated in the initiation of the fertilization wave. In frogs, ascidians and echinoderms, Src-family kinases (SFK) activate $\text{PLC}\gamma$ to produce InsP_3 and trigger the calcium waves (blue pathway); in sea urchins, there is good evidence that sperm–egg fusion is required for egg activation, but in frogs this is less certain. In mammals, sperm–egg fusion introduces $\text{PLC}\zeta$ into the egg cytoplasm, so producing InsP_3 (yellow pathway). Sperm–egg fusion may also introduce NAADP in echinoderms; NAADP activates plasma membrane calcium channels (red pathway). In ascidians, NAADP *inactivates* plasma membrane channels, while cADPr triggers local calcium release to trigger cortical granule exocytosis (red pathway). In sea urchins, calcium activates nitric oxide production, which generates cADPr via cGMP (green pathway). This postulated activation pathway in fact operates later during the fertilization calcium wave.

initiation of the fertilization wave are shown in figure 1. NAADP contributes to calcium signalling at fertilization by modulating calcium channels (Albrieux *et al.* 1998; Churchill *et al.* 2003; Moccia *et al.* 2004). Calcium influx through voltage-gated channels is an important element of the fertilization response across a wide range of species studies, but extracellular calcium is not necessary for the initiation of the fertilization calcium wave (summarized in Stricker 1999). cADPr and RyRs also act to control calcium concentrations at the egg cortex and control cortical granule exocytosis in some species (Ayabe *et al.* 1995; Albrieux *et al.* 1997, 1998). Both NAADP and cADPr have parts to play in the calcium wave itself, as we shall see in Act II. It looks though as if the activation of a phospholipase C is the wave-initiating event at fertilization. Again, Stricker (1999) summarizes the data across all the phyla studied and without exception the InsP_3 signalling pathway is active and essential at fertilization. The two key PLCs are $\text{PLC}\zeta$ in mammals and $\text{PLC}\gamma$ in frogs, echinoderms and ascidians. It appears that $\text{PLC}\gamma$ plays no role in mammals (Mehlmann *et al.* 1998) and there is as yet no evidence that $\text{PLC}\zeta$ plays any part in other species. It seems clear that $\text{PLC}\zeta$ enters the egg to activate it as a consequence of sperm–egg fusion. Whether the Src pathway also uses this mechanism remains ambiguous, as the activator that might enter

the egg from the sperm has not been identified in species other than mammals. On balance, it seems probable as no signal transduction mechanism involving receptors has been clearly identified, although it remains possible that an integrin receptor mechanism may be involved in frog oocytes at fertilization (Iwao & Fujimura 1996).

(c) *Act II: calcium wave propagation*

Fertilization calcium wave velocities are constant in the range of 5–30 $\mu\text{m s}^{-1}$ (Jaffe & Crton 1998; Stricker 1999; Jaffe 2008). Fertilization calcium waves are a subset of the class of calcium waves of this velocity, which are found widely in both germ line and somatic cells (Jaffe 1995, 2008). Jaffe presents the evidence that these are, in terms of chemistry, reaction–diffusion waves analogous to the well-known Belousov–Zhabotinsky reaction (Speksnijder *et al.* 1989; Jaffe 1991, 2008). The major source of calcium for the wave is the ER (Galione *et al.* 1991; Terasaki & Jaffe 1991; Kline 2000), which is charged with calcium by a SERCA pump (Kline & Kline 1992b; Jones *et al.* 1995; Lawrence & Cuthbertson 1995; Lee & Aarhus 2000). One proof of this is that the wave can propagate in the absence of external calcium (Chambers 1980; Chambers & Angeloni 1981; Schmidt *et al.* 1982; Crossley *et al.* 1988). Calcium waves are built up from many individual calcium release events from one or a very few clustered calcium channels in the ER membrane, be they InsP_3 or RyRs (Cheng *et al.* 1993; Lipp *et al.* 1997; Keizer & Smith 1998; Marchant *et al.* 1999). Propagation occurs by reaction (calcium-triggered activation of the release channel) and diffusion (of calcium to neighbouring receptors), a mechanism of CICR (Endo *et al.* 1970; Keizer & Smith 1998; Solov'yova *et al.* 2002). Fertilization calcium waves are in general carried by the InsP_3 receptor (Stricker 1999). For example, the mammalian fertilization wave is abolished by the microinjection of an InsP_3 receptor antibody (Miyazaki *et al.* 1992, 1993). The InsP_3 receptor is sensitized to CICR by InsP_3 (Adkins & Taylor 1999); in the absence of InsP_3 , calcium inhibits the receptor, while in its presence, calcium provokes further calcium release. Production and diffusion of InsP_3 can also participate in the reaction–diffusion mechanism underlying InsP_3 R-mediated calcium waves (Allbritton & Meyer 1993; Keizer *et al.* 1995). Calcium may trigger further production of InsP_3 (Whitaker & Aitchison 1985; Ciapa & Whitaker 1986; Ciapa *et al.* 1992; Stith *et al.* 1994). In support of this idea, a dominant negative PH domain inhibitor of $\text{PLC}\gamma$ not only delays the onset of the calcium wave, but also appears to diminish its propagation (Shearer *et al.* 1999). On the other hand, heparin, an InsP_3 antagonist, delays the onset of the calcium wave without appearing to alter its velocity (Crossley *et al.* 1991); perhaps this is explained by the fact that heparin is a competitive inhibitor: if, once the wave is initiated, InsP_3 increases markedly, it may be sufficient to overcome inhibition by heparin.

RyRs appear to contribute to the calcium wave only in fish, nemertean and echinoderm eggs, as these are the only eggs studied that are sensitive to caffeine or ryanodine, RyR agonists (Galione *et al.* 1993;

Miyazaki *et al.* 1993; Fluck *et al.* 1999; Santella *et al.* 1999b; Stricker 1999; Polzonetti *et al.* 2002). In echinoderms, the microinjection of calcium can trigger a propagating calcium wave (Hamaguchi & Hiramoto 1981; Mohri & Hamaguchi 1991) and ruthenium red, an RyR antagonist, reduces the propagation velocity of the wave (Miyazaki 1988; Galione *et al.* 1993). A calcium wave can be triggered in sea urchin eggs by calcium influx after sensitizing the RyR (McDougall *et al.* 1993). The sea urchin egg RyR (Shiwa *et al.* 2002) plays a contributory role in calcium wave propagation. Eggs of species lacking functional RyR responses at fertilization nonetheless possess RyRs (Swann 1992; Ayabe *et al.* 1995). In these mammalian eggs, calcium release from RyR at the egg periphery may nonetheless contribute to cortical granule exocytosis (Kline & Kline 1994) and to sustaining the multiple calcium oscillations that follow fertilization (Swann 1992).

In eggs with RyR receptors but lacking a functional RyR response, cADPr does not cause global calcium release when microinjected (Kline & Kline 1994; Ayabe *et al.* 1995). The receptor for cADPr has not yet been identified but its action requires calmodulin, which appears to increase affinity for cADPr and to increase the sensitivity of the RyR to CICR by several orders of magnitude (Lee *et al.* 1994, 1995; Thomas *et al.* 2001). It is not known why some RyR-containing eggs are insensitive to cADPr—perhaps they lack a receptor. In sea urchin and starfish eggs, cADPr and RyR contribute to the calcium wave (Galione *et al.* 1993; Santella *et al.* 1999b). Antagonists of cADPr do not alter the propagation of the fertilization calcium wave in sea urchin eggs (Lee *et al.* 1996), but appear to curtail the long falling tail of the transient (Leckie *et al.* 2003), indicating that cADPr acts late during the fertilization calcium response, mediated by NO (Leckie *et al.* 2003).

6. SINGLE AND MULTIPLE FERTILIZATION WAVES AND TRANSIENTS

Fishes, frogs, jellyfish, some molluscs, echinuran worms, sea urchins and starfish produce a single large calcium transient at fertilization (Stricker 1999); the others including nemertean worms, annelid worms, ascidians and mammals produce multiple calcium transients (Stricker 1999). Single transients are found in species with eggs and oocytes that arrest in interphase either before or after meiosis, while multiple transients occur in species in which oocytes are arrested during meiotic metaphase (the exceptions are the frogs and the fishes, whose oocytes undergo a single large calcium transient despite being arrested in the second meiotic metaphase). These observations underline the importance of understanding calcium signalling in the context of cell-cycle regulation.

Calcium signals have a natural propensity for oscillation, as both the InsP_3 and RyRs have both positive and negative feedback mechanisms that favour calcium oscillations (Berridge & Galione 1988). CICR, where increased cytoplasmic calcium concentrations enhance the open probability of the channels, is a property of both InsP_3 R and RyR and both receptors

have bell-shaped responses to cytoplasmic calcium: at higher concentrations, the effect of a further increase is inhibitory (Ehrlich 1995). These characteristics are themselves sufficient to generate oscillatory calcium release; depletion of ER calcium stores adds a further negative feedback element (Berridge & Galione 1988) and, for the InsP_3 receptor, additional positive feedback can come from calcium-stimulated hydrolysis of PtdInsP_2 . Of the oocytes that undergo repetitive cytoplasmic calcium spikes after fertilization, ascidian and mammalian oocytes are best studied. The detail of their calcium spiking is distinct: in ascidians, the spikes immediately after fertilization are superimposed on a larger sustained transient (McDougall & Sardet 1995), whereas in mammals, each spike is separate and the interval between spikes can last for several minutes (Swann & Parrington 1999). The second phase of spikes in ascidians more closely resembles that in mammalian oocytes and may involve NAADP (Albrieux *et al.* 1998). Nonetheless, both spiking patterns can be mimicked reasonably well by slow infusion or release of InsP_3 into the oocytes (Miyazaki 1991, 1995; Fissore & Robl 1993, 1994; Galione *et al.* 1994; Fissore *et al.* 1995; McDougall & Sardet 1995; Jones & Whittingham 1996; Swann 1996; Albrieux *et al.* 1997, 1998; Takahashi *et al.* 2000), suggesting that the spiking pattern may be governed by the basic feedback properties of the InsP_3 receptor (Adkins & Taylor 1999).

In some species, InsP_3 production leads to a single calcium transient, while in others multiple transients occur; there is a broad correlation, as we have seen, with the cell-cycle stage. This can be shown experimentally. Calcium spikes can be sustained indefinitely by preventing exit from meiosis. One way to achieve this is to use microtubule inhibitors, for example colcemid, to invoke the metaphase checkpoint in fertilized eggs and maintain a metaphase state (Kono *et al.* 1996). Another approach has been to use exogenous cyclin B or non-degradable cyclin B to defeat the APC/proteasome and maintain metaphase levels of cdk1/cyclin B kinase activity (Levasseur & McDougall 2000; Nixon *et al.* 2000). The mitotic kinase may maintain the oscillations by stimulating the continued production of InsP_3 , sensitizing the InsP_3 receptor itself or maintaining high levels of ER calcium by stimulating the SERCA pump. However, a simple hypothesis to explain the data presupposes that calcium spike generation is caused by a factor from sperm introduced after sperm-egg fusion and sequestered into the nucleus; if the fertilized egg is in mitosis, the factor will not be sequestered and spikes will persist, whereas interphase eggs will sequester the factor and a single calcium spike/wave will occur.

It is clear that in both ascidians (McDougall & Sardet 1995; McDougall *et al.* 1995; Roegiers *et al.* 1995) and mouse eggs (Deguchi *et al.* 2000; Dumollard *et al.* 2002), calcium spikes are generated at foci associated with the sperm entry point and thus a sperm-activating factor. The only evidence that the interphase nucleus can sequester a sperm-activating factor comes from mammalian eggs. For example, the transfer of the interphase nucleus of the one-celled embryo to an unfertilized oocyte can activate it: the nucleus breaks down in response to the elevated cdk1/cyclin B kinase activity and

the initiation of the calcium spikes correlates with the breakdown of the nuclear envelope (Kono *et al.* 1995). In fertilized eggs, spikes cease at the time of reformation of the pronucleus; inhibiting the reformation of the nuclear envelope with a lectin sustains the calcium spikes (Marangos *et al.* 2003). PLC ζ , the sperm-activating factor in the mouse, has been shown to be sequestered into the interphase nucleus (Kouchi *et al.* 2004; Larman *et al.* 2004; Yoda *et al.* 2004). There does appear to be virtue in the simple idea that repetitive calcium spiking occurs unless and until an interphase nucleus is present to sequester the calcium wave-generating activity introduced by the sperm at fertilization, at least in the mouse: in ascidian no nuclear sequestration occurs and calcium spikes are controlled solely by cdk1 activity (Levasseur *et al.* 2007).

What about the fish and the frog eggs? These exhibit a single calcium transient, despite being arrested in meiotic metaphase II, not interphase. Fish and frog eggs are large and have very prolonged fertilization calcium transients as a consequence. They exit metaphase and move into interphase within tens of minutes, as their calcium transients are subsiding (Stricker 1999), leaving no time for multiple calcium spikes.

7. FERTILIZATION AND THE CELL DIVISION CYCLE

A major role of the fertilization calcium signal is to activate the cell-cycle regulators, cdk1 and cyclin. Eggs and oocytes of different species are arrested at different points in the cell cycle (Whitaker 1996). Species differ in their point of arrest during meiotic maturation as they await fertilization. Ascidian oocytes, for example, arrest during first meiotic metaphase; mammalian and frog oocytes arrest during second meiotic metaphase while sea urchin eggs have completed meiosis and are arrested in G1 of the first post-meiotic cell cycle. Meiotic metaphase arrest is maintained by the sustained activity of the mitotic kinase, cdk1/cyclin, which maintains chromatin in its condensed state and stabilizes the meiotic spindle (Whitaker 1996); the interphase arrest in sea urchins is maintained by the suppression of cyclin synthesis by a cytoplasmic pH 0.5 units more acidic than that of fertilized eggs (Winkler *et al.* 1980; Whitaker & Steinhardt 1982; Evans *et al.* 1983).

Cyclins are synthesized during interphase and destroyed abruptly during mitosis (Evans *et al.* 1983; Hunt 1991). Protein synthesis is markedly pH dependent in sea urchin eggs and homogenates (Grainger *et al.* 1979; Winkler *et al.* 1980) and can, for example, be stimulated by weak bases that alkalinize the egg cytoplasm to levels comparable with those measured after fertilization (Grainger *et al.* 1979). The rapid alkalization after fertilization is achieved by the activation of a Na/H antiporter (Johnson & Epel 1976). The antiporter is activated by PKC, which in turn is stimulated by both the fertilization calcium transient and the diacylglycerol produced by the activation of phospholipase C (Swann & Whitaker 1985; Epel 1990). In sea urchin eggs, fertilization in interphase stimulates cyclin synthesis, but the calcium transient also directly stimulates DNA synthesis by

activating an ERK pathway (Philipova & Whitaker 1998, 2005; Philipova *et al.* 2005).

In oocytes that are arrested in meiosis awaiting fertilization, the oocyte protein *mos* appears to maintain a metaphase arrest before fertilization (Verlhac *et al.* 1996; Tunquist *et al.* 2002), preventing cyclin degradation. The fertilization calcium signal does not immediately interact with *mos* signalling (Lorca *et al.* 1991, 1993). Instead, it bypasses the checkpoint by stimulating cyclin degradation via CaMkinase II-mediated stimulation of cyclin ubiquitination (Lorca *et al.* 1991; Reimann & Jackson 2002) and stimulation of the proteasome degradation machinery (Kawahara & Yokosawa 1994; Aizawa *et al.* 1996). The major role of calcium in frog and mouse eggs at fertilization is to reinitiate anaphase onset by activating the anaphase-promoting complex/cyclosome (APC/C), relieving inhibition of cyclin degradation and stimulating proteasome activity to allow the cell cycle to proceed.

Mature but unfertilized mammalian oocytes continue to synthesize proteins. Continuing cyclin synthesis is essential for the maintenance of the meiotic arrest, as transcription inhibitors will relieve the metaphase arrest when added to unfertilized mammalian eggs (Moses & Kline 1995). Repetitive calcium spiking leads to progressive reduction in cyclin/cdk1 activity in the face of continuing cyclin synthesis (Ozil & Swann 1995; Swann & Parrington 1999; Cheung *et al.* 2000). Successive calcium spikes lead to the progressive destruction of a cyclin B–GFP fusion protein in ascidian and mouse oocytes and spike frequency declines with cyclin levels (McDougall & Levasseur 1998; Levasseur & McDougall 2000; Nixon *et al.* 2000, 2002).

The fertilization calcium signal must restart the cell cycle. In oocytes in metaphase, this is brought about by the CaMkinase II activation of cyclin degradation; in interphase oocytes, the converse is true: cyclin synthesis is restarted through a PKC-mediated pH increase.

8. CALCIUM AND THE EARLY EMBRYONIC CELL CYCLES

An argument by generalization suggests that if fertilization calcium signals control cell-cycle transitions, then calcium signals will also be important in the ensuing cell-cycle transitions in early embryos (Whitaker & Patel 1990). This idea has met with supportive data from experiments on the rapidly dividing embryos of sea urchins, frogs and flies. Sea urchin embryos undergo eight cycles of cell division within 4 hours, frog embryos, 8 cycles within 6 hours and fruit fly embryos, 13 nuclear divisions within 3 hours. During these divisions, the embryo relies heavily on maternal stores of metabolic substrates, mRNA and protein; there is little or no gene transcription (Gurdon *et al.* 1992; Davidson *et al.* 2002). These rapid embryonic mitoses involve three events in each cycle: dissolution of the nuclear envelope, chromatin condensation and formation of the mitotic spindle; separation and segregation of the chromosomes by spindle elongation; and separation of daughter cells by the formation of the cleavage furrow. Each of these events in sea urchin embryos is blocked by

lithium treatment and the block rescued by the addition of *myo*-inositol (Becchetti & Whitaker 1997), demonstrating that phosphoinositide signalling is involved (Berridge *et al.* 1982). Cell-cycle progression is also blocked by L690,330, a bisphosphonate inhibitor of inositol monophosphatase that acts similarly to lithium (Sconzo *et al.* 1998).

(a) Calcium signals and nuclear envelope breakdown

A calcium transient precedes nuclear envelope dissolution in the first cell cycle of the sea urchin embryo (Poenie *et al.* 1986). Microinjection of calcium or InsP_3 induces NEB precociously (Steinhardt & Alderton 1988; Twigg *et al.* 1988) and calcium chelators block NEB reversibly (Wilding *et al.* 1996). NEB in starfish embryos is also preceded by a calcium spike and blocked by heparin injection (Stricker 1995). The NEB calcium transient is very localized in the perinuclear region in both sea urchin (Wilding *et al.* 1996) and *Drosophila* embryos (Parry *et al.* 2005).

(b) Calcium signals and chromosome segregation

The separation of sister chromatids in sea urchin embryos is preceded by a small calcium transient (Groigno & Whitaker 1998). The transient is blocked by the calcium chelator dibromo-BAPTA or the InsP_3 receptor antagonist and blocking the calcium transient prevents the separation of sister chromatids. Uncaging calcium from NP-EGTA using a UV pulse or uncaging InsP_3 in the presence of heparin leads to normal chromatid segregation (Groigno & Whitaker 1998). Calcium signals accompany mitosis in syncytial *Drosophila* embryos restricted to the spindle region (Parry *et al.* 2005). Two InsP_3 antagonists, one the InsP_3 sponge, a recombinant protein consisting of the InsP_3 -binding domain of the type 1 InsP_3 receptor (Walker *et al.* 2002), and the other p130, an inactive phospholipase that binds InsP_3 (Takeuchi *et al.* 2000), prevented chromatid segregation (Parry *et al.* 2005). In frog embryos, microinjection of heparin (Han *et al.* 1992) and an InsP_3 R antibody (Muto *et al.* 1996) prevents the division of the one-celled embryo and EGTA and dibromo-BAPTA to prevent division in the two-celled embryo (Noguchi & Mabuchi 2002).

(c) Calcium and formation of the cleavage furrow

Calcium signals have been measured in the cleavage furrow of Medaka (Fluck *et al.* 1991), zebrafish (Chang & Meng 1995) and frog (Muto *et al.* 1996) embryos. These signals depend on InsP_3 -induced calcium release from internal stores (Chang & Meng 1995; Muto *et al.* 1996). Distinct calcium signals have been identified to be associated with lateral extension of the furrow and furrow deepening was very evident (Webb *et al.* 1997, 2008; Crton *et al.* 1998). A furrow-positioning calcium signal has also been identified (Chang & Lu 2000). Furrowing is blocked by the calcium chelator BAPTA and the InsP_3 antagonist heparin (Chang & Lu 2000), but is unaffected by RyR and NAADP antagonists (Lee *et al.* 2003). In sea urchin embryos, local increases in calcium concentration and calmodulin activation beneath and around

the cleavage furrow have been observed (Stricker 1995; Wilding *et al.* 1995; Groigno & Whitaker 1998; Török *et al.* 1998). In early human embryos, ER accumulates in the cleavage furrow (Goud *et al.* 1999). In *Drosophila* embryos, very superficial calcium increases closely correlated with actin dynamics in space and time occur in cleavage furrows (Parry *et al.* 2005).

(d) Cell-cycle calcium signals in early embryos

There seems then to be some substance in the idea that calcium signals control cell-cycle transitions in early embryos. Perhaps, though, this is the case only in embryos that undergo rapid cell divisions immediately after fertilization. Mouse embryos' cell cycles are measured in hours rather than minutes. Calcium transients are seen by some in mouse embryos around the time of NEB (Kono *et al.* 1996), but others have reported either a weak correlation of calcium signals with NEB (Tombes *et al.* 1992) or the absence of NEB- (Day *et al.* 2000; Tang *et al.* 2000; Gordo *et al.* 2002) and cleavage-associated (Tombes *et al.* 1992) calcium signals. The team that originally reported NEB-associated calcium signals in the mouse (Kono *et al.* 1996) later found that the calcium signals were detected only once NEB had occurred (Marangos *et al.* 2003). This raises the possibility that cell-cycle calcium signals are important only in rapidly dividing embryos.

9. CALCIUM SIGNALS IN EARLY EMBRYOS

The importance of calcium signalling at fertilization is beyond dispute. It is on the whole well understood and sets out the repertoire of calcium-signalling mechanisms that may be important later in development. There are strong indications that calcium signals also have an essential role in regulating cell-cycle transitions in rapidly dividing embryos.

Other papers in this issue (Freisinger *et al.* 2008; Moreau *et al.* 2008; Spitzer 2008) explore the role of calcium signalling in developmental patterning. The key challenge is to understand how the protean calcium signal can fulfil such disparate functions during development: fertilization; cell-cycle control; axis formation; and neuronal specification. There are some clues about how these different outcomes may be intertwined. One instance is the ascidian embryo.

The repetitive calcium spikes in fertilized ascidian embryos progressively induce cyclin destruction and hence promote exit from meiosis and cell-cycle progression (McDougall & Levasseur 1998; Levasseur & McDougall 2000). At the same time, these calcium spikes determine the profound cytoplasmic reorganization, by initiating marked cortical contractions in which ER is transported to a nipple-shaped extrusion in the vegetal hemisphere known as the contraction pole (Speksnijder *et al.* 1990, 1993; Speksnijder 1992; McDougall & Sardet 1995; Dumollard & Sardet 2001). In the same reorganization, mitochondria are concentrated towards the equator on one side of the embryo. The mitochondrial region is known as myoplasm, as it contains both the mitochondria and the cytoplasmic determinants that will specify and fuel the muscular tail of the

swimming embryo (Jeffery & Swalla 1990). Since the cortical, actin-based contractions are dependent on the calcium spikes and the ooplasmic segregation dependent on the contractions (Jeffery & Swalla 1990; Speksnijder *et al.* 1993; Roegiers *et al.* 1995), it is firmly established that the calcium waves are essential for embryonic pattern formation. This set of calcium spikes thus has at least two functions: to induce cell-cycle progression and to establish an embryonic axis.

Another example is the existence of fate-determining calcium signals in late blastulae and whether or how calcium signals can have intertwined roles at this stage of development. Can calcium signals control cell-cycle progression at the same time as they contribute to axis specification? In syncytial blastomeres of *Drosophila* embryos, a dorsoventral gradient of calcium concentration coexists with cell-cycle calcium signals (Créton *et al.* 2000; Parry *et al.* 2005). In zebrafish, one effect of XWnt-5A expression is to bring the late blastula calcium spikes into closer synchrony with the global cell division cycle of the blastula (Slusarski *et al.* 1997), so that they more clearly occur at the time of mitosis and cell division. It appears to be an effect not of altering the synchrony of spikes and cell division in single blastomeres, but of improving global cell-cycle synchrony in the blastula, so that the association of the calcium spikes with cell division becomes more apparent.

We may need to get used to the idea that calcium signals in early embryos can do more than one thing at once.

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