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Lighting the fuse at fertilization

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SUMMARY

In most deuterostome eggs, fertilization is marked by an abrupt and transient increase in intracellular calcium concentration. The transient takes the form of a propagating wave and is the signal for the onset of development. For those interested in cell signalling, the two obvious questions to ask are how the wave is initiated and how it propagates through the egg cytoplasm. Answers have come largely from experiments in frog, hamster, mouse and sea urchin eggs. One explanation of signal transduction at fertilization makes an analogy with transmembrane signalling in somatic cells, where a family of G-protein-linked receptors pass activating signals across the plasma membrane. Another, older idea is that it is the fusion of sperm and egg that is responsible for detonating the calcium explosion at fertilization. We discuss the relative merits of the two ideas. Both are plausible; the creative tension between them has led to experiments that broaden our view of signal transduction at fertilization.

Key words: fertilization, calcium concentration, cell signalling, egg cytoplasm, transmembrane signalling, G-protein

INTRODUCTION

Lionel Jaffe is responsible for the fundamental idea that the sperm detonates a calcium explosion at fertilization in deuterostome eggs (Jaffe, 1980; 1983). Others have demonstrated that the global calcium signal is necessary and sufficient for the onset of development (Steinhardt et al., 1974; Lorca et al., 1991; see Whitaker and Steinhardt, 1982 and Whitaker and Patel, 1990 for reviews). The analogy of the black, round anarchists' bomb with a fizzing fuse is a very useful one. It nicely illustrates that the fertilization calcium transient is an explosive, autocatalytic process; it also makes the point that these explosive processes must be detonated with a fuse of some sort. In sea urchin eggs, as we shall see, one can measure the time it takes for the fuse to burn: about 15 seconds elapses from the time when it is lit by the sperm to detonation. A third insight offered by the analogy is that the fuse and the high explosive need not be made of the same stuff. Equally, it may be important to make a distinction at fertilization between the triggering mechanism and the calcium explosion itself.

BANG

Calcium explosions of the sort that were first seen in eggs (Gilkey et al., 1978) are a general property of calcium signalling systems in somatic cells too (Berridge and Galione, 1988; Berridge, 1991). Calcium signalling systems appear to have built into them explosive positive feedback mechanisms that give rise to spatial waves and temporal oscillations of free calcium concentration when the signalling system is stimulated by a fixed amount of external agonist. Indeed, the waves and oscillations are two sides of the same coin (Jaffe, 1991). The unifying feature of calcium waves and oscillations is that they primarily involve the modulated release and re-uptake of calcium from intracellular stores. The site of calcium release and resequestration is generally accepted to be the endoplasmic reticulum (Terasaki and Sardet, 1991). Release of calcium occurs through a family of related calcium channels that are gated by the phosphoinositide messenger, inositol trisphosphate (InsP_3; Ross et al., 1989; Furuichi et al., 1989; Mignery et al., 1989; Südhof et al., 1991), by calcium itself (Lai et al., 1988; Finch et al., 1991) and possibly by a newly discovered and potent calcium-mobilizing agent cyclic-ADPribose (cADPr; Galione et al., 1991). Explosive positive feedback could occur as the result of calcium-stimulated production of InsP_3 (Whitaker and Irvine, 1984; Meyer and Stryer, 1988), through stimulation of ryanodine-sensitive calcium release channels by calcium itself (calcium-induced calcium release: CICR; Endo, 1977; Lai et al., 1988; Takeshima et al., 1989) or through InsP_3-induced alterations in the sensitivity of InsP_3-gated release channels to calcium (Finch et al., 1991; Bezprovzanny et al., 1991; Missiaen et al., 1991).

The fertilization calcium waves in frog and sea urchin eggs originate at the point of sperm attachment and cross the egg at a velocity of 5-10 μm/seconds (L. F. Jaffe, 1983; Eisen et al., 1984; Yoshimoto et al., 1987; Kubota et al., 1987; McCulloh and Chambers, 1991; Fig. 1). Two distinct positive feedback mechanisms were suggested as the basis for the wave: CICR in frog (Busa et al., 1985) and calcium-

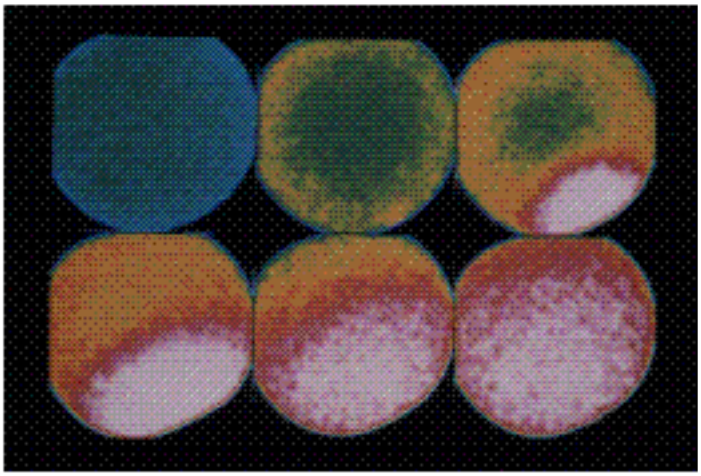

Fig. 1. Confocal image of changes in the cytoplasmic calcium concentration during fertilization in sea urchin eggs in equatorial section using the fluorescent indicator dye fluo3. The images read left to right in two rows and were taken every five seconds. The increase in Ca_i beneath the plasma membrane (image 2) is due to depolarization-induced calcium entry through voltage-gated channels. The calcium influx sets off a small, yellow, spherically symmetric CICR wave. The egg depolarizes at the same time as cytoplasmic continuity is established between egg and sperm, marking the onset of fertilization. However, though the calcium influx is very useful as a marker of the initial sperm-egg interaction, it is not an essential component of the fertilization response (Chambers, 1989); indeed, the fertilization calcium transient is unaffected by its absence (Fig. 2). The large fertilization calcium explosion proper shows up in pink and white. It is initiated at the point of sperm egg fusion (5 o'clock) and crosses the egg as a wave in some 20 seconds. Blue/green represents 0. 2 μM Ca_i, yellow, 0. 4 μM Ca_i and red 1 μM Ca_i. Concentrations higher than 1 μM are shown in white. The egg is 100 μm in diameter. *Lytechinus pictus*, 20°C.

stimulated InsP_3 production in sea urchin (Whitaker and Irvine, 1984; Whitaker and Aitchison, 1985; Swann and Whitaker, 1986). Ironically, the situation is now reversed: microinjecting heparin, an InsP_3-receptor antagonist (Hill et al., 1987; Cullen et al., 1988), abolishes the egg's response to InsP_3 but does not prevent the fertilization calcium transient in sea urchin eggs, suggesting that InsP_3 receptors do not contribute substantially to the calcium wave (Crossley et al., 1991); in contrast, heparin blocks activation and both InsP_3- and calcium-induced calcium release in frog eggs and homogenates (A. Galione and M. J. Whitaker, unpublished results) and an InsP_3 sponge blocks the fertilization calcium transient (Nuccitelli et al., 1992). Moreover, cADPr, a putative calcium-releasing messenger whose target has been suggested to be a ryanodine sensitive channel (Galione, 1992) causes substantial calcium release in the sea urchin egg (Clapper et al., 1987; Lee et al., 1989; Dargie et al., 1990) and in calcium-sequestering sea urchin egg homogenates (Clapper et al., 1987; Galione et al., 1991), while being ineffective in the frog. Ryanodine, an agonist of CICR channels, stimulates calcium release in sea urchin eggs (Galione et al., 1991; Sardet et al., 1992; McPherson et al., 1992) and in mouse eggs (Swann, 1992).

A reasonable case can now be made, then, for a CICR-based mechanism in the sea urchin egg and a mechanism based on the InsP_3 receptor in the frog. Whether the latter is due to calcium-stimulated InsP_3 production (CSIP) is another matter. The difficulty with CSIP as a mechanism is that PtdInsP_2, the precursor of InsP_3, is located predominantly in the plasma membrane in most cell types, while the fertilization calcium wave appears to propagate through the cytoplasm. This is a serious difficulty for an egg as big as the frog's (1 mm diameter). Diffusion of InsP_3 from the plasma membrane could never account for calcium increases deep within the egg. It is possible that PtdInsP_2 is present in the egg's internal membranes (Helms et al., 1991); it would be relatively easy to check this in the frog. Because of these difficulties for the CSIP hypothesis in the frog egg, it seems more likely that a positive feedback mechanism involving calcium-stimulated release via the InsP_3 receptor (Finch et al., 1991) operates in frog eggs. This is just a variant of CICR, but involves the InsP_3 receptor in place of the ryanodine receptor that mediates classical CICR in skeletal muscle (Lai et al., 1988).

Hamster eggs experience both calcium waves and calcium oscillations at fertilization (Igusa and Miyazaki, 1986; Miyazaki et al., 1986). The first fertilization calcium transient takes the form of a wave originating at the point of sperm entry, as in frog or sea urchin eggs. Subsequent transients are spaced at 1-10 minute intervals and continue for several hours. The early transients echo the first and begin at the site of sperm attachment and penetration; after three or four such transients, the echo has died away and the transients remain sharply defined temporally but are spatially homogeneous.

The multiple calcium transients after fertilization in hamster eggs can be reproduced with varying degrees of fidelity by artificial activating agents. Thimerosal, a sulphydryl reagent, mimics the large and persistent Ca^{2+} oscillations seen at fertilization most successfully, in both hamster and mouse eggs (Swann, 1991; 1992). It is not clear how thimerosal causes the Ca^{2+} oscillations; it may enhance the sensitivity of a CICR channel in a manner analogous to the effects of caffeine on the sarcoplasmic reticulum Ca^{2+} release channel (Swann,1991; 1992). Thimerosal may affect either or both of the calcium release channel types. A sensitizing effect on the InsP_3 channel (Finch et al., 1991) is quite possible, but note that in mouse oocytes injection of the InsP_3 receptor antagonist heparin blocks InsP_3-mediated Ca^{2+} oscillations but does not affect thimerosal-induced Ca^{2+} oscillations (Carroll and Swann, 1992); it has also been shown in permeabilized somatic cells that thimerosal releases calcium when the cells no longer respond to InsP_3 (Islam et al., 1992). The effects of thimerosal suggest that altering the sensitivity of Ca^{2+} channels may of itself be sufficient to cause calcium oscillations. This is one explanation of the fertilization response in hamster eggs, as we shall see later.

Raising intracellular calcium concentrations in hamster eggs triggers a calcium wave (Igusa and Miyazaki, 1983). This manifestation of CICR disappears when eggs have been injected with an antibody to the InsP_3 receptor (Miyazaki et al., 1992). Provided one accepts that the antibody reacts only with the InsP_3 receptor and not with other

members of the calcium release channel family, this is a very useful result. It seems to tell us that CICR in hamster eggs operates via the InsP_3 receptor.

Mouse eggs also undergo calcium oscillations at fertilization (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992). Their properties are very similar to those of hamster eggs: a calcium wave can be stimulated by calcium or InsP_3 injection and they respond to thimerosal with calcium oscillations, just as at fertilization (Swann, 1992).

The fertilization calcium explosions in frog, sea urchin and mammalian eggs look remarkably similar. Nonetheless, it seems likely that they are generated by mechanisms that differ markedly in their particulars, though a case can be made that all involve positive feedback mechanisms sited at calcium release channels in the endoplasmic reticulum. One set of explosions (frog, mouse, hamster) may depend on the InsP_3 receptor-channel, the other (sea urchin) may involve the ryanodine receptor-channel; both may owe their explosive power to the direct positive feedback of calcium ions on the calcium release channels of the endoplasmic reticulum.

LIGHTING THE FUSE

What happens when the fertilizing sperm contacts the egg to detonate the fertilization wave? The fuse can burn quite slowly. The time between sperm-egg interaction and the calcium explosion (the *latent period*) can be as long as 7 minutes in mouse eggs (Jaffe et al., 1983) and may be as short as about 10 seconds in hamster (Miyazaki and Igusa, 1981). Its duration is determined by the sperm: mouse sperm have a 7 minute latent period when fertilizing hamster eggs (Igusa et al., 1983).

An early hypothesis for detonation suggested that calcium, carried in the sperm cytoplasm, is injected into the egg to set off the calcium wave when the plasma membranes of sperm and egg fuse (L. F. Jaffe, 1980;1983). The idea was swept aside by the discovery of the polyphosphoinositide messenger system and its very evident presence in eggs (Ciapa and Whitaker, 1986). These data led to the view that the sperm behaved as an honorary hormone and caused the local production of phosphoinositide messengers by interacting with a receptor and GTP-binding protein coupled to phosphoinositidase C (L. A. Jaffe et al., 1988; L. A. Jaffe, 1990). The second hypothesis was enthusiastically received and good evidence in its favour accumulated from sea urchin, frog and hamster eggs. Now, though, there are data that argue against this idea, particularly in sea urchin eggs. In addition, electrical measurements of the process and properties of sperm-egg fusion have pushed the pendulum back a little, towards variants of the earlier hypothesis.

Phosphoinositide messengers at fertilization

Interest in the parts played by the phosphoinositide lipids was stimulated by the discovery that this class of lipid turned over rapidly in sea urchin eggs at fertilization (Turner et al., 1984). The observation more or less coincided with the discovery that InsP_3, one of the two hydrolysis products of PtdInsP_2, released calcium from membrane stores in cell homogenates (Streb et al., 1983). It was then a small step to show that InsP_3 activated sea urchin eggs when it was microinjected (Whitaker and Irvine, 1984; Swann and Whitaker, 1986). The production of InsP_3 and its conjugate messenger diacylglycerol (DAG) rises and falls with the same time course as the fertilization Ca_i transient and can be stimulated by A23187, the calcium ionophore (Ciapa and Whitaker, 1986; Ciapa et al., 1992), consistent with calcium-stimulated hydrolysis of PtdInsP_2 (Whitaker and Aitchison, 1985). Sea urchin egg homogenates have a non-mitochondrial membranous calcium-sequestration system (Clapper et al., 1987) that corresponds in the intact egg to the endoplasmic reticulum (Henson et al., 1989; 1990; Terasaki et al., 1991; Terasaki and Sardet, 1991) and responds to InsP_3 by releasing calcium. Injecting neomycin, an inhibitor of PtdInsP_2 hydrolysis (Swann and Whitaker, 1986), blocks the fertilization Ca_i wave, though note that neomycin affects calcium-stimulated processes in sea urchin eggs in other ways, too (McLaughlin and Whitaker, 1988), and also inhibits CICR in sarcoplasmic reticulum isolated from muscle (Palade, 1987). However, injecting heparin, the InsP_3 receptor antagonist, blocks InsP_3-induced calcium release, but not the fertilization calcium transient (Rakow and Shen, 1990; Crossley et al., 1991).

So the story in sea urchin eggs looks like this: they have a competent phosphoinositide messenger system that is activated at fertilization; some or all of the phosphoinositide messenger production is an effect of the Ca_i increase, not a cause; the fertilization calcium explosion is not prevented by blocking InsP_3-sensitive calcium release channels with heparin. There must be another calcium mobilizing system that is activated at fertilization. It may even be that InsP_3 plays no large part in the lighting and burning of the fuse at fertilization, nor in the explosion: a startling and unexpected conclusion.

Data from frog and mammalian eggs are less extensive. Micro-injecting InsP_3 activates frog (Busa et al., 1985) and hamster eggs (Miyazaki, 1988; Cran et al., 1988), causing calcium transients that look very much like the fertilization Ca_i transients. In hamster eggs, continuous infusion of InsP_3 can give rise to repetitive Ca_i transients that approximate to the repetitive fertilization Ca_i transients, but are shorter-lived (Swann et al., 1989). Frog egg homogenates sequester calcium and release it in response to InsP_3 (Galione and Whitaker, unpublished data). These observations suggest an analogy with the mobilization of phosphoinositide messengers in sea urchin eggs, but in no other eggs has measurement of phosphoinositide metabolism been reported. Yet, the best and most telling evidence for an essential role for the InsP_3 receptor at fertilization comes from an experiment in hamster eggs. Miyazaki has shown that injecting a monoclonal antibody directed against an InsP_3-receptor/channel protein blocks the calcium wave at fertilization (Miyazaki et al., 1992).

The receptor-G protein idea

The phosphoinositide messenger system in somatic cells is controlled by plasma membrane receptors linked to phosphoinositidase C by multi-subunit GTP-binding proteins (G-proteins; Gilman, 1987). Receptor control of InsP_3 pro-

duction can be by-passed by stimulating the G-protein with guanosine 5′-triphosphothioate (GTPγS). Exploiting this trick, it was shown that sea urchin eggs were activated by microinjection of GTPγS (Turner et al., 1986) and that G-proteins (identified by their susceptibility to ribosylation by cholera and pertussis toxins) were present in sea urchin egg plasma membrane (Turner et al., 1987). These experiments led to the suggestion that the phosphoinositide messenger system in sea urchin eggs might be activated by a receptor-G-protein mechanism similar to the one in somatic cells, an idea strengthened by the observation that sea urchin eggs could be activated by injecting cholera toxin, which interacts specifically with certain G-proteins (Turner et al., 1987). However, pertussis toxin does not inhibit fertilization in sea urchin (Turner et al., 1987), frog (Kline et al., 1991), starfish (Shilling et al., 1989) or hamster (Miyazaki, 1988).

There followed some very elegant experiments in frog and starfish in which cDNA made from brain RNA and cDNA coding for a G-protein-linked 5-HT receptor were introduced into immature oocytes (Kline et al., 1988; Shilling et al., 1990). After hormone-induced maturation, the receptors themselves had become incorporated into the egg membrane and treatment with 5-HT led to activation of the egg, much as at fertilization. These data beautifully confirmed that frog and starfish eggs possessed G-protein capable of stimulating the phosphoinositide messenger system. This is not equivalent, of course, to showing that a G-protein linked mechanism operates at fertilization. On this score, the G-protein idea is less convincing. For example, pertussis toxin (a G-protein antagonist) blocks hormone-induced maturation of starfish oocytes, but not fertilization (Shilling et al., 1989). The missing part of the G-protein story is the putative G-protein linked sperm receptor.

Positive support for the G-protein hypothesis was provided by the observation that GDPßS, a G-protein antagonist, blocked activation (judged by fertilization envelope elevation) after insemination in sea urchin eggs (Turner et al., 1986). However, the effect is due to inhibition of fertilization envelope elevation, not inhibition of the calcium wave (Crossley et al., 1991). This finding now argues against obligatory participation of G-proteins in the fertilization calcium wave in sea urchin eggs. Taking a different tack, if PPI hydrolysis is responsible for initiating the fertilization calcium wave, then it should persist when the wave is blocked by a calcium chelator. Using the pH_i increase as a measure of DAG production (Schwann and Whitaker, 1985), it is clear that while GTPγS will stimulate the production of DAG when the calcium wave is stifled by BAPTA injection, insemination will not, despite multiple sperm-egg encounters that result in sperm incorporation and a considerable degree of polyspermy (Crossley et al., 1991). It appears, then, that mobilization of PPI messengers is a consequence, not a cause, of the calcium wave. The effects of heparin underline this. As we mentioned above, heparin does not block the fertilization wave in sea urchin eggs, despite its effectiveness as a blocker of InsP_3-induced calcium release in sea urchin eggs (Crossley et al., 1991).

In mammalian eggs there is no need for difficult and elegant experiments with receptor cDNA, since there is already a PPI-linked G-protein coupled to a 5-HT receptor in the membrane in hamster (Miyazaki et al., 1990) and a G-protein-linked acetylcholine receptor in mouse (Swann, 1992) that generate calcium oscillations; this latter fact encouraged Williams and others (1992), who introduced mRNA coding for the ACh receptor into mouse oocytes to show that further acetylcholine receptors would result in ACh-induced egg activation. Applying 5-HT to hamster eggs triggers a series of repetitive Ca^{2+} oscillations, although, as with GTPγS, the oscillations die away much more rapidly than at fertilization (Miyazaki, 1991). There is also the rather more subtle observation that multiple 5-HT responses have a sharp threshold and unvarying frequency, while the frequency of the fertilization response is markedly dose-dependent: the more sperm fuse, the higher the frequency of oscillation. The G-protein idea has perhaps received its strongest support from experiments in hamster eggs using the G-protein antagonist, GDPβS. Injecting GDPβS has been shown to block the Ca^{2+} response during hamster egg fertilization, without abolishing the single response to InsP_3 injection (Miyazaki, 1988). This is the strongest evidence to date that a GTP-binding protein may transduce the fertilization response. As yet, the effects of GDPβS on the explosive calcium oscillations themselves have not been reported: G-proteins may also be involved in the translocation of calcium between different internal compartments (Ghosh et al., 1989). It is important to establish precisely where GDPβS acts, because experiments in frog oocytes have shown that GDPβS does not inhibit an initial Ca^{2+} release caused by InsP_3 injection, but does abolish subsequent oscillations (Sealfon et al., 1990). The idea here was that GDPβS inhibits Ca^{2+} movements between InsP_3-sensitive and -insensitive pools. This may also be true of hamster eggs since GDPβS blocks thimerosal-induced (CICR) Ca^{2+} oscillations (Swann, unpublished data).

A series of Ca^{2+} oscillations can be triggered in hamster eggs either by G-protein agonists that appear to stimulate phosphoinositide turnover or by continuous injection of InsP_3 (Miyazaki, 1988; Swann et al., 1989; Miyazaki et al., 1990). Whether these stimuli trigger propagating Ca^{2+} waves too has not yet been decided (Miyazaki et al., 1990; Miyazaki, 1991). Neither of them produces the long-lasting oscillations associated with fertilization; the G-protein-mediated Ca^{2+} oscillations are heavily damped and die away within tens of minutes and the InsP_3-induced oscillations are also of much shorter duration than those that occur during fertilization (Swann et al., 1989; Miyazaki et al., 1990; Swann, 1991). With InsP_3, as with 5-HT and GTPγS, there is a sharp threshold for the generation of calcium oscillations; the frequency is relatively insensitive to dose. Another difference noted between InsP_3-induced oscillations and the transients at fertilization is that they are not accompanied by much sensitization of CICR (Swann, 1991). In this they resemble frog oocytes, where InsP_3 promotes minor episodes of CICR, but does not give rise to a full-blown calcium explosion (DeLisle et al., 1992).

The major difficulty for the G-protein hypothesis in hamster eggs comes from a different source. The ability of GTPγS to trigger damped Ca^{2+} oscillations is one of the strongest pieces of evidence for the existence of appropri-

ate G-protein-mediated signal transduction (Miyazaki, 1988). However, stimulating protein kinase C by adding phorbol esters to eggs completely abolishes the GTPγS-triggered Ca^{2+} oscillations (Swann et al., 1989), while phorbol esters cause only slight reductions in the frequency of Ca^{2+} oscillations triggered by the sperm (Swann et al., 1989). There does not appear to be a simple explanation for these data in terms of the receptor/G protein idea of fertilization signal transduction, though it has occurred to us that a natural fertilization agonist that behaved like the ADP-ribosylating agent cholera toxin might activate a G-protein in a way that makes it insensitive to down-regulation by PKC. Once again, there is good evidence for the existence of PPI-linked G-proteins, but there is also a clear indication that the G-protein linked Ca_i response is entirely blocked by an inhibitor that has very little effect on the Ca_i response at fertilization.

Summing up

Looking at the data from experiments on sea urchin, frog and hamster eggs, it seems almost a certainty that the PPI-linked messengers InsP_3 and DAG are produced in concert with the fertilization calcium explosion. Cause and effect are still difficult to separate. In sea urchin, blocking the InsP_3 receptor does not materially alter the calcium explosion, while in frog and hamster, the same manoeuvre prevents it. Even in the latter case, where the experiments with receptor antibody make the strongest case in favour of participation of the InsP_3 receptor, we still do not know whether InsP_3 is part of the fuse, or the explosive or indeed, part of both. And again, it seems certain that these three different eggs contain G-proteins that link to a phosphoinositidase C. Yet, there is a strong possibility, given the experiments in sea urchin and hamster eggs that block the G-protein link to PPI without affecting the calcium explosion, that the pathway is silent at fertilization.

Sperm-egg binding

What is missing in our account so far is a description of how sperm and egg recognize and bind to one another. In one hypothesis, the sperm receptor on the egg would bind sperm and activate the phosphoinositide messenger system; this might lead to sperm-egg fusion. In the other, older, hypothesis, interaction of the sperm with its receptor on the egg must lead directly to fusion, prior to egg activation. A sperm receptor protein has very recently been identified in sea urchin eggs: it is a 350 $\times 10^3$ M_r membrane glycoprotein (Foltz and Lennarz, 1992). Proteolytic digestion of egg surface complex gave a 70×10^3 M_r fragment that binds to homologous, not heterologous, acrosome-reacted sperm (Foltz and Lennarz, 1990). Antiserum raised against the fragment cross-reacts with the native 350×10^3 M_r protein. Fab fragments block homologous fertilization and sperm binding.

The complementary molecule on the sperm appears to be bindin, a very hydrophobic protein which coats the acrosomal process (Vacquier and Moy, 1977). Bindins cause species-specific agglutination of eggs (Glabe and Vacquier, 1977). The 70×10^3 M_r sperm receptor fragment from *Strongylocentrotus purpuratus* eggs binds to *S. purpuratus* bindin, but not to the bindin of other species (Foltz and Lennarz, 1990). There is now a very good case to be made in favour of a bindin-glycoprotein interaction (Glabe and Clarke, 1991; Glabe et al., 1991; Lopez et al., 1993) as the species-specific recognition, binding and activation step at fertilization.

Similar sorts of experiments in guinea-pig have identified a sperm surface protein (PH-30, an integral membrane glycoprotein heterodimer) that is involved in sperm-egg fusion. The primary sequence of the α sub-unit shows it to contain a helical motif analogous to the fusion peptide of viral fusion proteins (Blobel et al., 1992); that of the β sub-unit suggests an integrin-binding domain. Interaction of sperm with zona-free hamster eggs is blocked by micromolar concentrations of RGD (Arg-Gly-Asp)-containing peptides that characteristically inhibit ligand-integrin interactions (Bronson and Fusi, 1990). The idea is that sperm-egg binding in mammals involves an integrin receptor (Blobel et al., 1992).

The identification of the sperm receptor is a major advance in our understanding of fertilization, but, for the moment, it does not help us choose G-proteins over other signal transduction pathways. It is evenhanded. One hand holds the interesting observation that in sea urchin eggs, in a small proportion of egg batches, the Fab fragment causes a small amount of activation (Foltz and Lennarz, 1992). This might seem to argue in favour of a genuine receptor function for the 350×10^3 M_r protein, perhaps linkage to a signal transduction system, though the effect is small. In mammals, integrins may transduce a membrane signal, though integrin signal transduction is unlikely to involve G-proteins, on present evidence. Integrin receptors may trigger calcium oscillations in neutrophils (Jaconi et al., 1991), perhaps by stimulating a tyrosine kinase (Kornberg et al., 1991); tyrosine kinase-linked receptors can trigger PPI turnover via phosphoinositidase Cγ (Rhee, 1991). Moreover, in sea urchin eggs, activation of a tyrosine kinase precedes the calcium explosion (Ciapa et al., 1991). These data are pointing in the direction of *echt* transmembrane signal transduction, not *via* G-proteins, but through a receptor with tyrosine-kinase activity. The other hand, though, holds the finding that PH-30 contains a viral fusion peptide motif and that bindin itself is a fusogen (Glabe, 1985a,b). Binding of bindin or PH-30 to its receptor on the egg plasma membrane should promote fusion of egg and sperm plasma membranes, seeming to argue in favour of sperm-egg fusion as the activating event.

Sperm-egg fusion

The G-protein idea presents some obvious difficulties and the PPI messengers, though important, may have lost their status as the sole mediators of the fertilization calcium explosion. If the data now begin to point away from the G-protein hypothesis, it is worth considering carefully whether they might now be pointing more in the direction of the older idea, that something in the sperm cytoplasm sets off the calcium wave when sperm and egg fuse (Loeb, 1913; L. F. Jaffe, 1983; Whitaker et al., 1989; Whitaker and Crossley, 1990).

To pursue this line of thought, we need to know when and how the sperm fuses with the egg. There is information about this in experiments from sea urchin eggs. Two

approaches have been used. The first uses chemical fixation to freeze the fusion process. The formation of a fusion pore between sperm and egg can then be followed using transmission electron microscopy (Longo, 1986) or the diffusion of dye from egg to sperm (Hinckley et al., 1986). These experiments are more sophisticated than their simple description may indicate, since it is crucially important to determine the timing of fertilization events precisely. After insemination of an egg suspension, sperm must collide with and attach to the egg. Many sperm can interact with an egg, some unsuccessfully. It is impossible in these circumstances to identify the precise moment at which the fertilizing sperm begins to interact productively with the egg. However, it *is* possible to detect this moment by making electrical measurements on sea urchin eggs.

The fertilizing sperm induces electrical currents in the egg membrane. The first of these is an inward current with a very rapid step-like onset (Dale et al., 1978). It is the first detectable event at fertilization. It precedes the fertilization calcium transient by 15-20 seconds (the latent period in sea urchin eggs: Whitaker et al., 1989; Swann et al., 1992; Fig. 2). The current step appears to mark the moment of productive sperm-egg interaction; for example, it corresponds to the time at which fertilization is no longer affected by addition of spermicides (Whitaker et al., 1989); it also corresponds to the point at which the electrical block to polyspermy (L. A. Jaffe, 1976) operates in sea urchin eggs (Shen and Steinhardt, 1984). It is not too fanciful to think of the current step as indicating the moment of fertilization. The aim, then, has been to relate the time at which sperm-egg fusion occurs to this current step, the fertilization landmark.

Both sets of experiments that looked at the timing of fusion with chemical fixation used this landmark. Single, voltage-clamped eggs were fixed at various times after the current step was observed. In the dye-transfer experiments, eggs were pre-loaded with a dye that stains DNA, the point

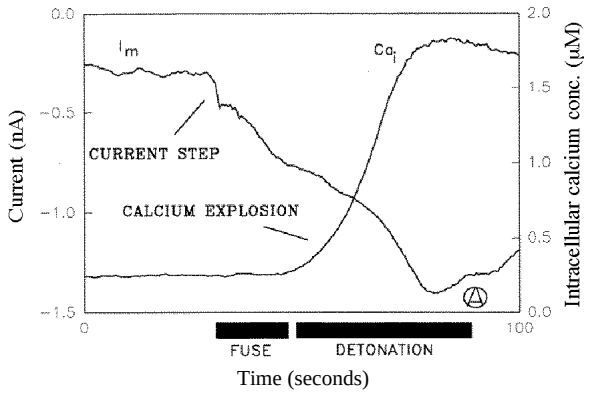

Fig. 2. Simultaneous recording of membrane current and Ca_i during fertilization in a single sea urchin egg. The inward current step occurs when cytoplasmic continuity is established between egg and sperm (McCulloh and Chambers, 1992). The calcium explosion occurs some 15 seconds later. Membrane voltage was clamped using a single-electrode technique to prevent activation of voltage-gated calcium channels and Ca_i was measured with the fluorescent indicator dye fura2 (Swann et al., 1992). *L. pictus*, 16°C.

being that the sperm head will become fluorescent only when an aqueous diffusion path is formed between sperm and egg cytoplasm, that is after sperm-egg fusion (Hinckley et al., 1986). Chemical fixation is essential in these experiments to provide the required time resolution, because the dye diffusion step takes many minutes. This technique finds dye diffusion after fixation within 4-8 seconds of the current step. The ultrastructural variant of this method is a technical tour-de-force in which single voltage-clamped eggs are chemically fixed at times after the current step, removed from the microscope, embedded individually and serial-sectioned to find the point of sperm-egg contact. Fusion (continuity of gamete plasma membranes: Longo et al., 1990) occurs 5 seconds or more after the current step.

The second approach to the question of when the sperm fuses with the egg is an all-electrical method that dispenses entirely with chemical fixation (McCulloh and Chambers, 1992). It relies on the fact that the electrical capacitance of biological membranes can be used to estimate the area of a patch of membrane. A sperm swims up to the surface of a voltage-clamped sea urchin egg within a large patch pipette that is also used to measure the membrane capacitance of the patch of egg membrane immediately beneath the tip of the pipette. When the sperm and egg fuse, the sperm membrane is added to that of the egg and total membrane capacitance increases. The results using this approach are striking: the capacitance increase occurs *at the same time* as the current step and has an equally rapid onset. In other words, this method suggests that fusion is the first thing that happens at fertilization in sea urchin eggs.

The two approaches do not quite agree about timing, but it is clear that fusion occurs 10-15 seconds before the onset of the calcium wave. In order to be more precise about timing, we need a better picture of what happens during membrane fusion. The best studied example of membrane fusion biophysics is that of secretory exocytosis, where secretory granules fuse with the inner surface of the plasma membrane. Here, as at fertilization, the membrane capacitance technique detects an aqueous pore that forms during fusion (Zimmerberg et al., 1987; Breckenridge and Almers, 1987). Once membrane fusion has occurred, the walls of the fusion pore are formed by a cylinder of bilayer membrane (see, for example, Chandler, 1884). There is still a debate, however, about what form the aqueous pore takes when it comes into being. In one hypothesis, it is always a lipid-dominated structure, stabilized by protein (Zimmerberg et al., 1991); in the other, it is initially a protein channel, analogous to the gap junction channel, that expands by sub-unit addition until a point is reached at which a stable lipid-lined pore can take its place (Spruce et al., 1990). Sperm-egg fusion is topologically equivalent to exocytosis, with inside and outside reversed and the debate about the structure of the fertilization fusion pore echoes the debate over exocytosis. The differences in timing between the fixation and capacitance methods could be explained if the pore were a protein channel during the first few seconds of its existence, since then, ultrastructurally, membrane fusion defined as lipid bilayer confluence would not yet have occurred (Longo et al., 1990). Alternatively, chemical fixation may be inadequate to preserve the initial lipid-based

pore, so that a lipid-based fusion pore is detected only when its structure has become robust (Whitaker et al., 1989). We favour the latter view, since chemical fixation is so obviously inadequate in preserving fusion pores during exocytosis (Chandler, 1984), but both views are plausible explanations of the early stages of sperm-egg fusion. The point for our purposes here is that the *earliest detectable event* at fertilization is the formation of an aqueous pore between egg and sperm cytoplasm that may permit the diffusion of an activating messenger from sperm to egg. Without this pore, the idea of a diffusible messenger would be beside the point. So far, early formation of an aqueous pore has been demonstrated only in sea urchin eggs.

Putative activating messengers

If a messenger from the sperm cytoplasm is what activates an egg (Dale et al., 1985), then it can be identified using three obvious criteria: it should be present in sufficient concentration in the sperm; it should perfectly mimic the fertilization response when microinjected and its specific antagonists should prevent egg activation. None of the putative activating messengers yet fulfils all these requirements, though some come close.

It might seem straightforward to demonstrate that a candidate messenger activates eggs and is present in sufficient concentration in sperm to act as the fertilization activator. For example, InsP_3 will activate a sea urchin egg at a pipette concentration of 1 μM; about 5×10^{-18} mole of InsP_3 is necessary (Whitaker and Irvine, 1984; Swann and Whitaker, 1986). Sea urchin sperm contain about 10^{-18}-10^{-17} mole InsP_3: this seems at first sight just sufficient (Iwasa et al., 1990). However, the activating messenger has to diffuse through an initially narrow aqueous pore from sperm to egg. Application of the diffusion equation shows that the local concentration in the egg at the point of sperm egg contact will be only about 1% of the concentration in the sperm (Whitaker et al., 1989). Bearing this in mind, the case for InsP_3 as the activating messenger on the first two criteria is less compelling; on the third criterion it founders: recall that heparin, a specific InsP_3 antagonist, does not prevent initiation of the calcium wave (Crossley et al., 1991). Another candidate messenger, cGMP, fares less well quantitatively. 5-10 μM cGMP is necessary for sea urchin egg activation (Whalley et al., 1992) and sperm contain only around 1 μmole/kg wet weight (Kopf et al., 1979). However, sperm contain a very active guanylate cyclase activity, raising the possibility that large quantities of cGMP can be generated at the point of sperm egg contact (Whalley et al., 1992). cGMP does not seem to activate eggs by stimulating the phosphoinositide messenger system directly, nor by activating a cGMP-dependent kinase (Whalley et al., 1992). It is hard to come up with a cGMP-specific antagonist in these circumstances, so the third criterion has not been met because it has not been tested. The most potent activating agent so far found for sea urchin eggs is cyclic ADP-ribose (Clapper et al., 1987; Lee et al., 1989; Galione et al., 1991; Galione, 1992). Its very potency suggests that it might be important at fertilization. As yet it has not been looked for in sperm, nor is there yet a specific antagonist to its actions, though it is blocked by ruthenium red, the CICR antagonist (Galione et al., 1991).

It was calcium itself that was originally put forward as the activating messenger that diffused from sperm to egg at fertilization (L. F. Jaffe, 1983). Calcium concentrations are high (several micromolar) in the sperm after the acrosome reaction (Schackmann, 1989) and the fertilization calcium wave might be triggered directly by a CICR mechanism. The idea is that calcium influx into the egg through channels in the sperm plasma membrane might, after sperm-egg fusion, load up the calcium store locally (L. F. Jaffe, 1990), giving rise to a calcium overload CICR response of the sort described in cardiac muscle (Fabiato, 1985). On the other hand, calcium microinjection does not trigger a calcium wave in sea urchin eggs and manoevres that *decrease* the calcium concentration in egg cytoplasm in the region of sperm-egg contact *increase* the probability of successful fertilization (Chambers et al., 1989; McCulloh et al., 1989; 1990). The idea of CICR as the direct initial trigger of the fertilization calcium wave looks more promising in frog and hamster eggs, where injection of calcium does trigger calcium transients and a calcium wave (Busa and Nuccitelli, 1985; L. F. Jaffe, 1990), but remember that frog egg homogenates show no clear evidence of the existence of a ryanodine-binding CICR channel (Galione and Whitaker, unpublished data; Table 1). In mammalian eggs, moreover, there is a plausible alternative to a mechanism involving low molecular mass factors.

Evidence for CICR exists in hamster eggs (Igusa and Miyazaki, 1983) and CICR has been investigated quite extensively. Calcium injection or influx triggers a single Ca_i transient. Thereafter, further calcium influx or injection does not promote further calcium release; instead, the egg shows an irreversible desensitization of CICR (Igusa and Miyazaki, 1983; McNiven et al., 1988; Swann, 1991). These results contrast quite markedly with what is found after fertilization, when there is an order of magnitude enhancement of CICR sensitivity that is maintained in the face of multiple calcium injections and is independent of external Ca^{2+} (Igusa and Miyazaki, 1983). Here is a strong clue to one of the effects of the activating signal. Whatever messenger the sperm may use in mammals, it must alter the sensitivity of CICR. Calcium itself cannot do it, as we have seen. InsP_3 seems unlikely on these grounds too, since it has only a minimal effect on CICR sensitivity (Swann, 1991). Others, such as cyclic GMP and cyclic ADP-ribose do not fit the bill in hamster eggs: they do not trigger a calcium release sufficient to cause the characteristic Ca^{2+}-dependent membrane responses (Swann, unpublished data). The most promising candidate for the job of sperm messenger in hamster eggs is a protein factor isolated from sperm cytosol (Swann and Whitaker, 1990; Swann 1990). Injecting the factor has been shown to trigger Ca^{2+} oscillations in hamster eggs identical to those seen at fertilization (Swann, 1990). This is particularly remarkable because, as we have already mentioned, G-protein agonists and InsP_3 are unable to reproduce the fertilization calcium response faithfully. Alone among all the agents we have listed, the factor sensitizes CICR in the egg, as at fertilization (Swann, 1990), suggesting that sensitizing a CICR mechanism may be sufficient to cause calcium oscillations. It also stimulates repetitive calcium oscillations in mouse eggs, causing activation (Swann, 1992). The

Table 1. Messengers at fertilization at a glance

BASICS	*Frog*	*Mouse–Hamster*		*Sea Urchin*
Fusion before Ca_i release	?	?YES(*a*)	?YES(*b*)	YES(*c*)
Latent period	YES-60s(*d*)	YES-400s(*a*)	YES-1-10s(*e*)	YES-20s(*f*)
Polyphosphoinositide turnover	?	?	?	YES(*g*)
Ca_i transient in response to cGMP	NO(*k*,*h*)	?	NO(*i*)	YES(*j*)
Thimerosal-sensitized CICR	YES(*k*)	YES(*l*)	YES(*m*)	YES(*k*,*n*)
Ca_i transient in response to hamster sperm factor	YES(*k*)	YES(*l*)	YES(*m*)	YES(*k*,*n*)
G PROTEINS	*Frog*	*Mouse–Hamster*		*Sea Urchin*
Response to GTPγS	YES(*o*)	YES(*l*)	YES(*p*)	YES(*q*)
Hormone response gives Ca_i via G-protein	YES(*r*)	YES(*l*,*s*)	YES(*t*)	?
Fertilization Ca_i blocked by GDPβS	?	?	YES(*p*)	NO(*u*)
InsP_3 *RECEPTOR/CHANNEL*	*Frog*	*Mouse–Hamster*		*Sea Urchin*
InsP_3-sensitive Ca store	YES(*v*)	YES(*l*)	YES(*t*)	YES(*w*)
Fertilization Ca_i blocked by heparin	YES(*k*)	?	?	NO(*u*,*x*)
Fertilization Ca_i blocked by InsP_3R-Ab	YES(*y*)	?	YES(*z*)	?
CICR blocked by heparin	YES(*k*)	?	?	NO(*k*)
'RYANODINE' RECEPTOR/CHANNEL	*Frog*	*Mouse–Hamster*		*Sea Urchin*
cADPr-sensitive Ca store	NO(*k*)	NO(α)	NO(*i*)	YES(β)
CICR on Ca injection	YES(*d*)	YES(*l*)	YES(γ)	NO(δ)
CICR blocked by ruthenium red	NO(*k*)	?	?	YES(*n*)
Ca release by 200 μM ryanodine	NO(*k*)	YES(*l*)	?	YES(ε)
CICR caused by caffeine	NO(*k*)	NO(*l*)	NO(ζ)	YES(ε)
Fertilization Ca_i blocked by ruthenium red	NO(*k*)	?	?	YES(*n*)

Highlighting indicates the similarities between species, double underlining the disparities. Two generalizations emerge: (1) the fertilization calcium wave is sensitive to blockers of the InsP_3 receptor in frog and hamster, but insensitive in sea urchin eggs; (2) the ryanodine receptor/channel is functionally-significant only in sea urchin eggs. These comparisons imply that the fertilization calcium-induced calcium release wave operates on the InsP_3 receptor in frog, mouse and hamster and on the ryanodine receptor in sea urchin eggs.

a,b Jaffe et al., 1983; Igusa et al., 1983. *Here the sudden cessation of the sperm tail beat (sperm stop) is taken to indicate sperm-egg fusion.*
c McCulloh and Chambers, 1992
d Busa and Nuccitelli, 1985
e Miyazaki and Igusa, 1981
f Shen and Steinhardt, 1984
g Turner et al.,1984; Kamel et al., 1985; Ciapa and Whitaker, 1986; Ciapa et al., 1992.
h Kline et al., 1990
i Swann, *unpublished observation*
j Whalley et al., 1992
k Galione, *unpublished observations in homogenates*
l Swann, 1992
m Swann, 1990; 1991
n McDougall and Whitaker, *unpublished observations*
o Kline et al., 1991
p Miyazaki, 1988
q Turner et al., 1986
r Kline et al., 1991
s Williams et al., 1992
t Miyazaki, 1990
u Crossley et al., 1991
v Busa et al., 1985
w Whitaker and Irvine, 1984
x Rakow and Shen, 1990
y Nuccitelli et al., 1992
z Miyazaki et al., 1992
α Kline et al., 1992
β Clapper et al., 1987; Lee et al., 1989
γ Igusa and Miyazaki, 1983
δ Swann and Whitaker, 1986, *but see figure 1; unpublished observations show this CICR to be potentiated by thimerosal*
ε Galione et al., 1991
ζ Miyazaki, 1990

frequency of calcium oscillations varies markedly with the amount of factor injected, as at fertilization and unlike InsP_3 and the G-protein agonists. The protein responsible has yet to be isolated. Cytosolic sperm factors have been shown to activate mouse, rabbit (Stice and Robl, 1990) and hamster eggs (Swann, 1990).

There are several contenders, but no obvious winner for the title of activating messenger. Perhaps the most interesting of the contestants is the protein factor that can be isolated from mammalian sperm, not least because a diffusible activating protein messenger is a novelty.

MULTIPLE SIGNAL PATHWAYS AT FERTILIZATION

Because fertilization in deuterostomes is accompanied by a large (if silent) calcium explosion that looks much the same from one species to another, we have tended to assume that a single signalling mechanism is responsible for setting it off. There may be more diversity in the response than we think, not only from species to species, but also in the fuse as opposed to in the explosion itself. The unfertilized eggs that we have spoken of all have competent G-protein-linked

phosphoinositide messenger systems and also show CICR. cADPr and cGMP may stimulate or modulate CICR independently of InsP_3. This is a profusion of mechanisms (summarized in Table 1) from which to make a choice. The message that the data in Table 1 seem to spell out is that CICR is probably linked to the InsP_3 receptor in frog, mouse and hamster eggs, but to the ryanodine receptor in sea urchin eggs. Indeed, in the broad sense, the calcium explosions may be very similar, differing only in the identity and properties of the calcium release channel.

We have identified these release channels very crudely by using the effects of agonists and antagonists to classify them as one of two channel types. It seems very likely that there are, in fact, more than two types of channel in the family (McPherson et al., 1991; Ross et al., 1992). We doubtless need agonists that distinguish more subtly between family members. Heparin, for example, is used as an InsP_3 receptor antagonist, but may also inhibit ryanodine-induced calcium release (Sardet et al., 1992). Conversely, ruthenium red is often used as an inhibitor of the ryanodine-sensitive channel, but it can, on occasion, inhibit InsP_3-sensitive calcium release at similar concentrations (Fujiwara et al., 1990; McPherson et al., 1992). On a different tack, a CICR wave propagates through the sea urchin egg cytoplasm (Fig. 1) and sea urchin egg homogenates respond well to ryanodine channel agonists (Galione et al., 1991), but ryanodine receptor antibody binding is concentrated just beneath the plasma membrane, apparently binding to only a very small fraction of the egg's calcium store (McPherson et al., 1992). The easiest way to understand these sorts of contradictions is to imagine an array of calcium release channels that show a spectrum of response to various antagonists (Ross et al., 1992; McPherson et al., 1991), rather than just two, one sensitive to InsP_3, the other to ryanodine. With this perspective, it should not be too surprizing to find that the channels involved in calcium release vary from egg to egg, or from fuse to bomb.

Set in contrast to subtle distinctions between calcium release channels is the clear divide between two ideas of how the fertilization signal is transmitted across the plasma membrane at fertilization. On one side of the divide is the notion that there is a sperm surface receptor linked to a G-protein, on the other the suggestion that the sperm may introduce a diffusible activating factor after gamete fusion. As yet, neither of these ideas is proved or disproved in any egg, but we see no compelling reason why the mechanism of fertilization at this level should vary from one deuterostome egg to the next.

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