

Mammalian membrane block to polyspermy: new insights into how mammalian eggs prevent fertilisation by multiple sperm

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Abstract. To inhibit fertilisation by more than one sperm (a condition known as polyspermy), eggs have developed preventative mechanisms known as blocks to polyspermy. The block at the level of the egg extracellular coat (the zona pellucida in mammals, the vitelline envelope in non-mammals) has been well characterised in many different animal species and the block at the level of the egg plasma membrane is understood in some non-mammalian species. However, virtually nothing is known about the membrane block to polyspermy in mammalian eggs, despite data dating back 50–90 years that provide evidence for its existence. In the present review, we will discuss the background on blocks to polyspermy used by animal eggs and then focus on the membrane block to polyspermy in mammalian eggs. This will include a summary of classical studies that provide evidence for this block in mammalian eggs, assays used to study the mammalian membrane block and what has been elucidated from recent experimental studies about the cellular signalling events that lead to membrane block establishment and the mechanism of how the membrane block may prevent additional fertilisation.

Polyspermy in mammals and its consequences

Polyploidy will generally be fatal to an embryo and has been detected in approximately 10–20% of spontaneously aborted human conceptuses (Jacobs *et al.* 1978; Hassold *et al.* 1980; Michelmann *et al.* 1986). Polyploid embryos can result from either digyny (due to errors in meiosis I during oocyte maturation or meiosis II during egg activation) or diandry (due to fertilisation by a diploid sperm or by two sperm). The majority of triploid human embryos appear to be the result of polyspermic fertilisation, with two sperm fertilising an egg (Jacobs *et al.* 1978; Zaragoza *et al.* 2000). Mammals use several mechanisms to prevent polyspermy (addressed below), although, even with these in place, the incidence of polyspermy is 1–2% when mating occurs around the time of ovulation. A variety of factors can contribute to increased rates of polyspermy in different species; these have been the subject of a recent review (Wang *et al.* 2003). For example, the incidence of polyspermy can increase, to up to 30% or even higher, if mating is delayed after ovulation until slightly later in the oestrous cycle (Austin 1961; Hunter 1991a, 1991b; Wang *et al.* 2003). There are also some species, most notably the pig, that have increased rates of polyspermy, *in vivo* to some extent and especially with *in vitro* inseminations (Hunter 1991a; Wang *et al.* 2003).

Triploid embryos die at various times after conception. Although most triploid embryos die early in embryogenesis, it is possible for some to survive into post-implantation development; studies of human embryos have found that

a subset of triploid embryos survived well into gestation (average approximately 111 days in one study of 1000 abortions; Hassold *et al.* 1980). A small number of triploid embryos has survived into the third trimester or to full term, but died shortly after birth (Jacobs *et al.* 1978; Hassold *et al.* 1980). It is thought that survival rates depend on the extent of mosaicism or what proportion of cells are diploid (Michelmann *et al.* 1986).

Blocks to polyspermy

As noted above, mammals use a variety of mechanisms to reduce the incidence of polyspermy. It is thought that capacitation, sperm transit through regions of the female tract (e.g. cervix, uterotubal junction) and sperm oviducal reservoirs serve to regulate the number of sperm that reach the site of fertilisation in a variety of species (Yanagimachi 1994; Suarez 1999). In the pig, in particular, exposure of gametes to oviducal epithelial cells and/or oviducal secretions can reduce polyspermy (Hunter 1991a; Wang *et al.* 2003). In addition to these processes involving the female reproductive tract, the eggs themselves mount blocks to polyspermy to prevent fertilisation by additional sperm after the egg has already been fertilised. Blocks to polyspermy occur primarily at two levels on the egg: the egg plasma membrane and the egg coat (the zona pellucida (ZP) in mammals and the vitelline envelope in non-mammals).

The egg coat block to polyspermy has been studied extensively in both non-mammalian and mammalian animals.

The egg coat block (also called the 'slow' block) in many animal species involves the exocytosis of cortical granules (CGs) from the cortex of the egg. In echinoderms and anuran amphibians, CG exocytosis results in the elevation of the vitelline envelope from the plasma membrane of the egg and the loss of the ability of the vitelline envelope to support sperm binding (Wolf 1974; Jaffe and Gould 1985; Hedrick and Hardy 1991). The block to polyspermy at the ZP of mammalian eggs is very similar to this, with the exocytosis of CGs causing conversion of the ZP to a form that cannot support sperm binding (Yanagimachi 1994; Abbott and Ducibella 2001).

In contrast with the egg coat block, membrane block to polyspermy differs significantly between mammalian and non-mammalian eggs. In non-mammals (such as sea urchins and frogs, two of the species most extensively studied), the membrane block involves a rapid and transient depolarisation of the egg plasma membrane potential so that sperm penetration of the egg is not favoured (although not completely inhibited; Jaffe and Gould 1985). Post-fertilisation membrane depolarisation occurs within a few seconds of fertilisation (and, thus, is also referred to as the 'fast block to polyspermy'), lasts up to several minutes and is observed in sea urchins, starfish, the marine worm *Urechis*, some ascidians, molluscs, annelids, nemerteans and anuran amphibians (Jaffe and Gould 1985). In contrast, significant membrane depolarisation has not been observed in mouse, hamster or rabbit eggs (Miyazaki and Igusa 1981; Igusa *et al.* 1983; Jaffe *et al.* 1983; McCulloh *et al.* 1983). Thus, the mechanism of the mammalian membrane block does not appear to be mediated by a change in membrane potential. It should also be noted that the mammalian membrane block is established in approximately the same time frame as the ZP block (0.5–1 h), whereas in non-mammalian species the electrical membrane block occurs within seconds of fertilisation and the egg coat block takes 30–60 s (sea urchin) to several minutes (frogs; Jaffe and Gould 1985). Thus, the mammalian blocks are distinguished spatially (membrane or ZP), whereas the non-mammalian blocks are distinguished spatially and temporally (fast or slow).

As noted above, a variety of factors can contribute to increased polyspermy rates, one of these being delayed mating leading to conception occurring at times later than ovulation (Austin 1961; Hunter 1991a, 1991b; Wang *et al.* 2003). We have shown recently that aged eggs have a reduced ability to establish a membrane block to polyspermy (Wortzman and Evans 2005), suggesting that this may be one cause of increased polyspermy in aged eggs. Post-ovulatory ageing may also affect CG exocytosis (and, thus, the ZP block to polyspermy), although in different ways. Aged eggs in a variety of species (mouse, rabbit, rat, hamster) have been reported to undergo spontaneous CG loss (i.e. without fertilisation; Szollosi 1967; Longo 1974a, 1974b; Xu *et al.* 1997). It is not clear that aged eggs have a reduced ability

to exocytose CGs if fertilised, although this is a possibility because sperm-induced Ca^{2+} oscillations are abnormal in aged mouse eggs compared with eggs collected near the time of ovulation (Igarashi *et al.* 1997; Gordo *et al.* 2002) and this abnormal Ca^{2+} signalling may affect egg activation responses, such as CG exocytosis.

Historical perspective: evidence for the membrane block to polyspermy in mammalian eggs

Little is known about the molecular or mechanistic basis of the membrane block in mammalian eggs, although evidence for its existence comes from several findings that date back several decades. Classic studies of fertilised, monospermic eggs recovered from natural matings found that there are extra sperm in the perivitelline space (PVS; between the ZP and the plasma membrane) that are apparently unable to fertilise the egg, despite being right next to the membrane (see Lewis and Wright 1935; Odor and Blandau 1949; Austin 1961). Numbers of supernumerary perivitelline sperm vary by species. The eggs of some species (rabbit, pika, pocket gopher, mole) have tens to hundreds of sperm in the PVS, suggestive of a highly effective membrane block and very little ZP block. In eggs from other species (dog, sheep, field vole), PVS sperm are rare, suggestive of a highly effective ZP block. Most species (mouse, human, rat, guinea-pig, cat, ferret, pig, cattle) appear to use both blocks to polyspermy; in these eggs, approximately one to 10 supernumerary sperm are found in the PVS, suggestive of an effective membrane block and also a ZP block to limit the number of sperm reaching the PVS (Lewis and Wright 1935; Odor and Blandau 1949; Austin 1961; Hunter 1990; Sengoku *et al.* 1995; Hunter *et al.* 1998). With regard to human eggs, there is one retrospective analysis of clinical data from subzonal inseminations that concluded that human eggs lack a membrane block (Wolf *et al.* 1997), but data from experimental studies provide convincing evidence that human eggs use a membrane block in addition to a ZP block to prevent polyspermy (Sengoku *et al.* 1995).

Complementing these data from studies of *in vivo*-fertilised eggs are data from *in vitro* fertilisation (IVF) experiments. These experiments use ZP-free eggs, allowing one to distinguish between effects at the level of the ZP and at the plasma membrane. Studies of eggs from a variety of mammalian species (e.g. mouse, hamster, human) have shown that ZP-free fertilised eggs are penetrated by few, if any, additional sperm when challenged with a second insemination, suggesting that the zygote membrane is somehow different from the membrane of the unfertilised egg and is unable to support sperm interactions (Wolf 1978; Zuccotti *et al.* 1991; Horvath *et al.* 1993; Maluchnik and Borsuk 1994; Sengoku *et al.* 1995). Furthermore, the number of sperm that fuse with ZP-free eggs plateaus (rather than increases steadily) when the eggs are cocultured continuously with sperm for several hours (Wolf 1978; Binor *et al.* 1982; McAvey *et al.* 2002),

raising the possibility that the egg membrane changes in some way that then prevents sperm penetration at later time-points after insemination.

Experimental assessments of the membrane block to polyspermy in mammalian eggs

Assays used to study the membrane block to polyspermy are based on classical studies that provided evidence for use of this block by mammalian eggs. Because a molecular marker for the establishment of the membrane block has not yet been identified, functional assays are used to assess the membrane block under various experimental conditions. Experiments to evaluate the membrane block are performed with ZP-free eggs in order to eliminate the contribution of the ZP block in preventing polyspermy. Two types of designs used to investigate the membrane block will be discussed here: (1) sperm incorporation over time; and (2) re-insemination. The end-point for both experiments is the average number of sperm fused per egg, as determined by staining fixed eggs with a DNA dye, such as 4',6'-diamidino-2-phenylindole (DAPI), to visualise decondensing sperm heads in the egg cytoplasm (McAvey *et al.* 2002).

Assay 1: sperm incorporation over time

When a normal membrane block is established, the number of sperm that fuse with an egg plateaus with time and, thus, examining sperm incorporation over time is a method for studying the membrane block. Figure 1 shows the data from a sample of this type of experiment, assessing sperm incorporation over time in mouse eggs inseminated with two different sperm concentrations. As shown in Fig. 1, eggs inseminated with a high sperm concentration ($100\,000\text{ sperm mL}^{-1}$) may become slightly polyspermic (an average of approximately two sperm per egg), likely due to the fact that more sperm-egg contacts are occurring with a higher sperm concentration than with a low sperm concentration, particularly before the full establishment of the membrane block. Nevertheless, the plateau in the number of sperm that fuse with ZP-free eggs suggests that the egg membrane is modified in some way, resulting in a zygote membrane that does not favour sperm penetrations at time-points after insemination. In a typical assay of sperm incorporation over time, relative levels of establishment of the membrane block are monitored by comparing the extent of sperm fusion between different experimental groups (see McAvey *et al.* 2002).

Assay 2: re-insemination

A second type of bioassay for the membrane block is a re-insemination experiment, which is outlined in Fig. 2. In this type of experiment, eggs that have been fertilised or experimentally manipulated are challenged with a second round of insemination at various times after the first insemination and the ability of the eggs to prevent further fertilisation is

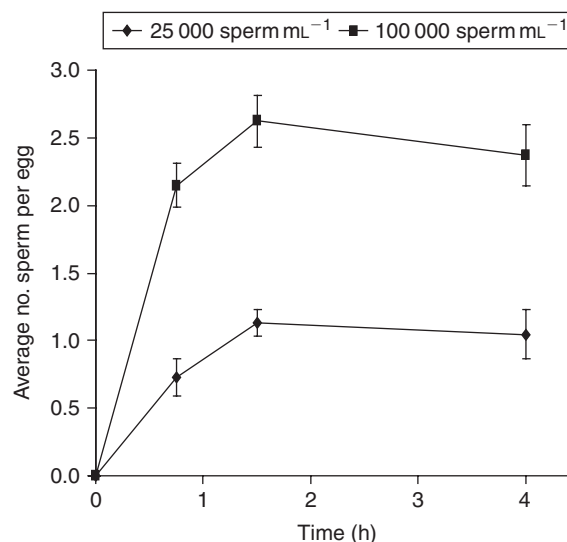


Fig. 1. Analysis of sperm incorporation over time, using two different sperm concentrations. Zona pellucida (ZP)-free mouse eggs were collected as described previously (McAvey *et al.* 2002) and inseminated with either $25\,000\text{ sperm mL}^{-1}$ (◆) or $100\,000\text{ sperm mL}^{-1}$ (■). Eggs were removed from the insemination drops at either 0.75, 1.5 or 4.0 h and then fixed to assess the average number of sperm fused per egg, as described previously (McAvey *et al.* 2002). Each data point represents approximately 40–60 eggs from at least two experiments. This study illustrates how the number of sperm fused per ZP-free egg will plateau, taken as evidence of the establishment of a membrane block to polyspermy, which prevents excess sperm penetration (see text). These data also illustrate how the level of the plateau is affected by the insemination conditions, most notably the concentration of sperm used. This is likely due to the fact that more sperm-egg contacts are occurring with a higher sperm concentration than with a low sperm concentration. These sperm-egg contacts occurring early in the insemination, before the time when the membrane block is fully established (approximately 1–2 h after insemination (Wolf 1978; Maluchnik and Borsuk 1994; Sengoku *et al.* 1995; Redkar and Olds-Clarke 1999; McAvey *et al.* 2002)), could likely lead to sperm-egg fusion, thus resulting in a higher number of sperm penetrating the eggs.

assessed in each of the groups. The extent of sperm penetration after this second insemination is indicative of the effectiveness of the membrane block. An important consideration for this assay is a means to distinguish the two populations of fertilising sperm. If the recovery period between the first and second insemination is long enough ($>3\text{ h}$), sperm from the first IVF will be forming male pronuclei and, thus, can be distinguished morphologically from sperm from the second insemination, which will still be decondensing (Wortzman and Evans 2005). In addition, labelling the sperm to be used in one of the inseminations is another means of distinguishing sperm from the first and second inseminations; this method can be used when the recovery period is less than 3 h or when a particular treatment disrupts male pronuclear formation. In our studies of mouse fertilisation, we have labelled sperm for the second insemination with the membrane-permeable dye MitoTracker Green (Invitrogen, Carlsbad, CA, USA)

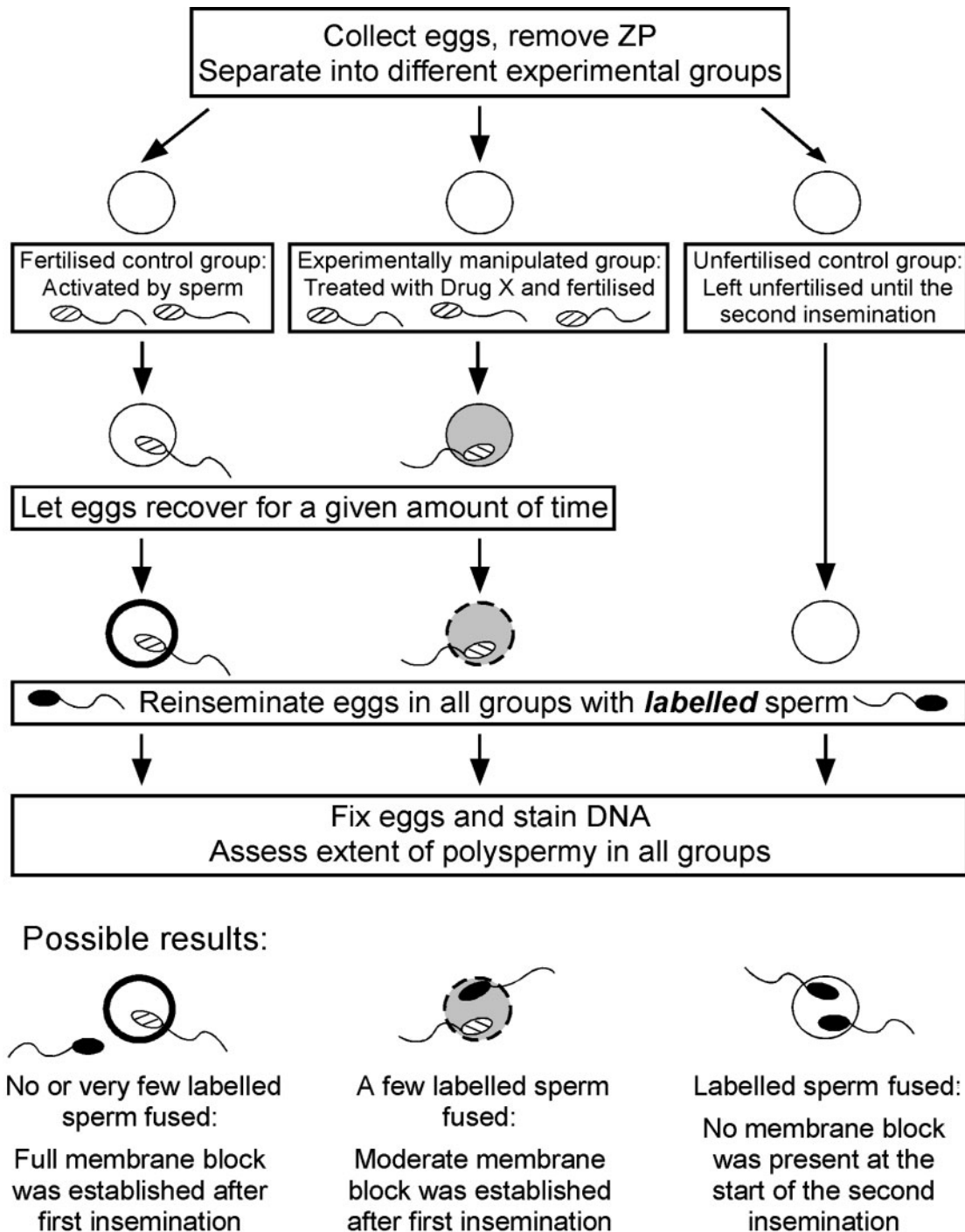


Fig. 2. Experimental design of a re-insemination assay. Zona pellucida (ZP)-free eggs were prepared as described previously (McAvey *et al.* 2002) and then split into different experimental groups: a fertilised control group, an unfertilised control group and an experimentally manipulated group. Examples of different experimental manipulations include treatment with a particular drug before fertilisation or parthenogenetic activation (e.g. Ca^{2+} ionophore or SrCl_2). (Note: if eggs are parthenogenetically activated, then they are not fertilised in the first step of the experiment, because the parthenogenesis is intended to mimic fertilisation. A control group activated by sperm should also still be included.) After insemination, eggs are allowed to recover for a specified amount of time to allow a membrane block to be established in the fertilised control group (indicated in the schematic diagram as a thicker line for the plasma membrane on the fertilised egg control). This incubation period is followed by insemination of all groups with a batch of labelled sperm (including the unfertilised control eggs that are kept in culture up to this point). After this second insemination, eggs are fixed and DNA is stained with DAPI (McAvey *et al.* 2002) so that the number of labelled sperm that have fused with the eggs can be determined. The possible results diagrammed in this figure would apply to eggs treated with a drug that gives rise to a moderate membrane block after the first insemination (such as eggs treated with low concentrations of BAPTA-AM, as discussed in the text).

(A. J. Gardner and J. P. Evans, unpublished observations). This dye has also been used to label bovine sperm (Sutovsky *et al.* 1997). MitoTracker Green selectively accumulates in mitochondria, which are localised in the mid-piece of the sperm.

It should be noted that it is essential to include appropriate controls in the second insemination step of these re-insemination assays. At one end of the spectrum are unfertilised 'naïve' eggs; these eggs indicate the extent of sperm fusion with eggs that have not yet established a membrane block. At the other end of the spectrum are fertilised eggs, namely eggs that were fertilised and activated in the first insemination step. These eggs will have established the membrane block and, thus, will indicate the extent to which penetration by sperm from the second insemination can be prevented by the membrane block. Through the inclusion of these controls, the extent of membrane block establishment in experimentally manipulated eggs can be assessed relative to normally fertilised eggs that have established a normal membrane block and to unfertilised eggs that have not established a membrane block.

Recent experimental insight into the mechanisms underlying the mammalian membrane block

Since the classical studies of *in vivo* fertilised eggs provided evidence that mammalian eggs use a membrane block to prevent polyspermy, more recent investigations have used the assays described above and other experimental approaches to examine the mammalian membrane block and how it is established following fertilisation. The section below summarises some of these more recent findings, including some of our own unpublished work. Unless otherwise noted, the majority of the work mentioned in this section uses the mouse as an experimental model system.

Calcium signalling and the membrane block to polyspermy

Shortly after fertilisation takes place, Ca^{2+} release activity is delivered from the sperm into the egg cytoplasm which triggers transient increases, or oscillations, in intracellular Ca^{2+} levels in the fertilised egg (Kurokawa *et al.* 2004). These Ca^{2+} oscillations give rise to a set of events known collectively as egg activation, which includes CG exocytosis, giving rise to the ZP block to polyspermy (see above), and cell cycle resumption (i.e. exit from meiotic arrest and progression to embryonic interphase). The Ca^{2+} oscillations triggered either by fertilisation or parthenogenetic activation are disrupted by the intracellular Ca^{2+} chelator BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetra-acetic acid acetoxymethyl ester) and lead to the inhibition of downstream egg activation events (Kline and Kline 1992). In addition, mouse eggs treated with 10 μM BAPTA-AM to completely abolish all post-fertilisation Ca^{2+} oscillations (Kline and Kline 1992) become increasingly polyspermic over time compared with control eggs, placing membrane

block establishment among the Ca^{2+} -dependent egg activation events (McAvey *et al.* 2002).

Although treatment of mouse eggs with 10 μM BAPTA-AM abolishes sperm-induced Ca^{2+} oscillations completely, treatment of eggs with lower concentrations of BAPTA-AM leads to the attenuation of sperm-induced Ca^{2+} signalling; eggs that experienced these moderated Ca^{2+} signals undergo partial egg activation responses (Kline and Kline 1992; A. J. Gardner, C. J. Williams and J. P. Evans, unpublished observations). Similar results have been obtained from studies using electroporomeabilisation to activate mouse eggs parthenogenetically by inducing controlled pulses of Ca^{2+} influx, thus mimicking post-fertilisation Ca^{2+} oscillations (Ducibella *et al.* 2002). This work shows that there are distinct Ca^{2+} requirements for the initiation and subsequent completion of specific egg activation responses (Ducibella *et al.* 2002). These studies did not examine Ca^{2+} dependence of the membrane block and so our laboratory has investigated how the treatment of eggs with a range of concentrations of BAPTA-AM to attenuate sperm-induced Ca^{2+} signalling impacts the ability of eggs to establish a membrane block. We have found that, as post-fertilisation Ca^{2+} signalling is gradually attenuated with increasing concentrations of BAPTA-AM, eggs become increasingly polyspermic when inseminated (A. J. Gardner, C. J. Williams and J. P. Evans, unpublished data). These results suggest that the membrane block is a graded response, rather than an all-or-none event where the membrane is either completely receptive or completely unreceptive to sperm.

Parthenogenesis and the membrane block to polyspermy

Eggs can be induced to activate in the absence of sperm by treatment with SrCl_2 or Ca^{2+} ionophore, a phenomenon known as parthenogenesis. The SrCl_2 and Ca^{2+} ionophore cause an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ that is sufficient to induce low to moderate extents of egg activation events, such as CG exocytosis and cell cycle resumption, but it remains unclear whether parthenogenetically activated eggs are able to establish a membrane block to polyspermy. Although there are previous reports that ZP-free eggs activated parthenogenetically with SrCl_2 or the Ca^{2+} ionophore A23187 did not establish a membrane block to polyspermy (Wolf *et al.* 1979; Horvath *et al.* 1993), there are caveats to aspects of these studies. First, some experiments did not include fertilised eggs as a control. This control is crucial (discussed above), particularly in light of our finding that the membrane block is not an on/off response and that, instead, there are varying levels of robustness. Second, these studies did not take the post-ovulatory age of the eggs into account. At later post-ovulatory ages, eggs have improved abilities to undergo egg activation responses in response to parthenogenetic stimuli (Fulton and Whittingham 1978; Nagai 1987; Collas *et al.* 1989; Ware *et al.* 1989; Fissore and Robl 1992; Abbott *et al.* 1998). Therefore, our laboratory has examined the question of whether

parthenogenetic activation with SrCl_2 or the Ca^{2+} ionophore A23187 can induce the establishment of membrane block in mouse eggs, testing eggs that were collected 13 and 17 h after human chorionic gonadotrophin (hCG), the latter of which have been reported to undergo greater extents of cell cycle resumption in response to inositol 1,4,5-trisphosphate injection (Xu *et al.* 1997). Our studies show that sperm penetrated SrCl_2 - and A23187-activated eggs to similar extents as unfertilised eggs, whereas virtually no sperm penetrated eggs that had been activated by a fertilising sperm. The results were similar with eggs collected at 13 and 17 h after hCG, indicating that parthenogenetic activation with SrCl_2 and A23187 did not induce membrane block establishment and that post-ovulatory age did not affect the ability of mouse eggs to establish a membrane block in response to these stimuli (A. J. Gardner and J. P. Evans, unpublished observations).

Intracytoplasmic sperm injection and the membrane block to polyspermy

Eggs of many (although not all) species can be fertilised by injection of a sperm into the egg cytoplasm, a procedure known as intracytoplasmic sperm injection (ICSI; Bedford *et al.* 2004; Yanagimachi 2005). Intracytoplasmic sperm injection-fertilised eggs undergo activation events, such as CG exocytosis and cell cycle resumption, and give rise to live offspring in several species (for a review, see Yanagimachi 2005), but evidence from studies of mouse and human eggs suggests that ICSI does not induce establishment of a membrane block to polyspermy (Maleszewski *et al.* 1996; Sengoku *et al.* 1999). We have re-examined this issue because advances in mouse ICSI techniques that improve post-ICSI Ca^{2+} signalling have been made since 1996, when the first studies of the membrane block in ICSI-fertilised mouse eggs were performed (Kurokawa and Fissore 2003). However, even with various modifications to the ICSI protocols to improve Ca^{2+} signalling, we find that ICSI-fertilised mouse eggs incorporate just as many sperm as do unfertilised eggs (G. B. Wortzman, M. Kurokawa, R. A. Fissore and J. P. Evans, unpublished observations), suggesting that ICSI did not trigger the establishment of a membrane block to polyspermy.

Taken together, these data raise the question of what is different between conventionally fertilised eggs and eggs that are fertilised by ICSI, as well as eggs that are activated parthenogenetically (discussed above), and how do these differences contribute to the failure of ICSI-fertilised or parthenogenetically activated eggs to establish a membrane block to polyspermy. One candidate is Ca^{2+} signalling. It is known that Ca^{2+} oscillations generated by ICSI or parthenogenesis differ from those generated by conventional fertilisation. Calcium imaging studies of eggs fertilised *in vitro* show that the first Ca^{2+} oscillation begins 1–4 min after sperm–egg fusion and originate at the sperm entry point, with later oscillations beginning in the vegetal pole (Lawrence *et al.* 1997; Deguchi *et al.* 2000), whereas the timing and/or spatial

organisation of Ca^{2+} signals induced by ICSI, SrCl_2 or Ca^{2+} ionophore differ from these induced during conventional IVF (Kline and Kline 1992; Nakano *et al.* 1997; Sato *et al.* 1999; Kurokawa and Fissore 2003). As noted above, our data indicate that Ca^{2+} signalling plays a role in the establishment of the membrane block (McAvey *et al.* 2002; A. J. Gardner, C. J. Williams and J. P. Evans, unpublished observations). With this in mind, it seems possible that there are qualitative differences between the signalling triggered when eggs are activated in the absence of sperm and the signalling triggered by *bona fide* fertilisation. We have tested the hypothesis that more ‘fertilisation-like’ oscillations are required for the establishment of the membrane block by examining whether injection of an extract of soluble sperm proteins would trigger the establishment of membrane block in mouse eggs. Injection of sperm extract is known to induce Ca^{2+} oscillations with spatial and temporal characteristics very similar to those induced by a fertilising sperm (Wu *et al.* 1998a, 1998b; Oda *et al.* 1999). However, we found that sperm extract-injected eggs did not appear to establish a membrane block to polyspermy, because just as many sperm penetrated these eggs as fused with unfertilised ‘naïve’ control eggs (G. B. Wortzman, M. Kurokawa, R. A. Fissore and J. P. Evans, unpublished observations). Therefore, it is likely that additional signalling from the sperm contributes to the initiation of the membrane block after fertilisation.

Another candidate is sperm-induced remodelling of the egg cortical cytoskeleton, which appears to be abnormal in ICSI-fertilised eggs (G. B. Wortzman, M. Kurokawa, R. A. Fissore and J. P. Evans, unpublished observations) and is lacking in parthenogenetically activated eggs. It is interesting to note that treating eggs with cytochalasin D, a drug that disrupts actin microfilaments and, thus, inhibits cortical remodelling, leads to a moderate increase in the extent of polyspermy (McAvey *et al.* 2002). Based on these observations, post-fertilisation rearrangement of the cortical cytoskeleton could be a contributing factor to the post-fertilisation modifications in the egg that culminate in a membrane block to polyspermy. Finally, an additional candidate is the sperm–egg membrane interaction. Sperm–egg membrane interaction is a step-wise process including: (1) sperm–egg binding (which may include a signalling event triggered by the binding of an extracellular sperm ligand to an egg receptor); and (2) membrane fusion between the gametes. Experiments are underway in our laboratory to examine how these numerous possibilities may play a role in the establishment of membrane block.

Differences in the membranes of unfertilised and fertilised eggs

In addition to the question of how the fertilising sperm triggers the establishment of membrane block is the issue of how the plasma membranes of unfertilised and fertilised eggs differ and how these differences render the membrane of a

fertilised egg unable to be penetrated by additional sperm. Relatively little is known in this area. The membrane block appears to be established approximately 1–2 h after insemination (Wolf 1978; Maluchnik and Borsuk 1994; Sengoku *et al.* 1995; Redkar and Olds-Clarke 1999; McAvey *et al.* 2002) and persists for many hours, or even days, into the early embryonic cleavage-stage embryos (Zuccotti *et al.* 1991; Maleszewski and Bielak 1993; Sengoku *et al.* 1995, 1999; Redkar and Olds-Clarke 1999). The failure of additional sperm to penetrate already fertilised eggs may be due to a decrease in sperm adhesion to the egg plasma membrane or perhaps sperm detachment after brief or weak adhesion (Wolf and Hamada 1979; Horvath *et al.* 1993; Sengoku *et al.* 1995; Redkar and Olds-Clarke 1999). Other than this, little is known about the molecular differences between unfertilised and fertilised egg membranes. Experiments using fluorescent tags to track certain membrane lipids in mouse eggs suggest that there are fertilisation-induced changes in the organisation of membrane lipids (Wolf *et al.* 1981). Similar techniques were used to track the diffusion of two labelled membrane proteins in mouse eggs; it was observed that the fluidity of one labelled protein in the membrane increased after fertilisation, whereas the fluidity of the other tagged protein did not change (Wolf and Ziomek 1983). Other than these bits of data, little is known about the differences in the organisation or composition of protein and lipid domains of membranes in unfertilised and fertilised eggs. A better understanding of the changes in membrane dynamics that occur after fertilisation should lead to the identification of a molecular marker(s) for the establishment of the membrane block and facilitate the development of additional assays for the membrane block to complement the functional assays examining whether eggs can prevent subsequent sperm penetration.

Perspectives and future directions

Although there is clearly much we still have to learn about the mammalian membrane block to polyspermy, there are some interesting implications from what we do know. The finding that ICSI does not trigger the establishment of the membrane block suggests that although ICSI can induce several egg activation events and support embryonic development, it does not induce all egg activation events, namely the membrane block. Although this has few ramifications in the clinic (because ICSI-fertilised eggs are not going to be exposed to additional sperm), it does indicate that ICSI is not the full equivalent of conventional IVF. The different utilisation of ZP and membrane blocks to polyspermy by different mammalian species is also a point of interest. These differences could be exploited as a means to perform a comparative study of fertilisation in different species, as well as to gain insights into molecular mechanisms underlying the membrane block. Finally, approaches such as proteomics and analyses of knockout mice with abnormalities in egg activation should allow us

to gain insights into the molecular players that contribute to the membrane block.

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