Calcium and sperm components in the establishment of the membrane block to polyspermy: studies of ICSI and activation with sperm factor

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One important result of egg activation is the establishment of blocks to prevent polyspermic fertilization; these blocks are established on the zona pellucida and the egg plasma membrane. This study examines what the sperm brings to the egg to induce the establishment of the membrane block to polyspermy, building on past evidence that membrane block establishment does not occur in response to parthenogenetic stimuli that induce a single transient increase in cytosolic Ca2+ or intracytoplasmic sperm injection (ICSI). We test the hypotheses that (i) sperm-associated Ca2+ release activity triggers membrane block establishment; (ii) introduction of sperm contents via variations on ICSI protocols (resulting in improved Ca2+ transients, egg activation and embryo development over traditional ICSI protocols) triggers membrane block establishment and (iii) sperm adhesion [binding of an extracellular sperm ligand(s) to an egg receptor(s)] combined with sperm-associated Ca2+ release activity triggers membrane block establishment. Interestingly, none of these stimuli induced establishment of the membrane block to polyspermy in mouse eggs. However, the sperm-associated remodeling of the egg cortical cytoskeleton differs between conventionally fertilized and ICSI-fertilized eggs; taken with our previous data implicating actin microfilaments in membrane block establishment, this raises the possibility that cortical reorganization may be a contributing factor. In sum, fertilization-like Ca2+ transients, either alone or combined with sperm–egg binding, are not sufficient for membrane block establishment, but that an event(s) associated with gamete interaction plays a role in this membrane function change.

Keywords: fertilization; ICSI; egg activation; polyspermy; calcium

Introduction

One of the keys for successful embryogenesis is the prevention of fertilization by more than one sperm, a condition known as polyspermy. Polyspermic fertilization will result in a triploid embryo, which typically die during early development (triploidy is detected in ~10% of spontaneously aborted human conceptuses (Jacobs et al., 1978; Hassold et al., 1980; Michelmann et al., 1986; Robinson et al., 2001; Stephenson et al., 2002). To prevent polyspermy, the mammalian egg utilizes blocks to prevent polyspermic fertilization; these blocks are established on the zona pellucida (ZP) and the egg plasma membrane. The ZP block is the result of Ca2+-triggered exocytosis of cortical granules, the contents of which modify the ZP to a form that does not support sperm interaction (Yanagimachi, 1994; Abbott and Ducibella, 2001). Much less is known about the membrane block in mammalian eggs. The goal of this study was to elucidate how the sperm induces the egg to establish the membrane block to polyspermy. Previous studies indicate that sperm-induced calcium transients play an important role in the establishment of membrane block to polyspermy (McAvey et al., 2002; Gardner et al., 2007). On the other hand, increased cytosolic Ca2+ concentration ([Ca2+]cyt) in the egg is not in itself sufficient to establish the membrane block, as eggs treated with parthenogenetic agents that increase [Ca2+]cyt can undergo some egg activation events but fail to establish a membrane block to polyspermy (Horvath et al., 1993; Maleszewski et al., 1996; Wolf et al., 1979; Sengoku et al., 1999; Gardner et al., 2007). Furthermore, additional sperm are able to fuse with the plasma membrane of intracytoplasmic sperm injection (ICSI)-generated mouse and human embryos (Maleszewski et al., 1996; Sengoku et al., 1999), suggesting that fertilization by ICSI does not induce membrane block establishment. These results from parthenogenetic activation and ICSI provide the foundation for the hypotheses tested in this work.

The increases in [Ca2+]cyt that occur in eggs in response to parthenogenetic agents or conventional ICSI methods differ significantly from the [Ca2+]cyt increase that occurs in response to a fertilizing sperm, as sperm-induced [Ca2+]cyt transients have distinct frequencies, amplitude and spatial organization (Endo, 1985; Kline and Kline, 1992; Swann and Ozil, 1994; Jones et al., 1995; Nakano et al., 1997; Deguchi et al., 2000; Jellerette et al., 2000; Kurokawa and Fissore, 2003). In contrast, [Ca2+]cyt transients in ICSI-fertilized...
eggs have can be significantly different, including delayed initiation, premature termination and complete failure of \([Ca^{2+}]_{cyt}\) increase, depending on the ICSI procedure and the species (Tesarik et al., 1994; Kurokawa and Fissore, 2003; Bedford et al., 2004; Malcuit et al., 2006b). Postulating that the \([Ca^{2+}]_{cyt}\) increase that results from parthenogenetic stimuli or ICSI is insufficient to trigger membrane block establishment, we hypothesize that ‘fertilization-like’ \([Ca^{2+}]_{cyt}\) transients trigger membrane block establishment.

To test our hypothesis, we activated eggs with the nonhydrolyzable IP3 receptor agonist adenophostin (Wu et al., 2001; Gordo et al., 2002) or a lysate of sperm proteins (known as sperm extract or sperm factor). These agents induce fertilization-like \([Ca^{2+}]_{cyt}\) release through an IP3 receptor-mediated pathway from the endoplasmic reticulum with similar spatial and temporal characteristics as \([Ca^{2+}]_{cyt}\) transients triggered by a fertilizing sperm (Oda et al., 1999; Wu et al., 2001).

The failure of ICSI-generated mouse embryos to establish a membrane block to polyspermy has been taken as evidence for sperm membrane fusion being linked with membrane block establishment (Maleszewski et al., 1996). However, there are multiple interpretations of this result that additional sperm are able to fuse with the plasma membrane of ICSI-generated embryos, and we address two specific possibilities in the work presented here. First, given that variations in ICSI procedures and sperm preparations can affect various end-points following ICSI (Kurokawa and Fissore, 2003; Morozumi et al., 2006), here we address the question of whether the ICSI methods used previously had been insufficient to induce membrane block establishment. Our studies here use improved methods of ICSI that result in improved quality of \([Ca^{2+}]_{cyt}\) transients, egg activation responses and rates of development to blastocyst stage as compared with conventional ICSI protocols (Kurokawa and Fissore, 2003). These ICSI studies also test a complementary hypothesis that components of the sperm that would be lacking in the sperm factor preparation could play a role in the establishment of a membrane block to polyspermy. Secondly, given that ICSI-fertilized eggs do not experience sperm–egg membrane interactions (i.e., binding or fusion), we also tested the hypothesis that signaling associated with sperm–egg binding, in combination with \(Ca^{2+}\) signaling, contributes to membrane block establishment. This hypothesis is based on one of the original models for how a sperm activates an egg, known as the receptor hypothesis or contact hypothesis, in which a sperm ligand binding an egg receptor triggers a signal transduction pathway(s) in the egg, (Foltz and Shilling, 1993; Schultz and Kopf, 1995; Runft et al., 2002). Current evidence indicates that the release of \(Ca^{2+}\) ions from the endoplasmic reticulum in mammalian eggs is triggered by phospholipase C (PLC) delivered from the sperm into the egg cytosol upon sperm–egg fusion (Runft et al., 2002; Saunders et al., 2002; Kurokawa et al., 2004; Malcuit et al., 2006a). However, there appear to be differences in how egg activation responses, including the membrane block, are affected downstream from the increase in \([Ca^{2+}]_{jos}\) (Ducibella et al., 2002, 2006; Ozil et al., 2005; Knott et al., 2006; Gardner et al., in press, 2007), and here, we tested the hypothesis that sperm–egg binding, which would allow an egg receptor(s) to interact with a sperm ligand(s), is working in concert with increased \([Ca^{2+}]_{cyt}\) in leading to membrane block establishment.

This study showed that fertilization-like \([Ca^{2+}]_{jos}\) transients, either alone or combined with sperm–egg binding, are not sufficient for membrane block establishment, but that an event(s) associated with gamete interaction plays a role in the establishment of the membrane block, and sperm-associated remodeling of the cortical cytoskeleton may be a contributing factor in this process.

### Materials and Methods

#### Egg collection and ZP removal

All work with animals was reviewed and approved by our Animal Care and Use Committee. Egg collection and ZP removal were performed as previously described (McAvey et al., 2002). Metaphase II (MII)-arrested eggs were collected from 6–8 week-old superfertilized CD-1 or CF-1 mice (Harlan, Indianapolis, IN, USA) or C57BL/6 x DBA/2J hybrid mice (hereafter referred to as B6D2F1) at 13 h post-ICSI injection to induce ovulation. Cumulus cells were removed by brief incubation (<5 min) in either Whitten’s medium \([109.5\text{ mM NaCl}, 4.7\text{ mM KCl}, 1.2\text{ mM KH}_2\text{PO}_4, 1.2\text{ mM MgSO}_4, 5.5\text{ mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid (as a hemichlamic salt; Ca}\_2\text{ concentration in the medium is 2.4 mM (Whitten, 1971)] with 7\text{ mM NaHCO}_3 and 15\text{ mM HEPES (hereafter referred to as }’\text{Whitten’s-HEPES’})\text{] and }0.04\%\text{ Type I-S hyalurondiase (Sigma, St Louis, MO, USA)}\text{ or in Whitten’s-HEPES medium containing 30 mg/ml bovine serum albumin (BSA) (Albunax M from Gibco-BRL, Gaithersburg, MD, USA) and 0.02}\%\text{ Type IV-S hyalurondiase (Sigma). After cumulus cell removal, the ZP was removed by a brief incubation (~15 s) in acidic culture medium-competitive buffer (acidic MEMCO; 10 mM HEPES, 1 mM NaHPO}_4, 0.8 mM MgSO}_4, 5.4 mM KCl, 116.4 mM NaCl, final pH 1.5) and then allowed to recover for 60 min in Whitten’s medium containing 22 mM NaHCO}_3 and 15 mg/ml BSA (W-B/15). Eggs were cultured in 5% \(\text{CO}_2\text{ in air.}\)

#### Sperm preparation for in vitro fertilization

Sperm were collected from the cauda epididymides of sacrificed CD-1 male mice (8-week-old or retired breeders, Harlan). Sperm from one epididymis were collected in 100 \(\mu\text{l W-B/15}.\) After 10–15 min, the tissue was removed from the droplet and the sperm were pipetted into the bottom of a tube containing 750 \(\mu\text{l W-B/15}.\) After 45 min, 225 \(\mu\text{l from the top of the swim-up culture was removed and placed in a culture dish and covered with light mineral oil. For sperm binding assays, sperm from the cauda epididymis were collected in 400 \(\mu\text{l W-B/15};\) upon removal of the tissue the sperm remained in the droplet. Sperm for in vitro fertilization (IVF) and adhesion assays were cultured for a total of 2.5–3 h in W-B/15 to allow the sperm to undergo capacitation and spontaneous acrosome exocytosis. Sperm concentration was determined using a hemocytometer.

#### Preparation of immobilized sperm and permeabilized sperm

Cauda epididymal sperm from B6D2F1 male mice were collected in 1 ml injection buffer (75 mM KCl, 20 mM HEPES, pH 7.0). The sperm suspension was washed three times and resuspended in 0.5 ml injection buffer. Prior to injection, the sperm suspension was diluted 1:1 with 12% polyvinylpyrrolidone (Sigma). In the assays with permeabilized sperm, the tails were separated by a brief sonication (3 min, 4.5 Hz) of the sperm in injection buffer submerged in ice water. These samples were then washed in injection buffer. The plasma membrane was disrupted by incubating in 5 ml of Dulbecco’s phosphate-buffered saline (DBPS) containing 0.1% (v/v) Triton X-100 for 15 min. These Triton X-100-treated sperm were then washed twice in injection buffer (75 mM KCl, 20 mM HEPES, pH 7.0) prior to injection.

#### Preparation of soluble sperm extract

Preparation of sperm extract from boar sperm has been previously described (Wu et al., 1997, 1998). Intact sperm (5–10 \(\times 10^6\) sperm/ml) were lysed by sonication (XL2020, Heat Systems Inc., Farmingdale, NY, USA) using a small probe at a setting of 3 for 15–25 min at 4°C; the insoluble fraction was then pelleted by ultracentrifugation. The clear supernatant was concentrated with ultrafiltration membranes (Centriprep-30; Amicon, Beverly, MA, USA) to final concentrations of 20–30 mg/ml protein. These extracts were then mixed for 30 min at 4°C with ammonium sulfate at 50% final saturation, the precipitates were collected by centrifugation (10 000 \(\times g\), 15 min, 4°C), and the pellets were stored at −20°C until use. Pellets were washed three times in injection buffer (75 mM KCl and 20 mM HEPES, pH 7.0) to remove the ammonium sulfate and concentrated with ultrafiltration membranes (Centricron-50, Amicon, Beverly, MA). Samples were aliquoted and frozen at −80°C. The stock concentration was 15 mg/ml.

558
Microinjection of eggs
B6D2F1 eggs were used for ICSI experiments and CD-1 eggs were used for adenophostin and sperm factor experiments (Kurokawa and Fissore, 2003). A drop of injection buffer was prepared containing either 1 mg/ml sperm extract, 10 μM adenophostin, whole sperm, immobilized sperm heads or permeabilized sperm heads. Eggs were placed in separate adjacent drops containing Chatot, Ziamok and Bavister (CBZ) medium (Chatot et al., 1989) containing 20 mM HEPES, 5 mM NaHCO3 and 0.1 mg/ml polyvinyl alcohol (PVA) (hCBZ) for ICSI with whole sperm, immobilized sperm heads or permeabilized sperm or hCBZ containing 1.5% sucrose for injection of sperm factor or adenophostin. For adenophostin or sperm factor injection, glass micropipettes were filled by suction from a drop containing the reagent. For ICSI, the sperm was aspirated into the pipette (tail first if intact sperm was used) and several piezo pulses were applied to separate the sperm head from the tail. The tip of the injection pipette containing the sample was moved until it reached the ZP, then several short, high-amplitude piezo pulses were applied to advance the pipette through the ZP. Once in the perivitelline space, the pipette was advanced against the membrane until the tip of the pipette was at the other side of the egg cortex; the egg membrane was penetrated by applying one or two long, low-amplitude piezo pulses. The contents of the pipette were injected into the egg cytoplasm with a minimum amount of fluid. The injection volume was ~5–10 pl, resulting in final intracellular concentrations of the compounds of 1–5% of the stock concentration in the injection pipette. After injection, the eggs were incubated in the injection droplet on a warming tray for 10–15 min prior to transfer into W-B/15.
Microinjected eggs were incubated for 3–4 h, an incubation period we have previously used to allow for establishment of the membrane block (Wortzman and Evans, 2005). The range in this recovery time was due to experiments in which multiple injection groups were examined. The recovery period started upon the completion of injections of the last group, and thus groups that were injected first received a longer recovery period. One hour prior to incubation with sperm, the ZP was removed with acidic MEMCO as described above. In order to exclude eggs that had failed to activate after microinjection, only eggs that emitted a second polar body were used for the studies described here.

IVF reimplantation assay for the establishment of the membrane block to polyspermy
As a control to be tested in parallel with the microinjected eggs, ZP-free eggs were activated by conventional IVF for 45 min with 50 000 sperm/ml to generate monospermic eggs as previously described (Wortzman and Evans, 2005). After insemination, the eggs were washed through one drop of Whitten’s medium containing 22 mM NaHCO3 and 0.05% PVA (W-B/PVA) and two drops of W-B/15 to remove any loosely attached sperm and then cultured in sperm-free W-B/15 for 3–4 h in parallel with the microinjected eggs. After this sperm-free incubation, eggs that had been fertilized (as determined by the emission of a second polar body) were selected for the second insemination.
To assess establishment of the membrane block to polyspermy, eggs were examined for their susceptibility to be fertilized when challenged with sperm after activation treatment. Eggs were inseminated in 10 μl insemination drops containing 10 eggs and 100 000 sperm/ml (sperm:egg ratio of 100:1) for 1.5 h. Control groups, IVF eggs (i.e. eggs fertilized and activated by conventional IVF) and unfertilized eggs, were challenged with sperm in parallel. [These insemination conditions were selected based on our previous experience, to result in an average of 80–100% of eggs fertilized with ~0.8–1.2 sperm fused per egg (Evans et al., 1995; McAvey et al., 2002); in the experiments presented here with CD-1 and B6D2F1 eggs, mean values for average number of sperm fused with unfertilized control eggs ranged from 0.8 to 2.0.] After 1.5 h, eggs were washed through three 100 μl drops of W-B/PVA to remove loosely attached sperm; washes for all experimental groups were done by the same person using the same pipette. Eggs were fixed in freshly prepared 3.7% paraformaldehyde in PBS, permeabilized in PBS containing 0.1% Triton-X 100 for 15 min and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 1.5 μg/ml 4’,6-diamidino-2-phenylindole (DAPI, Sigma). Sperm morphology, sperm–egg binding and sperm–egg fusion were assessed. Data are reported as the average number of IVF2 sperm fused per egg, focussing on the sperm from the second insemination that penetrated the eggs. Data from multiple experiments were analysed using ANOVA with Fisher’s PLSD post hoc testing as noted in the Results section, using StatView version 5.0 (SAS Institute, Cary, IN, USA). A value of P < 0.05 was considered significant.

Treatment of eggs with sperm factor and sperm adhesion
A schematic of the experimental design is depicted in Fig. 3A. Fertilized control eggs and sperm-factor-injected eggs were prepared as described above. The ‘sperm factor + adhesion’ eggs were generated as follows. ZP-intact eggs were microinjected with sperm factor as described above. Following injection, eggs were transferred to W-B/15 and cultured 45–60 min to allow recovery from the injection. The eggs were then subjected to ZP removal, followed by a 60 min culture. These ZP-free, sperm-factor injected eggs were then washed through 3–6 drops of Ca2+-deficient W-B/15 (Whitten’s medium made with 4.8 mM C2H8O4 1/2 Ca (Evans et al., 1995)) and then inseminated for 3.75 h with 100 000 sperm/ml (sperm:egg ratio of 100:1) in Ca2+-deficient W-B/15. Previous reports showed that insemination in Ca2+-deficient media supports sperm–egg binding, but not sperm–egg fusion (Miyamoto and Ishibashi, 1975; Yanagimachi, 1978; Fraser, 1987; Fujimoto et al., 1994; Evans et al., 1995). After the insemination in Ca2+-deficient medium, the eggs were vigorously washed through a drop of W-Bo/0.05% PVA to remove any loosely bound sperm and then washed through several drops of calcium-containing W-B/15. For each experiment, a subset of eggs was examined to confirm that no sperm–egg fusion occurred during this Ca2+-deficient incubation (data not shown).
Eggs from the four groups (sperm factor + adhesion, sperm factor only, fertilized and unfertilized) were challenged with a fresh batch of sperm as described above (sperm:egg ratio of 100:1 in W-B/15 for 1.5 h) and then examined for fertilization by this second batch of sperm, which could be distinguished from the sperm from the first insemination by morphology (first batch sperm would be pronuclear stage, whereas second batch sperm were decondensing (Wortzman and Evans, 2005)). As an additional control, some eggs that had been subjected to the first insemination in Ca2+-deficient medium were cultured for 1.5 h in the absence of additional sperm; this confirmed that sperm from the adhesion step did not remain on the egg surface and did not penetrate the eggs (data not shown).

Assessment of fertilization cone formation
ZP-intact eggs were injected with an immobilized sperm head or a permeabilized sperm head as described above. After microinjection, the eggs were allowed to recover in WB/15 for 45–60 min and the ZP was removed. As a control, ZP-free eggs were inseminated for 45 min with 50 000 sperm/ml and then washed in W-B/PVA to remove any loosely attached sperm. Eggs were assessed under a dissection microscope for extrusion of the second polar body and formation of the fertilization cone. Upon formation of the fertilization cone, eggs were fixed in freshly prepared 3.7–4% paraformaldehyde in PBS and stained with phalloidin as previously described (Wortzman and Evans, 2005).

Results
Analysis of the membrane block to polyspermy in eggs activated by sperm factor or adenophostin
As noted in the Introduction, previous studies demonstrate that mouse eggs activated by parthenogenesis and ICSI fail to establish a membrane block to polyspermy. Since the increases in [Ca2+]cyt in eggs activated by these means differ dramatically from those induced by normal fertilization, we hypothesized that only Ca2++ oscillations like those triggered by a fertilizing sperm could induce membrane block establishment. To test this hypothesis, eggs were microinjected with adenophostin or sperm factor [agents that have been shown to induce fertilization-like Ca2++ release (Oda et al., 1999; Wu et al., 2001)] and then cultured for 3–4 h, to allow time to manifest signs of normal egg activation (i.e. second polar body emission) and to establish a membrane block to polyspermy (Wolf, 1978; Maluchnik and Borsuk, 1994; Sengoku et al., 1995; Redkar and Olds-Clarke, 1999, McAvey et al., 2002; Wortzman and Evans, 2005). Then, to
determine if these eggs had established a membrane block to polyspermy, the ZP were removed and the eggs were inseminated. Controls, which were inseminated in parallel, were unfertilized/unactivated ‘naïve’ eggs and fertilized eggs (i.e. that had been activated by a fertilizing sperm). These experiments revealed that sperm were able to fuse with adenophosphitin-activated eggs and sperm factor-activated eggs to the same extent as with unfertilized ‘naïve’ eggs. In contrast, sperm from this second insemination did not fuse with eggs that had been activated by a fertilized sperm (Fig. 1). These results indicate that egg activation by either the soluble sperm extract or adenophosphitin was not sufficient to establish the membrane block to polyspermy.

**Analysis of the membrane block to polyspermy in eggs fertilized by four variations of ICSI**

Since induction of the fertilization-like Ca\(^{2+}\) transient with sperm factor was not sufficient to induce the membrane block to polyspermy, we next examined if other sperm components could be involved in the establishment of the membrane block. Sperm factor is a preparation of soluble sperm components and induces fertilization-like Ca\(^{2+}\) transients when injected into eggs, but we reasoned that components of the sperm lacking in the sperm factor preparation could also play a role in the establishment of a membrane block to polyspermy. Previous work reported that additional sperm are able to fuse with the plasma membrane of ICSI-generated mouse embryos (Maleszewski et al., 1996), but we examined this in further detail by fertilizing eggs using several variations of protocols for mouse ICSI (Kurokawa and Fissore, 2003), including immobilizing the sperm head and removing the tail prior to injection. This ICSI method resulted in improved egg activation responses and embryo development as compared with ICSI using a whole sperm (Kurokawa and Fissore, 2003). We also tested an additional ICSI protocol modification, injecting a permeabilized sperm head. Permeabilization of the sperm with Triton X-100 results in faster initiation and increased frequency of Ca\(^{2+}\) transients, indicating that permeabilization facilitates the release of sperm contents into the egg cytoplasm (Kurokawa and Fissore, 2003). We hypothesized that these ICSI variations might be more effective than conventional ICSI to induce the membrane block to polyspermy. However, we found that sperm were able to fuse with eggs fertilized by ICSI with an immobilized sperm head or a permeabilized sperm head to the same extent as they were able to fuse with unfertilized ‘naïve’ eggs. In contrast, sperm did not fuse with eggs that had been activated in conventional IVF by a fertilizing sperm (Fig. 2A). These results indicate that egg activation by injection of an immobilized sperm head or a permeabilized sperm head was not sufficient to establish the membrane block to polyspermy, despite the fact that these eggs undergo other events of egg activation (e.g. completion of meiosis and entry into embryonic interphase).

Upon finding that ICSI with an immobilized sperm head or a permeabilized sperm head was not sufficient for membrane block establishment, we tested two additional ICSI methods: (i) ICSI with a whole sperm (head and tail) to assess if injection of tail components might aid membrane block establishment and (ii) ICSI with three immobilized sperm heads, to test if increasing the ‘dose’ of sperm components

![Figure 1](http://molehr.oxfordjournals.org/)

**Figure 1:** Effect of egg activation by soluble sperm factor or the IP\(_3\) receptor agonist adenophosphitin on the establishment of the membrane block. Eggs were activated with either adenophosphitin or sperm factor or buffer then were cultured for 3—4 h. Eggs activated by fertilization were generated in parallel with the microinjected eggs by inseminating ZP-free eggs then washing to remove loosely attached sperm. After this 3—4 h culture period, the eggs were then challenged with sperm, in parallel with control unfertilized eggs. Results are based on two experiments, with a total of 30—65 eggs for each group, except for the buffer (mock)-injected which was done once (n = 9 eggs). Sperm from the first and second inseminations could be distinguished from each other as sperm from the second insemination were undergoing decondensation, whereas sperm from the first insemination had formed a pronucleus. The graph shows the average number of decondensing sperm fused per fertilized egg (sperm from the second insemination, or IVF2). Statistically significant differences from the control unfertilized egg group in the number of decondensing sperm are indicated with an asterisk (P < 0.0001).

![Figure 2](http://molehr.oxfordjournals.org/)

**Figure 2:** Effects of microinjection of an immobilized sperm head, permeabilized sperm head, whole sperm or multiple sperm heads in the establishment of the membrane block. Eggs were injected with either an immobilized sperm head or a permeabilized sperm head (A) or a whole sperm or three sperm heads (B). Following injection the eggs were incubated for 3—4 h. Eggs activated by conventional fertilization were generated in parallel with the ICSI-fertilized eggs, by inseminating ZP-free eggs then washing to remove loosely attached sperm. After this 3—4 h culture period, the eggs were challenged with sperm in parallel with unfertilized eggs. [These insemination conditions were selected based on our previous experience (Evans et al., 1995; McAvey et al., 2002); in the experiments here, mean values for average number of sperm fused with unfertilized control eggs ranged from 0.8 to 2.0.] The graphs show the average number of sperm from the second insemination fused per fertilized egg for each group. Statistically significant differences from the control unfertilized egg group in the number of sperm are indicated with an asterisk (P < 0.0001). Results in A are based on four experiments for each control group and the intact sperm head group and on two experiments for the permeabilized sperm head group, with a total of 30—65 eggs for each group. Results in B are based on one experiment, with a total of 4—30 eggs for each group.
in the egg cytoplasm could facilitate establishment of the membrane block (it has previously been shown that Ca\(^{2+}\) transients have a higher frequency in eggs injected with three sperm heads as compared to eggs injected with one sperm head (Kurokawa and Fissore, 2003). However, our experiments demonstrated that the number of additional sperm could fuse with eggs injected with a whole sperm or with three sperm heads was not statistically different from the number of sperm that fused with control unfertilized eggs (Fig. 2B), indicating that these versions of the ICSI protocols were not sufficient to trigger membrane block establishment in mouse eggs.

**Analysis of the membrane block to polyspermy in eggs experiencing sperm adhesion and sperm factor-induced Ca\(^{2+}\) signaling**

Since none of the variations on ICSI methods were sufficient to induce establishment of the membrane block to polyspermy, we next considered what deficiencies or abnormalities could be present in ICSI-fertilized eggs as compared with conventionally fertilized eggs. Abnormal Ca\(^{2+}\) signaling was one possibility [Ca\(^{2+}\) transients in ICSI-fertilized eggs initiate more slowly and terminate prematurely (Kurokawa and Fissore, 2003)], but our studies above using sperm factor suggested that fertilization-like Ca\(^{2+}\) transients were not sufficient for membrane block establishment. Therefore, we considered that some event associated with gamete membrane interaction, which would be lacking in ICSI-fertilized eggs, would contribute to membrane block establishment. We hypothesized that the binding of the sperm to the egg may contribute to membrane block establishment, such as by presenting a sperm ligand to an egg receptor. To test this hypothesis, we designed experiments in which eggs experienced Ca\(^{2+}\) signaling (triggered by sperm factor injection) and sperm–egg binding, but not sperm–egg fusion. This was achieved by allowing sperm to bind to eggs in Ca\(^{2+}\)-deficient medium [sperm can bind to eggs in Ca\(^{2+}\)-deficient medium but cannot fuse (Miyamoto and Ishibashi, 1975; Yanagimachi, 1978; Fraser, 1987; Fujimoto et al., 1994; Evans et al., 1995)]. These eggs that had been injected with sperm factor and then allowed to experience sperm adhesion were then challenged with sperm and assessed for the extent of fertilization from this second insemination (Fig. 3). Controls included unfertilized ‘naïve’ eggs, eggs activated by conventional IVF and eggs activated by sperm factor. The eggs activated by conventional IVF were not penetrated by additional sperm, whereas the ‘sperm extract + sperm adhesion’–treated eggs were penetrated to extents similar to the unfer-

**Cortical remodeling in eggs activated by IVF or ICSI with intact sperm or permeabilized sperm**

The data above indicated that sperm factor-induced fertilization-like Ca\(^{2+}\) transients, even combined with sperm binding, were not sufficient for establishment of the membrane block to polyspermy. We next examined remodeling of the cortical cytoskeleton. Significant remodeling of the egg’s cortical actin occurs following fertilization, resulting in the formation of an actin-rich fertilization cone over the sperm DNA (Lopata et al., 1980; Brunet and Maro, 2005). Processes involving the egg actin cytoskeleton could be involved in the membrane block (McAvey et al., 2002), and we speculated that ICSI-fertilized eggs may have abnormalities in the cortical actin. Indeed, we found that fertilization cone formation was noticeably delayed in eggs fertilized by ICSI with either a whole sperm or with a permeabi-

**IVF (Fig. 4).** The delay in fertilization cone formation was less pronounced in eggs that were ICSI-fertilized with a permeabilized sperm head, suggesting that permeabilization and/or use of an isolated sperm head facilitates the exposure of sperm DNA and/or other factors that contribute to the remodeling of the cortical cytoskeleton. This delay in fertilization cone formation was accompanied by a lag in extrusion of the second polar body (data not shown), in agreement with previous studies of ICSI-fertilized mouse eggs showing delayed cleavage rates (Kurokawa and Fissore, 2003) and suggestive of slower initiation and/or progression of egg activation. Phalloidin

Figure 3: Effect of fertilization-like Ca\(^{2+}\) signaling + sperm adhesion on the establishment of the membrane block to polyspermy

(A) This diagram shows the experimental design for egg activation by sperm factor extract followed by sperm adhesion. Eggs in the ‘sperm extract + sperm adhesion’ group were injected with sperm factor and then incubated with sperm in Ca\(^{2+}\)-deficient medium so that sperm bind but do not fuse to the eggs. These eggs would be activated as a result of the sperm factor injection, but not contain a male pronucleus. Controls were set up in parallel with the ‘sperm extract + sperm adhesion’–treated eggs; eggs in the sperm extract group were activated by sperm factor injection (as in Fig. 1) or eggs were activated by sperm via conventional fertilization (fertilized control). Eggs in the fertilized control group would establish a membrane block (indicated by the dark line for the plasma membrane) and contain a male pronucleus (hatched oval). All eggs were then challenged with sperm (IVF2), in parallel with unfertilized control eggs. (B) Results are based on two experiments, with a total of 10–25 eggs for each group. The graph shows the average number of sperm from the second insemination fused per fertilized egg for each group. Statistically signif-

561
staining of fertilization cones in these eggs revealed that although these actin-rich structures developed significantly more slowly in ICSI-fertilized eggs, there were no obvious differences between conventionally fertilized eggs, eggs fertilized by ICSI with a whole sperm or eggs fertilized by ICSI with a permeabilized sperm head (open circles).

Discussion
The event(s) that leads to establishment of the membrane block in mammalian eggs are largely unknown. The failure of ICSI-generated embryos to establish a membrane block to polyspermy has been taken as evidence for sperm membrane incorporation being linked with membrane block establishment (Maleszewski et al., 1996). It is clear that ICSI-fertilized eggs do not experience sperm–egg membrane interactions, but at the time these studies were performed, little was known about the characteristics of [Ca\(^{2+}\)]\(_{cyt}\) increase in ICSI-fertilized eggs. It has since been established that ICSI often triggers variable Ca\(^{2+}\) responses, the characteristics of which can be greatly altered depending on the ICSI procedure itself and sperm preparation (Kurokawa and Fissore, 2003; Morozumi et al., 2006); in large domestic species, ICSI consistently triggers underlying Ca\(^{2+}\) responses (Bedford et al., 2004; Malcuit et al., 2006b). ICSI-fertilized eggs do not experience sperm–egg binding and have abnormal Ca\(^{2+}\) oscillations (Nakano et al., 1997; Kurokawa and Fissore, 2003); therefore, we tested these as two events as possible contributors to membrane block establishment.

This work provides evidence that neither the abnormal Ca\(^{2+}\) signaling associated with ICSI nor the lack of sperm adhesion is to blame for the deficient membrane block to polyspermy. Eggs that experience ‘fertilization-like’ Ca\(^{2+}\) oscillations (due to activation with adenophostin, sperm factor or by improved ICSI protocols) are fertilized by sperm to the same extent as naïve unfertilized eggs, indicating that the plasma membranes of these eggs maintain their receptivity to sperm rather than converting to an unreceptive state. Our data also argue against the lack of sperm adhesion contributing to the failure of ICSI-fertilized eggs to establish a membrane block to polyspermy [although it is a formal possibility that an interaction between a sperm ligand and an egg receptor that specifically requires extracellular Ca\(^{2+}\) is involved in membrane block establishment, as these studies used Mg\(^{2+}\)-containing, Ca\(^{2+}\)-deficient medium (Evans et al., 1995)]. However, our studies do identify a new abnormality in ICSI-fertilized eggs. The formation of fertilization cones is substantially delayed in ICSI-fertilized eggs as compared with eggs fertilized by conventional IVF, and this appears to be linked with the exposure of factors in the sperm, as Triton X-100-permeabilized sperm induce cortical remodeling more quickly than unpereamblized sperm. This finding, taken together with our previous evidence for a role of the actin cytoskeleton in the membrane block (McAvey et al., 2002) and our observation of other relations between abnormalities in cortical organization and impaired membrane block establishment [(McAvey et al., 2002; Wortzman and Evans, 2005) and unpublished data], raises the possibility that abnormalities in cortical remodeling may be somehow linked with abnormalities in establishing a membrane block to polyspermy.

These results, combined with previous studies, provide insights into the events that contribute to the membrane block to polyspermy, and in turn, what deficiencies in ICSI-fertilized eggs could contribute to the failure in membrane block establishment. Egg activation begins with the early events of membrane fusion creating membrane continuity between the sperm and the egg and releasing PLC activity and possibly additional factors into the egg. The PLC triggers IP\(_3\)-mediated Ca\(^{2+}\) release from the endoplasmic reticulum. Membrane fusion then progresses to sperm incorporation, including the sperm DNA and intracellular membrane components. The actin-rich fertilization cone then forms over the sperm DNA. Ca\(^{2+}\)-dependent and other signaling pathways appear to be involved in membrane block establishment. Increased [Ca\(^{2+}\)]\(_{cyt}\) in the egg contributes to membrane block establishment (McAvey et al., 2002; Gardner et al., 2007), but, as we show here, fertilization-like Ca\(^{2+}\) transients are not sufficient for membrane block establishment (Fig. 1), and we also have evidence that a sperm-associated, Ca\(^{2+}\)-independent pathway can also contribute to the membrane block (Gardner et al., 2007). These results suggest that other events of fertilization-associated signaling, in addition to increased [Ca\(^{2+}\)]\(_{cyt}\), mediate this change in the egg membrane. Downstream targets for these Ca\(^{2+}\)-dependent or other signaling pathways could be in the egg membrane and/or cortex, with the changes mediated by the signaling pathways resulting in modifications in the egg membrane so that it was now unreceptive to sperm, thus blocking additional fertilization. The targets of these signaling pathways could also include components associated with the sperm membrane, the sperm DNA and/or egg-derived factor(s) that associate with some sperm component once this is exposed to egg cytosol.

On the basis of data shown here and those from other studies, there are several considerations with regard to the failure of ICSI-fertilized eggs to mount a membrane block to polyspermy. Our data here argue against sperm–egg adhesion contributing to membrane block establishment. The possibility remains that an event(s) associated with gamete fusion does contribute to the membrane block, although the mechanism by which such an event could affect the egg membrane remains unclear. One previous study fused zygotes to either MII eggs, parthenogenetically activated eggs or germinal vesicle-stage oocytes, using either electric field- or polyethylene glycol-mediated fusion; a time-associated decline in the percentage of these hybrid cells that were penetrated by sperm was observed (Krukowska et al., 1998). These results show that fusion with a sperm-modified membrane of a zygote can create a hybrid membrane i.e. less receptive to sperm, but the data do not definitively demonstrate that the membrane block is due to a membrane fusion event per se. Moreover, the changes that occur in these hybrid cells as a result of artificially induced fusion are likely to be different from those triggered by a fertilizing sperm. Ultrastructural studies reveal that similar hybrid cells created from fused oocytes temporarily have an altered surface morphology (Fulka et al., 1989), which could affect the membrane’s receptivity to sperm. Lastly, the relative equal membrane contribution when fusing zygotes to eggs is clearly different from the dissimilar
membrane ratio with sperm–egg fusion. We calculate that the surface area of a mouse sperm head is only 0.14% of the surface area of a mouse egg, and so dilution of the egg membrane by the sperm membrane would be only a minor contributor to membrane block establishment, if at all.

Besides the actual event of membrane fusion itself, it is possible that membrane block establishment occurs as a result of changes in the egg occurring with or downstream from the process of gamete fusion. One possibility is the introduction of factors from the sperm that in turn induce changes in the egg. It should be noted that these putative sperm-associated factors could be enzymes (similar to how the sperm delivers PLCζ to the egg), substrates for egg enzymes or activators or cofactors for egg enzymes. The delivery system via a precise point of membrane fusion may be important, since we show here that the injection of soluble components of sperm into the egg cytosol does not induce membrane block establishment. These

Figure 5: Fertilization cones in eggs fertilized by conventional IVF or by ICSI with whole sperm or permeabilized sperm head. Eggs were fertilized by conventional IVF (A and B) or by ICSI with whole sperm (B and C) or by ICSI with permeabilized sperm (E and F). A, C and E show the localization of F-actin, stained with phalloidin; the fertilization cone is indicated with the asterisk. B, D and F show DNA staining with DAPI. PB2, second polar body (somewhat out of the plane of focus in E and F); M, maternal DNA; S, sperm DNA.
sperm factors could be associated with structures in the sperm, such as the membrane, cytoskeleton, nucleus, perinuclear theca or DNA and thus may be lacking in the sperm extract preparation. The sperm DNA may also be part of the process; the presence of DNA in the egg cortex is part of the signal that leads to reorganization of cortical actin (Deng et al., 2003), and as we show here, fertilization cone is delayed in ICSI-fertilized eggs.

An additional and complementary possible explanation for the failure of ICSI-fertilized eggs to establish the membrane block is that the events leading to membrane block establishment in ICSI-fertilized eggs do not occur in a sufficiently coordinated fashion. The timing and coordination of multiple events of fertilization may be crucial for membrane block establishment, and thus the deficiencies in membrane block establishment in an ICSI-fertilized egg may be due to the aberrant timing of Ca\(^{2+}\) release and/or cortical remodeling, possibly also linked with the absence of key events associated with sperm–egg fusion.

In conclusion, this study provides important insights into both the membrane block to polyspermy and that ICSI. With these studies of ICSI-fertilized eggs taken together with previous work, we have an improved model for events that contribute to the establishment of the membrane block. Our results show that the Ca\(^{2+}\) oscillations associated with fertilization are not sufficient for the membrane block to polyspermy and suggest that events associated with or downstream from sperm–egg fusion may be an effector(s) of the membrane block to polyspermy. With regard to ICSI, it is clear that fertilization by ICSI leads to live births, but it is also evident that ICSI does not lead to all events of the egg-to-embryo transition, as the membrane block to polyspermy is lacking. Although this is inconsequential in infertility clinics (ICSI-fertilized eggs are not exposed to additional sperm), it does raise questions about what other events of egg activation and early embryogenesis may be modified in ICSI-fertilized eggs.

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