Establishment of the mammalian membrane block to polyspermy: evidence for calcium-dependent and -independent regulation

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Abstract

One crucial result of egg activation is the establishment of blocks on the zona pellucida and the egg plasma membrane to prevent fertilization by additional sperm. The mechanism(s) by which a mammalian egg regulates the establishment of the membrane block to polyspermy is largely unknown. Since Ca^{2+} signaling regulates several egg activation events, this study investigates how sperm-induced Ca^{2+} transients affect the membrane block to polyspermy, building on our previous work (Biology of Reproduction 67:1342). We demonstrate that mouse eggs that experience only one sperm-induced Ca^{2+} transient establish a membrane block that is less effective, than in eggs that experience normal sperm-induced Ca^{2+} transients but that is more effective than in eggs with completely suppressed $[\text{Ca}^{2+}]_{\text{cyt}}$ increases. Sperm-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ regulate the timing of membrane block establishment, as this block is established more slowly in eggs that experience one or no sperm-induced Ca^{2+} transients. Finally, our studies produce the intriguing discovery that there is also a Ca^{2+} -independent event that is associated with fertilization in the pathway leading to membrane block establishment. Taken together, these data indicate that Ca^{2+} plays a role in facilitating membrane block establishment by regulating the timing with which this change in egg membrane function occurs, and also that the membrane block differs from other post-fertilization egg activation responses as Ca^{2+} is not the only stimulus. The membrane block to polyspermy in mammalian eggs is likely to be the culmination of multiple post-fertilization events that together modify the egg membrane's receptivity to sperm.

Reproduction (2007) **133** 383–393

Introduction

Calcium signaling plays a key role in the earliest stages of mammalian development, with transient increases (also called oscillations) in cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{cyt}}$) in fertilized eggs inducing a cascade of cellular changes collectively known as egg activation (Runft *et al.* 2002, Malcuit *et al.* 2006). The egg activation responses triggered by Ca^{2+} signals serve to initiate embryonic development and to prevent polyspermy (fertilization by additional sperm). The repetitive transient increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ allow for the different events of mammalian egg activation to be encoded in a specific temporal order by the same upstream stimulus, as the characteristics of Ca^{2+} transients appear to be 'decoded' to generate multiple cellular responses (Ducibella *et al.* 2002, Berridge *et al.* 2003).

The goal of this study is to investigate how sperm-induced Ca^{2+} transients associated with egg activation affect the membrane block to polyspermy. Mammalian eggs use both a zona pellucida (ZP) block and a membrane block to prevent polyspermy (Yanagimachi 1994, Abbott & Ducibella 2001). Very little is known about the membrane block, although evidence demonstrating its existence comes from several studies (Austin 1961, Wolf 1978, Zuccotti *et al.* 1991, Horvath *et al.* 1993, Maluchnik & Borsuk 1994, Sengoku *et al.* 1995, McAvey *et al.* 2002, Gardner & Evans 2006). The mechanism by which a mammalian egg membrane decreases its receptivity to sperm is not known. While non-mammalian species (i.e. frogs, several marine invertebrates) use a membrane block that involves a rapid and transient depolarization of egg membrane

potential (Jaffe & Gould 1985, Gould & Stephano 2003), it appears that the mammalian membrane block works by a different mechanism as membrane depolarization is not observed in fertilized mouse, hamster, or rabbit eggs (Miyazaki & Igusa 1981, Igusa *et al.* 1983, Jaffe *et al.* 1983, McCulloh *et al.* 1983).

The mechanism by which a mammalian egg regulates the establishment of the membrane block also is largely unknown. Our previous work indicates that increased $[Ca^{2+}]_{cyt}$ is an important component of membrane block establishment, as complete suppression of sperm-induced Ca^{2+} transients with 10 μ M BAPTA-AM, an intracellular Ca^{2+} chelator, results in a dramatic increase in polyspermy in ZP-free mouse eggs (McAvey *et al.* 2002). Other events of egg activation such as cortical granule (CG) exocytosis and cell cycle resumption, also do not occur in eggs in which increased $[Ca^{2+}]_{cyt}$ is completely inhibited. It is further known from a variety of studies, such as those using either partial suppression of sperm-induced Ca^{2+} transients or parthenogenetic activation resulting from different types of manipulated $[Ca^{2+}]_{cyt}$ increases, that different egg activation responses require different 'levels' of calcium-mediated signals for initiation and then completion (Kline & Kline 1992, Ducibella *et al.* 2002, Ozil *et al.* 2005). For example, studies using electroporation of mouse eggs to induce pulses of Ca^{2+} influx that mimic post-fertilization Ca^{2+} transients show that one pulse is sufficient to initiate CG exocytosis, 4–8 pulses in mouse eggs induce exit from metaphase II arrest and pronuclear formation requires 8–24 pulses (Ducibella *et al.* 2002). Thus, we sought to define more clearly the Ca^{2+} dependence of membrane block establishment. Work presented here shows that mouse eggs that experience only one sperm-induced Ca^{2+} transient establish a membrane block that is intermediate in effectiveness – i.e. less effective at preventing multiple fertilizations than in eggs that experience normal sperm-induced Ca^{2+} transients, but more effective than in eggs treated with 10 μ M BAPTA-AM to suppress Ca^{2+} transients completely.

Another significant unknown is precisely how the membrane blocks in eggs experiencing little or no sperm-induced $[Ca^{2+}]_{cyt}$ increase are deficient in preventing additional fertilization. Insights into this mechanism would shed light on what role Ca^{2+} plays in membrane block establishment. We test the following possibilities: (1) the membrane block is less effective at preventing additional sperm fusion; (2) the membrane block is established more slowly and thus additional sperm have a larger time window to fertilize the egg after the first sperm has fused; or (3) the membrane block is transient and thus additional sperm can penetrate the egg after the initial block weakens. The studies here demonstrate that sperm-induced increases in $[Ca^{2+}]_{cyt}$ regulate the timing of membrane block establishment. This work also produced the interesting finding; there is a

Ca^{2+} -independent component in the pathway that contributes to membrane block establishment. These data suggest that Ca^{2+} plays a role in facilitating membrane block establishment, and that the membrane block is fundamentally different from other post-fertilization egg activation responses as Ca^{2+} is not the only driver.

Materials and Methods

Egg collection, BAPTA-AM treatment, and in vitro fertilization

Egg collection was performed as previously described (McAvey *et al.* 2002). Metaphase II-arrested eggs were collected from 6 to 8-week-old-superovulated CF-1 mice (Harlan, Indianapolis, USA) at 13 h after human chorionic gonadotropin (hCG) injection unless otherwise indicated. Cumulus cells were removed by brief incubation (<5 min) in either Whitten's medium (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt; Ca^{2+} concentration is 2.4 mM (Whitten 1971)) with 7 mM $NaHCO_3$ and 15 mM HEPES (hereafter referred to as 'Whitten's-HEPES') and 0.04% Type I-S hyaluronidase (Sigma), or in Whitten's-HEPES medium containing 30 mg/ml bovine serum albumin (BSA; Albumax I from Gibco-BRL) and 0.02% Type IV-S hyaluronidase (Sigma). After cumulus cell removal, the ZP were removed by a brief incubation (~15 s) in acidic culture medium compatible buffer (10 mM HEPES, 1 mM NaH_2PO_4 , 0.8 mM $MgSO_4$, 5.4 mM KCl, 116.4 mM NaCl; pH 1.5) and then the eggs were allowed to recover for 60 min in Whitten's medium containing 22 mM $NaHCO_3$ and 15 mg/ml BSA. Eggs were cultured in a humidified atmosphere of 5% CO_2 in air. Following the recovery period and prior to insemination, ZP-free eggs were incubated in Whitten's medium containing 15 mg/ml BSA and either 0.5, 1, 2, or 5 μ M 1,2-bis (*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM; Calbiochem, La Jolla, CA, USA; 10 mM stock in DMSO) for 60 min, then washed before insemination. Culture medium for control eggs contained 0.1% DMSO.

In vitro fertilization (IVF) of ZP-free eggs was performed essentially as described previously (Evans *et al.* 1995, McAvey *et al.* 2002) with the following additions. Sperm from one epididymis of a killed CD1 male mouse (8-week-old or retired breeders, Harlan) was collected in 125 μ l Whitten's medium containing 15 mg/ml BSA. After 10–15 min, tissue was removed from the droplet and the sperm were pipetted into the bottom of a tube containing 750 μ l Whitten's medium containing 15 mg/ml BSA ('swim-up'). After 45 min, 225 μ l from the top of the swim-up culture were removed and placed in a culture dish and covered with

light mineral oil. The sperm were cultured for a total of 2.5–3 h in Whitten's medium containing 15 mg/ml BSA to allow the sperm to undergo capacitation and spontaneous acrosome exocytosis. Ten eggs per 10 µl drop were inseminated for 0.75, 1.5 or 4 h with a sperm:egg ratio of 50:1 (50 000 sperm/ml) unless otherwise noted. These insemination conditions for the assessment of the membrane block to polyspermy (i.e. sperm incorporation over time) were chosen to achieve 100% fertilization in control eggs; this resulted in ~1–2.5 sperm fused per egg. After the indicated insemination time, the eggs were washed through three drops of Whitten's medium containing 15 mg/ml BSA using a thin bore pipette to detach any loosely attached sperm. Eggs were then fixed in 3.7% paraformaldehyde in PBS and stained with 1.5 µg/ml 4',6'-diamidino-2-phenylindole (DAPI) to assess the stage of maternal DNA and visualize decondensing sperm heads in the egg cytoplasm.

Calcium imaging

For analysis of sperm-induced [Ca²⁺]_{cyt} in eggs treated with BAPTA-AM, eggs were treated with 0, 0.5, 1, 2, 5, or 10 µM BAPTA-AM as described above. Just prior to imaging, ZP-free eggs were loaded with fura-2, acetoxymethyl ester (10 µM in Whitten's medium containing 15 mg/ml BSA and 0.025% Pluronic F-127; Molecular Probes, Eugene, OR, USA) at 37 °C in an atmosphere of 5% CO₂ in humidified air for 20 min. Inseminations were performed by placing 8–10 eggs from each treatment group in a 5 µl drop of Whitten's medium (without BSA) under light mineral oil in a Leiden chamber (Medical Systems, Greenvale, NY, USA), allowing the eggs to settle on a glass coverslip. The chamber was placed on a constant temperature stage (Model 5000, Micro Devices, Newtown, PA, USA) on an inverted microscope (TE 300, Nikon, Melville, NY, USA), with a laminar flow of 5% CO₂ in air over the chamber. Once the eggs adhered to the coverslip, 5 µl Whitten's medium containing 30 mg/ml BSA were added to bring the final concentration of BSA to 15 mg/ml. Two microliters of capacitated epididymal sperm of 6-week-old B6SJL/F₁/J males (Jackson Laboratories, Bar Harbor, ME; 1–2 × 10⁶ sperm/ml in Whitten's medium containing 15 mg/ml BSA) were then added to the adherent eggs in the 10 µl medium.

For analysis of [Ca²⁺]_{cyt} in Ca²⁺ ionophore-activated eggs, eggs were collected at 13 or 17 h post-hCG and loaded with fura-2-AM as described above and then washed through three drops of Ca²⁺/Mg²⁺-free medium (109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 0.05% PVA). About 15–20 eggs were transferred to a 12 µl drop of Ca²⁺/Mg²⁺-free Whitten's medium under light mineral oil in a Leiden chamber and placed on a constant temperature stage on an inverted microscope as above. Immediately prior to imaging, 4 µl of a 40 µM

A23187 solution was added to the drop to bring the final concentration of A23187 to 10 µM.

Eggs were illuminated with a 100 W xenon arc lamp; light output was passed through a Lambda 10-2 filter wheel (Sutter Instrument Co., Novato, CA, USA) to alternate excitation wavelengths between 340 and 380 nm. Emitted light passed through a fura-2 bandpass filter cube and was recorded with a Princeton Instruments MicroMAX CCD camera (Roper Scientific, Trenton, NJ, USA). The 340/380 emission ratios were obtained every 10 s. These data were collected for each egg in the field of view and then were analyzed using MetaFluor software (Universal Imaging Corp., West Chester, PA, USA) to assess alterations in whole-egg intracellular Ca²⁺ concentration.

Cortical granule labeling and quantification

Eggs were fixed in 3.7% paraformaldehyde in PBS for 30 min, permeabilized in 0.1% Triton X-100, and blocked in *Lens culinaris* agglutinin (LCA) block (3 mg/ml NaIO₄ – treated casein, 0.1 M glycine in PBS + 0.02% NaN₃). Intracellular CGs were stained with LCA-biotin (10 µg/ml, Sigma) followed by avidin-FITC (2 µg/ml, Molecular Probes). Eggs were then mounted in 4 µl Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) to flatten them between the slide and coverslip; mounting medium also contained 1.5 µg/ml DAPI to stain DNA. Eggs were imaged using a Nikon S Fluor 100× oil objective, N.A. 0.5–1.3 (total magnification including 10× ocular: 1000×), and CG density in the egg cortex was quantified by computer-assisted image quantification using either MetaMorph software (Universal Imaging Corp., West Chester, PA, USA) by counting CG density in measuring four separate ~184 µm² areas in each egg as previously described (Xu *et al.* 2003) or using IPLabs software (Scanalytics Inc., Fairfax, VA, USA) by counting a 50 µm² by hand and by computer-assisted image quantification, and a 300 µm² area by computer-assisted image quantification as previously described (Abbott *et al.* 1999, Wortzman & Evans 2005).

Parthenogenetic activation and subsequent challenge with sperm

For experiments with the calcium ionophore A23187, metaphase II eggs were collected as described above at 13 or 17 h post-hCG. After the 60 min recovery from ZP removal, eggs were washed through three 100 µl drops of Ca²⁺/Mg²⁺-free media, incubated in a 25 µl drop of Ca²⁺/Mg²⁺-free Whitten's medium containing 5 µM A23187 for 5 min, then washed through five 100 µl drops of Ca²⁺/Mg²⁺-free Whitten's medium. Activation treatment with A23187 occurred at 15 or 19 h post-hCG for eggs collected at 13 and 17 h post-hCG respectively.

As controls for these experiments, eggs were either left unactivated or were activated by fertilization. For sperm-induced activation, ZP-free eggs were inseminated with a sperm:egg ratio of 50:1 (10 eggs per 10 μ l drop with 50 000 sperm/ml) for 45 min, then washed through three 100 μ l drops of Whitten's medium containing 15 mg/ml BSA to remove loosely attached sperm. These conditions were optimized to generate monospermic eggs since polyspermic eggs show a higher frequency of Ca^{2+} transients (Faure *et al.* 1999) and this altered Ca^{2+} signaling could potentially affect egg activation events, including the membrane block to polyspermy.

Eggs were cultured for 3 h following activation by treatment with A23187 or by fertilization, as previous data suggest the membrane block is established in 0.75–2 h post-insemination (Wolf 1978, Horvath *et al.* 1993, Sengoku *et al.* 1995, Redkar & Olds-Clarke 1999, McAvey *et al.* 2002). Previous work had allowed 40 min after ionophore treatment before challenging the eggs with sperm (Wolf *et al.* 1979). The 3-h incubation used here also allowed the sperm DNA in the fertilized eggs to progress to pronuclear stage so that this sperm could be distinguished from any fertilizing sperm from the second insemination (Wortzman & Evans 2005). After this incubation, eggs in all groups were inseminated for 45 min with a sperm:egg ratio of 50–100:1 (10 eggs added to a 10 μ l drop containing 50 000 or 100 000 sperm/ml, freshly collected and capacitated), then washed through three 100 μ l drops of Whitten's medium with 15 mg/ml BSA and fixed in 3.7% paraformaldehyde. Eggs were scored for the number of sperm fused per egg from the second insemination, which could easily be distinguished from the pronuclear stage sperm from the first insemination (Wortzman & Evans 2005).

For experiments with A23187 in which CG exocytosis and cell cycle resumption were assessed, eggs were cultured for 4-h post-activation treatment or insemination, then fixed and stained with DAPI to assess cell cycle resumption and with LCA to stain CGs (described in more detail below; eggs for these analyses were not subjected to the challenge with a second batch of sperm).

Reinsemination assay – challenging zygotes with a second batch of sperm

The design of the reinsemination assay was modified from Wortzman & Evans (2005). The first insemination of the reinsemination experiment (IVF1) was optimized to generate monospermic eggs. The sperm:egg ratios for IVF1 that produced primarily monospermic eggs for each of the experimental groups were as follows: control eggs, 100:1 for 20 min; 1 μ M BAPTA-AM-treated eggs, 50:1 for 20 min; 10 μ M BAPTA-AM-treated eggs, 30:1 for 20 min. After the first insemination, eggs were washed through three drops of Whitten's medium containing 15 mg/ml BSA using a thin bore pipette to detach any loosely attached sperm. The fertilized eggs were incubated for 45

or 90 min in a sperm-free drop to allow time for membrane block establishment. After this incubation, eggs were challenged with a second round of insemination (1 h with a sperm:egg ratio of 250:1). Sperm for this second insemination were freshly collected, capacitated for 2–3 h, and labeled with the mitochondrial marker MitoTracker Green (Molecular Probes, Carlsbad, CA, USA). Fifteen minutes before the start of the second insemination, 1×10^6 sperm/ml were incubated with 100 nM MitoTracker Green (diluted from a 1 mM stock in DMSO) for 10 min in the dark at 5% CO_2 in atmosphere; this resulted in 95–100% of the sperm being labeled, and this labeling was not lost in ethanol- or aldehyde-fixed samples (data not shown). After labeling, the sperm were diluted to 250 000 sperm/ml with Whitten's medium containing 15 mg/ml BSA. As a control, unfertilized eggs were inseminated in parallel with the fertilized egg groups to assess the baseline level of the average number of sperm fused per egg from the second insemination (IVF2 control eggs). Eggs were washed through three drops of Whitten's medium containing 15 mg/ml BSA using a thin bore pipette to detach any loosely attached sperm. Eggs were fixed in 3.7% paraformaldehyde in PBS and stained with DAPI to assess the stage of maternal DNA and visualize decondensing sperm heads in the egg cytoplasm. Decondensing sperm heads with no detectable green fluorescence in the midpiece (where mitochondria are localized in the sperm) were from the first insemination, and decondensing sperm heads with an associated MitoTracker Green-labeled midpiece were from the second insemination.

Statistical analyses

Statistical analyses (ANOVA with Fishers protected least significant difference *post-hoc* testing or χ^2 analyses, as indicated) were performed using Statview 5.0 (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered significant unless otherwise noted. Error bars in figures represent the S.E.M.

Results

The effects of BAPTA-AM treatment on sperm-induced Ca^{2+} transients

We treated eggs with a range of concentrations of BAPTA-AM, as previously described by Kline & Kline (1992), to attenuate sperm-induced Ca^{2+} transients. We focused on 0.5–5 μ M BAPTA-AM, based on preliminary experiments that revealed that eggs treated with BAPTA-AM concentrations in this range showed greater extents of polyspermy when compared with untreated control eggs (data not shown). Ca^{2+} imaging experiments were performed on ZP-free eggs treated with 0, 0.5, 1, 2, 5, or 10 μ M BAPTA-AM in order to characterize how these BAPTA-AM treatments affected sperm-induced

Ca²⁺ transients. A representative Ca²⁺ trace from an egg in each treatment group is shown in Fig. 1, and Table 1 summarizes data on six characteristics of increases in [Ca²⁺]_{cyt} in all eggs analyzed: the percentage of eggs that had 0, 1, 2, or 3+ transients; the average number of transients in 60 min after the start of insemination; the average amplitude of the first [Ca²⁺]_{cyt} increase; the average amplitude of subsequent transients (as applicable); the duration of the first [Ca²⁺]_{cyt} increase (i.e. total time from the start of the increase to the time when [Ca²⁺]_{cyt} returned to basal levels); and the time from the start of the insemination to the start of the increase in [Ca²⁺]_{cyt}. We observed mild attenuation of sperm-induced Ca²⁺ transients with low BAPTA-AM concentrations (0.5, 1 µM) and more severe attenuation at higher concentrations (2, 5, 10 µM). Eggs treated with 0.5 or 1 µM BAPTA-AM typically had one [Ca²⁺]_{cyt} increase that was lower in amplitude and longer in duration than the first transient in control eggs. All of the eggs treated with 0.5 or 1 µM BAPTA-AM experienced some increase in [Ca²⁺]_{cyt} whereas only 79% of eggs treated with 2 µM BAPTA-AM experienced an increase in [Ca²⁺]_{cyt}. The increases in [Ca²⁺]_{cyt} in the eggs in this 2 µM group were lower in amplitude and longer in duration than the first transient in control eggs; these decreases in amplitude and increases in duration were

Figure 1 Representative traces of [Ca²⁺]_{cyt} transients in fertilized BAPTA-AM-treated eggs. ZP-free eggs were loaded with fura 2-AM for 20 min, then loaded with 0.5, 1, 2, 5, or 10 µM BAPTA-AM for 60 min prior to insemination. Control eggs were incubated in medium containing 0.1% DMSO. The sperm were added at the start of imaging shown on each tracing (*t*=0). Fertilization-induced Ca²⁺ transients were detected by exciting fura-2 fluorescence and measuring the intensity of light emitted (340/380 nm). A shows a representative [Ca²⁺]_{cyt} profile for a control egg (DMSO-treated, 0 µM BAPTA-AM). B–F show representative [Ca²⁺]_{cyt} profiles for eggs treated with 0.5, 1, 2, 5, or 10 µM BAPTA-AM respectively.

Table 1 Characteristics of sperm-induced increases in [Ca²⁺]_{cyt} in BAPTA-AM-treated eggs.

BAPTA-AM (µM)	Number of eggs imaged	Percentage of eggs with a given number of Ca ²⁺ transients in the 60 min from the start of insemination					Average amplitude (340/380)			
		0 (%)	1 (%)	2 (%)	3+ (%)	Av. no. of Ca ²⁺ transients in the 60 min from the start of insemination	First transient	Subsequent transient	Duration of first transient (min)	Time to first transient (min)
0	11	0	0	0	100	4.73 ± 0.41	2.58 ± 0.10	1.92 ± 0.07	4.15 ± 0.15	14.63 ± 1.89
0.5	31	0	97	3	0	1.07 ± 0.05 ^a	2.18 ± 0.11 ^a	1.2 ^b	8.46 ± 0.39 ^a	23.90 ± 2.17 ^a
1	16	0	100	0	0	1.00 ± 0.00 ^a	1.41 ± 0.17 ^{a,c}	N/A	10.34 ± 0.76 ^{a,c,d}	16.75 ± 1.45
2	14	21	79	0	0	1.00 ± 0.00 ^a	1.14 ± 0.05 ^{a,c}	N/A	13.79 ± 0.54 ^{a,c,e}	22.61 ± 4.79
5	24	92	8	0	0	0.08 ± 0.06 ^{a,c,d,e}	0.93 ± 0.08 ^f	N/A	10.45 ± 0.35 ^f	42.05 ± 5.65 ^f
10	9	100	0	0	0	0.00 ^{a,c,e,d}	N/A	N/A	N/A	N/A

^aStatistically different from 0 µM. ^bOnly 1/31 eggs had more than one Ca²⁺ transient. ^cStatistically different from 0.5 µM. ^dStatistically different from 1 µM. ^eBased on only 2/24 eggs; n too small for statistical analysis.

more extensive than those observed in eggs treated with 0.5 or 1 μM BAPTA-AM. Eggs treated with 5 μM BAPTA-AM were distinguished from the other groups, in that only two of 24 eggs imaged showed an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. We verified that the eggs in the 5 μM BAPTA-AM treatment group were fertilized by staining them with DAPI to visualize decondensing sperm heads in the egg cytoplasm (data not shown). All the post-fertilization Ca^{2+} transients were completely abolished in eggs treated with 10 μM BAPTA-AM in our experiments, in agreement with previous studies (Kline & Kline 1992).

The effects of attenuated sperm-induced Ca^{2+} transients on the membrane block to polyspermy and other egg activation events

We next determined how attenuation of sperm-induced Ca^{2+} transients affected the establishment of the membrane block to polyspermy by examining the average number of sperm fused per egg over time in ZP-free eggs treated with 0, 0.5, 1, 2, or 5 μM BAPTA-AM. The number of sperm fused per ZP-free egg normally plateaus with increased time post-insemination, indicative of the establishment of a membrane block to polyspermy that prevents additional sperm from fusing with fertilized eggs (Wolf 1978, Sengoku *et al.* 1995, Redkar & Olds-Clarke 1999, McAvey *et al.* 2002). One experimental series examined sperm–egg fusion at 0.75 and 1.5 h post-insemination (Fig. 2A), and the second examined sperm–egg fusion at 1.5 and 4 h post-insemination (Fig. 2B). Increased attenuation of the post-fertilization Ca^{2+} transients with increasing concentrations of BAPTA-AM resulted in increased polyspermy of ZP-free eggs. In addition, the average number of sperm fused per egg increased significantly from 0.75 to 1.5 h post-insemination in eggs treated with 0.5, 1, 2, or 5 μM BAPTA-AM ($P < 0.01$) but not in control eggs. The average number of sperm fused per egg at each post-insemination timepoint was different between virtually all BAPTA-AM treatment groups when compared with untreated controls (figure legend details the statistical comparisons). These data indicate that eggs with increasingly attenuated sperm-induced Ca^{2+} transients establish less effective membrane blocks to polyspermy.

As a control and in order to understand completely the effects of BAPTA-AM-mediated attenuation of sperm-induced Ca^{2+} transients on egg activation, we also examined cell cycle resumption and CG exocytosis (Supplementary Figure 1, which can be viewed online at <http://www.reproduction-online.org/supplemental/>). Three different cell cycle resumption endpoints were assessed, as was done previously (Ducibella *et al.* 2002): (1) metaphase II arrest, (2) full progression to embryonic interphase, and (3) metaphase III which is an intermediate state in which the egg completes meiosis but does not continue to embryonic interphase (as a result of M-phase promoting factor (MPF) activity

Figure 2 Effect of attenuated Ca^{2+} transients on the extent of polyspermy. ZP-free eggs were incubated in either DMSO or 0.5, 1, 2, or 5 μM BAPTA-AM for 60 min prior to insemination and washed free of BAPTA-AM before fertilization. Eggs were inseminated with a sperm:egg ratio of 50:1, washed free of loosely bound sperm, and fixed to determine the extent of sperm–egg fusion. A shows the average number of sperm fused per egg when the eggs were inseminated for 45 min or 1.5 h; data are based on three experiments with 27–41 eggs per group per timepoint. Except for the 0 μM treatment group, the increase in the extent of polyspermy from 0.75 to 1.5 h within each concentration group is statistically significant ($P < 0.01$). B shows the average number of sperm fused per egg when the eggs were inseminated for 1.5 or 4 h; data are based on four experiments with 41–64 eggs per group per timepoint. Only the 0 μM treatment group had a statistically significant increase in the extent of polyspermy from 1.5 to 4 h ($P = 0.04$). All treatment groups are significantly different from one another within a given timepoint ($P < 0.05$) with the following exceptions: at 0.75 h post-insemination, the difference between the 0 and 0.5 μM groups is not significant; and at 4 h post-insemination, the difference between the 1 and 2 μM groups is not significant. Data in A and B are from experiments with both treatments performed in parallel on the same day. Error bars represent the S.E.M.

decreasing only transiently, due to insufficient $[\text{Ca}^{2+}]_{\text{cyt}}$ increases, and then returning to high levels (Kubiak 1989, Ducibella *et al.* 2002, Hyslop *et al.* 2004)). As the post-fertilization Ca^{2+} transients were increasingly attenuated, the proportion of eggs exiting metaphase II arrest gradually decreased, while the proportion of eggs remaining arrested at metaphase II increased (Supplementary Figure 1, which can be viewed online at <http://www.reproduction-online.org/supplemental/>). A subset of eggs (4–23%) in the 0.5–5 μM treatment groups resumed the cell cycle but regressed to metaphase III. BAPTA-AM attenuation of sperm-induced Ca^{2+} transients affected the kinetics as well as the overall extent of CG exocytosis (supplemental data). In eggs treated with 0.5 or 1 μM BAPTA-AM, CG exocytosis was slightly reduced at 0.75 h post-insemination but reached control levels by 4 h post-insemination. In eggs treated with 2 μM BAPTA-AM, both the timing and the extent of CG exocytosis were affected. Eggs treated with 5 μM BAPTA-AM had the overall extent of CG exocytosis reduced by $\sim 20\%$ at both 0.75 and 4 h post-insemination when compared with pre-insemination CG densities. Taken together, these data on eggs experiencing attenuated sperm-induced Ca^{2+} transients, like the data from electroporation studies (Ducibella *et al.* 2002), support the conclusion

that there are distinct Ca²⁺ requirements for the initiation and the completion of cell cycle resumption and CG exocytosis.

Effects of calcium ionophore-induced [Ca²⁺]_{cyt} increase on membrane block establishment

The data in Fig. 2 indicated that the membrane block to polyspermy was not an 'all or none' response by the egg; as sperm-induced Ca²⁺ transients were increasingly attenuated, the more sperm were fused with the eggs. Additionally, eggs treated with 0.5 µM BAPTA-AM were only slightly more polyspermic than control untreated eggs, but most of these eggs only experienced one transient increase in [Ca²⁺]_{cyt} (Fig. 1B, Table 1). This observation raised the question of whether a single increase in [Ca²⁺]_{cyt} was sufficient to establish a partially effective membrane block to polyspermy, and prompted us to examine the effects of parthenogenetic activation with the Ca²⁺ ionophore A23187, which induces a single increase in [Ca²⁺]_{cyt} on membrane block establishment. While it has been reported previously that parthenogenetically activated eggs did not establish a membrane block to polyspermy, our analyses here included some important variables not included in previous studies (Wolf *et al.* 1979, Horvath *et al.* 1993). First, we tested the hypothesis that post-ovulatory time could affect the ability of eggs to establish a membrane block to polyspermy in response to treatment with Ca²⁺ ionophore, as aged eggs are more responsive to parthenogenetic stimuli (Fulton & Whittingham 1978, Nagai 1987, Collas *et al.* 1989, Ware *et al.* 1989, Fissore & Robl 1992, Abbott *et al.* 1998). Previous studies of membrane block establishment in response to ionophore treatment only tested freshly ovulated eggs (Wolf *et al.* 1979). Secondly, we tested the hypothesis that Ca²⁺ ionophore may induce a partially effective membrane block. Our analyses here included eggs activated by a fertilizing sperm; this control had been omitted from previous studies but was crucial to include here in light of our data that membrane blocks may vary in effectiveness (e.g. in eggs experiencing different levels of attenuated Ca²⁺ transients; Fig. 2).

Ca²⁺ imaging showed that A23187 treatment, induced a single transient increase in [Ca²⁺]_{cyt} in eggs collected at 13 and 17 h after hCG injection (Table 2), as

previously reported (Kline & Kline 1992, Swann & Ozil 1994, Jellerette *et al.* 2000; note: we did not test eggs at > 17 h post-hCG since we have demonstrated that eggs collected at 22 h post-hCG have a reduced ability to be fertilized and to establish a membrane block (Wortzman & Evans 2005). To determine how an A23187-induced increase in [Ca²⁺]_{cyt} impacted downstream egg activation responses, eggs treated with 5 µM A23187 were compared with unfertilized/unactivated eggs and eggs activated by sperm, using insemination conditions optimized to obtain monospermic eggs. Activated eggs were cultured for 3 h and then challenged with sperm. As controls, CG exocytosis and cell cycle resumption were analyzed 4 h after activation treatment or insemination in a subset of eggs from each group.

None of the eggs treated with A23187 appeared to establish a membrane block to polyspermy, regardless of whether the eggs were collected 13 or 17 h post-hCG. The number of sperm fused per egg was similar for unfertilized eggs and A23187-treated eggs, whereas virtually no sperm fused with eggs that were activated by fertilization (Fig. 3C and F). Moderate levels of CG exocytosis and low levels of cell cycle resumption (similar to those observed previously with A23187 (Abbott *et al.* 1998)) occurred in A23187-treated eggs that were collected at 17 h post-hCG (Fig. 3D and E), demonstrating that A23187 treatment induced egg activation responses, albeit to a lesser extent than fertilization did, in agreement with previous studies. Little or no CG exocytosis or exit from metaphase II arrest occurred in A23187-treated eggs that had been collected at 13 h post-hCG (Fig. 3A and B), in agreement with previous demonstrations of low responsiveness of 'young' eggs to parthenogenetic stimuli (Fulton & Whittingham 1978, Nagai 1987, Collas *et al.* 1989, Ware *et al.* 1989, Fissore & Robl 1992, Abbott *et al.* 1998).

Investigating the deficiencies in the membrane blocks of Ca²⁺-suppressed eggs

As shown above, eggs experiencing attenuated sperm-induced Ca²⁺ transients establish a less effective membrane block to polyspermy (Fig. 2). We next sought to define more precisely how the membrane blocks of Ca²⁺-suppressed eggs were deficient in preventing additional fertilization, testing the following possibilities: (1) the membrane block is less robust and therefore

Table 2 Characteristics of increases in [Ca²⁺]_{cyt} after treatment with A23187.

Time of egg collection	Number of eggs analysed	Number of transients in 60 min of imaging		Average peak amplitude of transient (340/380)	Average duration of transient (min)
		1 (%)	2+ (%)		
13 h Post-hCG	14	100	0	1.85 ± 0.03	8.20 ± 0.42
17 h Post-hCG	31	100	0	1.76 ± 0.04	11.16 ± 0.55*

*Indicates statistically significantly different from the value for the 13 h post-hCG ($P < 0.05$).

Figure 3 Ability of A23187-treated eggs to undergo CG exocytosis, resume the cell cycle, and establish a membrane block to polyspermy. Eggs were collected 13 or 17 h post-hCG, then activated with either 5 μM A23187 or sperm (as detailed in the Materials and Methods), or left unactivated/unfertilized. A–C show data from eggs collected 13 h post-hCG, and D–F show data from eggs collected 17 h post-hCG. A and D show CG densities in unactivated control eggs and in eggs activated with A23187 or sperm (eggs were fixed at 4 h post-activation). Data are expressed as CGs/100 μm^2 ($n=27$ –47 eggs for each treatment group from four experiments). B and E show what percentages of A23187-treated or inseminated eggs had exited from metaphase II arrest by 4 h post-treatment. Note: inseminated eggs include a small number of eggs that were not fertilized and thus remained arrested at metaphase II. C and F show the average number of sperm fused per egg after insemination with a sperm:egg ratio of 50:1 for 45 min in unactivated/unfertilized eggs, A23187-treated eggs or previously fertilized eggs ($n=32$ –53 eggs for each treatment group from three experiments; note: values in C and F for eggs activated by sperm represent only decondensing sperm heads, excluding pronuclear stage sperm from the first insemination.)

less effective at preventing additional sperm fusion; (2) the membrane block is slower to establish and thus additional sperm can fertilize the egg after the first sperm has fused; or (3) the membrane block is transient and thus additional sperm can penetrate the egg after the initial block weakens.

To determine which of these deficiencies existed in Ca^{2+} -suppressed eggs, we used a 'reinsemination' assay, in which fertilized ZP-free eggs were challenged with a second batch of sperm and assessed for how many sperm from the second insemination were able to penetrate the eggs after either 45- or 90-min recovery period following the first insemination. Our controls were: (1) untreated fertilized eggs that experienced normal sperm-induced Ca^{2+} signaling; and (2) unfertilized 'naïve' eggs that were only challenged with sperm in parallel with the second insemination of the fertilized eggs. These controls were compared with eggs treated with 1 μM BAPTA-AM (one sperm-induced Ca^{2+} transient) or 10 μM BAPTA-AM (completely abolished sperm-induced Ca^{2+} transients; Fig. 1, Table 1).

When reinseminated after 45 min, both groups of Ca^{2+} -suppressed eggs (1 or 10 μM BAPTA-AM) became fertilized to the same extent as naïve, unfertilized control eggs (IVF2 control; Fig. 4A), demonstrating that the

Ca^{2+} -suppressed eggs had not established an effective membrane block at this time. However, when reinseminated after a 90 min recovery time, Ca^{2+} -suppressed eggs were as effective as control fertilized eggs in preventing additional sperm fusion (Fig. 4A). This finding suggested that the post-fertilization Ca^{2+} transients played a role in the proper timing of membrane block establishment, as the membrane block was established more slowly in Ca^{2+} -suppressed eggs. Additionally, Ca^{2+} transients were not solely responsible for membrane block establishment because eggs that had no post-fertilization Ca^{2+} transients were still able to prevent additional sperm fusion upon reinsemination, albeit more slowly.

We gained additional insights from comparing the subset of zygotes that were monospermic after IVF1 to the subset of zygotes that were polyspermic after IVF1. Interestingly, polyspermic zygotes had fewer Mito-Tracker-labeled sperm fused with them than did the monospermic eggs. This was observed in zygotes derived from control untreated eggs (experiencing normal sperm-induced Ca^{2+} transients) and from eggs treated with 1 or 10 μM BAPTA-AM (Fig. 4B and C). There was also moderate evidence for a correlation between the extent of polyspermy from IVF1 and whether eggs

Figure 4 Ability of Ca²⁺-suppressed eggs to prevent additional fertilization upon reinsemination. A shows the average number of sperm fused per egg from the second insemination (average number of MitoTracker green-labeled sperm per egg) in unfertilized eggs and fertilized eggs treated with 0–10 μM BAPTA-AM. Data are based on 39–75 eggs per treatment group from four experiments. B shows the average number of MitoTracker green-labeled sperm fused per egg in the subset of eggs from A that were monospermic after IVF1 (69–87% of the total group). C shows the average number of MitoTracker green-labeled sperm fused per egg in the subset of eggs from A that were polyspermic after IVF1 (13–31% of the total group). Data are from experiments with both timepoints performed in parallel on the same day. A statistically significant difference between unfertilized control levels (IVF2) and a treatment group is represented by an a ($P < 0.05$), and a statistically significant difference between monospermic and polyspermic eggs from IVF1 within a treatment group is represented by a b ($P < 0.012$). Error bars represent the s.e.m.

were penetrated by sperm from IVF2 (i.e. eggs that were dispermic or trispermic or more from IVF1 were increasingly less likely to contain sperm from IVF2; χ^2 analysis $P = 0.07$ for control eggs, $P = 0.01$ for eggs treated with 1 μM BAPTA-AM, and $P = 0.06$ for eggs treated with 10 μM BAPTA-AM).

Discussion

We have previously shown that complete suppression of $[Ca^{2+}]_{cyt}$ transients in fertilized ZP-free mouse eggs, leads to an increase in the number of fused sperm (McAvey *et al.* 2002), indicative of disruption in the ability of the eggs to establish a membrane block to prevent polyspermy. Here, we extend those findings by defining the effects of partial suppression of sperm-induced Ca²⁺ transients on the membrane block, by determining that Ca²⁺ plays a role in the timing of membrane block establishment, and by documenting that there is a Ca²⁺-independent component of the pathway leading to membrane block establishment.

Mouse eggs that experience only one sperm-induced Ca²⁺ transient establish a membrane block that is less effective at preventing multiple fertilizations than the membrane block in eggs that experience normal sperm-induced Ca²⁺ transients (Fig. 2). However, the membrane block in these eggs that experience only one sperm-induced Ca²⁺ transient is substantially more effective than the block in eggs treated with 5 or 10 μM BAPTA-AM to suppress Ca²⁺ transients completely, indicating that one sperm-induced transient increase in $[Ca^{2+}]_{cyt}$ can trigger the egg to establish a membrane block that is intermediate in effectiveness. This discovery is somewhat surprising since it had been previously reported that Ca²⁺ ionophore, which induces only one transient increase in $[Ca^{2+}]_{cyt}$, does not induce membrane block establishment (Wolf *et al.* 1979). This prompted us to re-examine the effects of Ca²⁺ ionophore treatment on membrane block establishment. Our studies here, include the important variable of post-ovulatory aged eggs (omitted from previous membrane block studies) because aged eggs are more responsive to parthenogenetic stimuli (Fulton & Whittingham 1978, Nagai 1987, Collas *et al.* 1989, Ware *et al.* 1989, Fissore & Robl 1992, Abbott *et al.* 1998). We find that neither freshly ovulated nor post-ovulatory aged eggs establish even a partially effective membrane block in response to ionophore treatment, despite the fact that the Ca²⁺ ionophore-induced transient in $[Ca^{2+}]_{cyt}$ has similar characteristics to the transient in eggs treated with 0.5 and 1.0 μM BAPTA-AM. These data provide evidence that some aspect of sperm-induced Ca²⁺ transients is required for membrane block establishment and/or that some event associated with fertilization (and lacking in parthenogenetic activation) is part of the pathway leading to membrane block establishment.

Additional insights into these issues come from our work here that defines why eggs with little or no sperm-induced $[Ca^{2+}]_{cyt}$ increase following fertilization become more polyspermic than control eggs. Our studies show that Ca^{2+} regulates the timing of membrane block establishment. The membrane block is established more slowly in eggs with attenuated or completely suppressed Ca^{2+} transients than in control eggs that experience normal sperm-induced Ca^{2+} transients, and thus additional sperm have a larger time window in which to fertilize an egg after the first sperm has fused with the egg. These studies also reveal that polyspermic zygotes have fewer MitoTracker-labeled sperm from the second insemination (IVF2) fused with them than do the monospermic eggs. Interestingly, this was observed for control untreated eggs (experiencing normal sperm-induced Ca^{2+} signaling) and eggs treated with 1 or 10 μ M BAPTA-AM (Fig. 4B and C). These results indicate that: (a) polyspermic zygotes establish a more effective membrane block and were better able to prevent additional fertilization events than were monospermic zygotes; and (b) the effect on the membrane block of multiple sperm–egg interaction events resulting from polyspermy is independent of increased $[Ca^{2+}]_{cyt}$ in the egg. Taken together, these data complement the conclusions from the studies noted above and provide evidence that a fertilization-associated event(s) is a part of the pathway leading to membrane block establishment, with Ca^{2+} playing a role to facilitate membrane block establishment.

This study demonstrates that the membrane block differs from other post-fertilization egg activation responses as Ca^{2+} plays a role in facilitating membrane block establishment but is not the only stimulus. The additional stimulus/stimuli appears to be associated with some event(s) occurring with fertilization. The role of Ca^{2+} in regulation of the timing of membrane block establishment is clearly important, since the assays of sperm incorporation over time (Fig. 2) show that an increased extent of polyspermy can be associated with this delay. An optimal, sperm-induced pathway of Ca^{2+} signaling may be important, as it is known that spatial and temporal characteristics of Ca^{2+} transients differ between eggs activated by IVF and eggs activated by parthenogenetic stimuli and also eggs fertilized by intracytoplasmic sperm injection (Nakano *et al.* 1997, Sato *et al.* 1999, Deguchi *et al.* 2000). ICSI is pertinent here as it too, like parthenogenesis, has been reported to be unable to induce establishment of the membrane block (Maleszewski *et al.* 1996). Several cellular and molecular events occur with fertilization and are different or lacking in eggs activated parthenogenetically or by ICSI. These include sperm–egg binding, sperm–egg fusion, and sperm-induced changes in the egg cortex and cytoplasm, and any of these may also function with the aforementioned spatial–temporal characteristics of Ca^{2+} transients to effect membrane

block establishment. The membrane block to polyspermy in mammalian eggs is likely to be the culmination of multiple events that together modify the egg membrane's receptivity to sperm.

Acknowledgements

This work was supported by grants from the NIH/NICHD (HD045671, HD037696) and the March of Dimes (6-FY-04-59) to J P E, and a Burroughs Wellcome Fund Career Award in the Biomedical Sciences to C J W. A J G has been supported by a training grant from the NIH (HD07276). We are grateful to Dr Karl Broman (Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health) and Matthew Marcello (lab of J P E) for assistance with statistical analyses, and to members of the Evans lab for discussions of the data. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 31 October 2006

Accepted 30 November 2006