PLC and IP$_3$-evoked Ca$^{2+}$ release initiate the fast block to polyspermy in *Xenopus laevis* eggs

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The prevention of polyspermy is essential for the successful progression of normal embryonic development in most sexually reproducing species. In external fertilizers, the process of fertilization induces a depolarization of the egg’s membrane within seconds, which inhibits supernumerary sperm from entering an already-fertilized egg. This fast block requires an increase of intracellular Ca$^{2+}$ in the African clawed frog, *Xenopus laevis*, which in turn activates an efflux of Cl$^-$ that depolarizes the cell. Here we seek to identify the source of this intracellular Ca$^{2+}$. Using electrophysiology, pharmacology, bioinformatics, and developmental biology, we explore the requirement for both Ca$^{2+}$ entry into the egg from the extracellular milieu and Ca$^{2+}$ release from an internal store, to mediate fertilization-induced depolarization. We report that although eggs express Ca$^{2+}$-permeant ion channels, blockade of these channels does not alter the fast block. In contrast, insemination of eggs in the presence of Xestospongin C—a potent inhibitor of inositol 1,4,5-trisphosphate (IP$_3$)-induced Ca$^{2+}$ release from the endoplasmic reticulum (ER)—completely inhibits fertilization-evoked depolarization and increases the incidence of polyspermy. Inhibition of the IP$_3$-generating enzyme phospholipase C (PLC) with U73122 similarly prevents fertilization-induced depolarization and increases polyspermy. Together, these results demonstrate that fast polyspermy block after fertilization in *X. laevis* eggs is mediated by activation of PLC, which increases IP$_3$ and evokes Ca$^{2+}$ release from the ER. This ER-derived Ca$^{2+}$ then activates a Cl$^-$ channel to induce the fast polyspermy block. The PLC-induced cascade of events represents one of the earliest known signaling pathways initiated by fertilization.

Introduction

Embryonic development is a tightly regulated series of events that begins with the fertilization of an egg by a single sperm. This remarkable process of gamete unification is surprisingly error prone. For example, the fertilization of an egg by more than one sperm, a condition known as polyspermy, is a significant barrier to successful reproduction (Wong and Wessel, 2006). Polyspermy causes chromosomal abnormalities that are embryonically lethal in nearly all sexually reproducing species. To avoid polyspermy-induced lethality, eggs have developed multiple mechanisms to prevent sperm entry into an already-fertilized egg (Stricker, 1999; Wong and Wessel, 2006); however, the molecular pathways driving these events are still poorly understood.

The two most common strategies for preventing polyspermy are the fast block and the slow block (Wong and Wessel, 2006). As their names imply, these mechanisms differ with respect to how quickly they occur. The fast block involves depolarization of the egg and occurs within seconds of fertilization (Jaffe, 1976). Cross-species fertilization experiments demonstrated that sperm possess a voltage sensor that prevents their entry into a depolarized egg (Jaffe et al., 1983a). The mechanism that enables sperm to detect the egg’s membrane potential remains unknown.

In contrast, the slow block involves the creation of a physical barrier surrounding the nascent zygote and takes several minutes to complete (Stricker, 1999; Wong and Wessel, 2006). Whereas the slow block occurs in nearly all sexually reproducing species, the fast block is limited to externally fertilizing organisms in which the sperm-to-egg ratio can be extremely high (Jaffe, 1976; Cross and Elinson, 1980; Jaffe et al., 1983b).

The fast block has been documented in diverse externally fertilizing organisms (Jaffe and Gould, 1985), including fucoid algae (Brawley, 1991), sea urchins (Jaffe, 1976), starfish (Miyazaki and Hirai, 1979), marine worms (Gould-Somero et al., 1979), and amphibians (Cross and Elinson, 1980; Charbonneau et al., 1983). The signaling pathways underlying the fast block remain elusive for most species. Because of evolutionary distance and differences in habitat among the species that use the fast block, the precise mechanisms are likely to vary. Nevertheless, eggs capable of undergoing the fast block share three characteristics: their fertilization-activated membrane depolarization, known as the fertilization potential, persists for 1 min or more (Jaffe, 1976; Grey...
et al., 1982), thereby distinguishing it from action potentials in other excitable cells such as neurons or cardiac myocytes (Hille, 2001); when held at their fertilization potential voltage, the eggs can be bound, but not entered, by sperm (Jaffe, 1976); and when held at hyperpolarized potentials, the eggs can be fertilized by multiple sperm (Jaffe, 1976).

A Cl− current depolarizes frog eggs at fertilization to mediate their fast polyspermy block (Cross and Elinson, 1980; Grey et al., 1982; Webb and Nuccitelli, 1985; Glahn and Nuccitelli, 2003). We have recently demonstrated that the Ca2+-activated Cl− channel, TMEM16A, conducts this current in eggs from the African clawed frog Xenopus laevis (see Wozniak et al. in this issue). Although we know increased intracellular Ca2+ is necessary for the fast block, how fertilization signals increased Ca2+ to open TMEM16A channels was unknown. A prominent role for elevated intracellular Ca2+ as a trigger for the fast block has previously been demonstrated by two independent experiments. First, fertilization failed to evoke a depolarization in X. laevis eggs loaded with the Ca2+-chelator BAPTA (Kline, 1988). Second, treating X. laevis eggs with a Ca2+ ionophore, a lipid-soluble compound that transports Ca2+ across the plasma membrane and increases the intracellular Ca2+ concentration, evoked a depolarization in the absence of fertilization (Grey et al., 1982). The ionophore-signaled depolarization demonstrated that increased intracellular Ca2+ is sufficient to trigger the fast block, and the BAPTA experiments demonstrated that an elevation of intracellular Ca2+ is necessary.

Here we sought to uncover the mechanisms by which fertilization signals increased intracellular Ca2+ in the X. laevis egg to activate the fast block. We reasoned that fertilization could signal the opening of Ca2+-permeant channels to allow Ca2+ entry into the egg or that Ca2+ could be released from an intracellular store. To distinguish between these two possibilities, we interrogated existing proteomics and RNA-seq data and found that two Ca2+-permeant channels, TrpV4 and PKD2, are expressed in X. laevis eggs (Wühr et al., 2014; Session et al., 2016). However, insemination in the presence of broad-spectrum Ca2+ channel inhibitors, at concentrations known to block the candidate channels, did not alter the fertilization-signaled depolarization. In contrast, inhibition of PLC and inositol 1,4,5-triphosphate (IP3) receptors (IP3Rs) on the ER successfully abolished any fertilization-signaled depolarization and increased the incidence of polyspermic fertilizations. Together these results indicate that fertilization activates PLC to release Ca2+ from the ER and signal the fast polyspermy block in X. laevis.

### Materials and methods

**Reagents**

Xestospongin C, GdCl₃, U-73343, and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Tocris, and human chorionic gonadotropin was purchased from Henry Schien. U-73122 hydrate was purchased from Sigma-Aldrich. SK&F-96365 was purchased from Cayman Chemical. All other materials, unless noted, were purchased from Thermo Fisher Scientific.

**Solutions**

Variations of modified Ringer’s (MR) solution were used for all experiments. MR contains (in mM) 100 NaCl, 1.8 KCl, 2.0 CaCl₂,

| Table 1. Biophysical properties of the fertilization-evoked depolarizations |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | Resting potential | Fertilization potential | Sperm addition to depolarization time | Depolarization rate |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | mV                          | mV                          | min                          | mV/ms                      |
| Control (MR/5)              | −17.9 ± 0.9                 | −2.6 ± 1.2                  | 5.3 ± 0.5                    | 3.9 ± 0.8                   |
| 10 µM GdCl₃                 | −17.0 ± 2.3                 | −2.6 ± 0.7                  | 3.5 ± 0.8                    | 10.6 ± 7.9                  |
| 20 µM SK&F-96365           | −13.6 ± 2.0                 | −6.4 ± 1.9                  | 3.8 ± 1.0                    | 6.1 ± 3.6                   |
| 500 nM Xestospongin C       | −11.0 ± 1.4                 | —                           | —                           | —                           |
| 2% DMSO                     | −21.5 ± 4.2                 | −6.6 ± 2.3                  | 8.0 ± 1.4                    | 5.8 ± 3.4                   |
| 100 µM 2-APB                | −19.6 ± 1.4                 | −4.8 ± 2.0                  | 6.6 ± 1.8                    | 0.6 ± 0.3                   |
| 1 µM U73122                 | −22.1 ± 4.1                 | —                           | —                           | —                           |
| 1 µM U73343                 | −20.8 ± 2.3                 | −3.7 ± 1.2                  | 1.9 ± 0.4                    | 14.9 ± 6.6                  |

Mean ± SEM for the indicated measurement before, during, and after the fertilization-signaled depolarization.

| Table 2. Development assays in various conditions |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | Undeveloped | Developed | Polyspermic | n |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Control                     | 3 ± 2                    | 97 ± 2                   | 13 ± 3                   | 6 |
| 10 µM Gd                     | 5 ± 5                    | 95 ± 5                   | 10 ± 7                   | 3 |
| 20 µM SK&F-96365            | 23 ± 11                  | 77 ± 11                  | 16 ± 7                   | 3 |
| Control                     | 46 ± 15                  | 54 ± 15                  | 13 ± 4                   | 3 |
| 500 nM Xestospongin C       | 32 ± 8                   | 68 ± 8                   | 34 ± 6                   | 3 |
| Control                     | 6 ± 5                    | 94 ± 5                   | 8 ± 4                    | 3 |
| 100 µM 2-APB                | 19 ± 10                  | 81 ± 10                  | 69 ± 17                  | 3 |
| Control                     | 38 ± 9                   | 82 ± 9                   | 11 ± 7                   | 3 |
| 1 µM U73122                 | 26 ± 16                  | 74 ± 16                  | 12 ± 3                   | 3 |
| 1 µM U73343                 | 35 ± 13                  | 65 ± 13                  | 60 ± 11                  | 3 |

Mean ± SEM are reported. Out of the zygotes that initiated embryonic development, embryos were categorized as monospermic or polyspermic based on cleavage furrow symmetry. Control development for Xestospongin C was MR/3 with 2% DMSO.
1.0 MgCl₂ and 5.0 HEPES, pH 7.8, and was filtered using a sterile, 0.2-µm polystyrene filter (Heasman et al., 1991).

**Fertilization solution**
Control fertilization recordings were made in our standard solution of 20% MR diluted in DDI₂O (also known as MR/5). Various inhibitors were added to MR/5 and used during fertilization recordings. Experimental conditions were made by diluting concentrated inhibitor stocks solutions, which were themselves made in water or DMSO. All recordings were made in solutions that contained a final DMSO concentration of <0.5%, except for those made in 500 nM Xestospongin C. The 500 nM Xestospongin C in MR/5 solution contained 2% DMSO; because we have found that fertilization in ≥2% DMSO alone can alter embryonic development (Tables 1 and 2), the control for the Xestospongin C treatment was MR/5 with 2% DMSO.

**Solutions for embryonic development and polyspermy assays**
Incidence of embryonic development and polyspermy were assayed in 33% MR (also known as MR/3). Various inhibitors were added to MR/3, which contained final DMSO concentrations of <0.5%, except for the 500-nM Xestospongin C trials. As described above, the 500-nM Xestospongin C treatment solution contained 2% DMSO; thus, we included MR/3 with 2% DMSO as a control solution for this trial.

**Animals**
All animal procedures were conducted using accepted standards of humane animal care and were approved by the Animal Care and Use Committee at the University of Pittsburgh. X. laevis adults were obtained commercially (Research Resource Identifier NXR_0.0031; NASCO) and housed at 18°C with a 12-h/12-h light/dark cycle.

**Collection of gametes, fertilization, and developmental assays**
As previously described (Wozniak et al., 2017), eggs were collected from sexually mature X. laevis females. In brief, females were injected 1,000 IU of human chorionic gonadotropin into their dorsal lymph sac and housed overnight for 12–16 h at 14–16°C. Within 2 h of being moved to room temperature, females began laying eggs, which were collected on dry Petri dishes. Eggs were then used within 10 min of being laid.

Sperm were collected from testes of X. laevis sexually mature males after euthanasia by a 30-min immersion in 3.6 g/liter tricaine-S (pH 7.4; Wozniak et al., 2017). Testes were manually cleaned by removing fat and vessels and were stored at 4°C in MR for usage on the day of dissection or in L-15 medium for use up to 1 wk later. For whole-cell recordings, sperm suspensions were created by mincing ~1/10 of a testis in MR/5; if not used immediately, this solution was stored on ice and used within 1 h. No more than three separate sperm additions were added to an egg during a whole-cell recording, and the total added sperm suspension volume never exceeded 7.5% of the recording-solution volume. After insemination during whole-cell recordings, eggs were transferred to MR/3 for up to 16 h to monitor development.

Initiation of embryonic development and the incidence of polyspermy were assessed as previously described (Grey et al., 1982).

Figure 1. Fertilization signals a depolarization in X. laevis eggs. (A) Representative whole-cell recordings made during fertilization in control conditions (in MR/5 solution). Dashed line denotes 0 mV. (B) Images of X. laevis (top) egg before sperm addition and (bottom) egg ~15 min after fertilization with animal pole contracted. Tukey box plot distributions of the (C) resting and fertilization potentials in control conditions and (D) depolarization rate (n = 31, recorded over 22 experiment days). The central line represents the median value, the box denotes the data spread from 25 and 75%, and the whiskers reflect 10–90%.

Approximately 20–40 eggs were placed in each treatment and incubated for 10 min before fertilization. Sperm were then minced in 1/10 testis in MR, and the sperm suspension was used to fertilize eggs. Approximately 90 min after insemination, the initiation of embryonic development was assessed based on the appearance of cleavage furrows. Polyspermic fertilization was determined based on the incidence of asymmetric and incomplete cleavage furrows during the first few cleavage events, whereas monospermic fertilization was noted based on symmetric, complete furrows.

**Electrophysiology**
Whole-cell recordings were made using TEV-200A amplifiers (Dagan) and digitized by Axon Digidata 1550A (Molecular Devices). The pClamp Software (Molecular Devices) was used for data acquisition at a rate of 5 kHz. Pipettes of 5–20 MΩ resistance were pulled from borosilicate glass and filled with 1 M KCl. Resting and fertilization potentials were generally stable and quantified ~10 s before and after the depolarization, respectively. Depolarization rates were quantified by determining the maximum velocity of a 1-mV shift in the membrane potential for each recording.
L and S versions of each gene were summed. then normalized to transcripts per million. Expression values for bute and allowing multimappers (-p -s 0 -M). Raw counts were v1.8.3.2 in unstranded, paired-end mode using the gene_ID attri-
tureCounts (Liao et al., 2014) on Xenbase primary gene models from Session et al. (2016). Protein concentrations are from Wühr et al. (2014) script levels were >1 TPM. Transcript levels (shown as TPM) were obtained from Session et al. (2016), and proteomics data are from Wühr et al. (2014) of Ca2+ channels in X. laevis eggs. Fig. S1 shows RNA and protein expression of Ca2+ channels in X. laevis eggs.

Imaging
Unfertilized and fertilized eggs were imaged with a stereoscope (Leica Microsystems) equipped with a Leica 10447157 IX objective and DFC310 FX camera. Images were analyzed using LAS (version 3.6.0 build 488) software and Photoshop (Adobe).

Proteomic and RNA-seq analysis
RNA-seq reads from Session et al. (2016) were downloaded from the NCBI Sequence Read Archive (accession no. SRX1287707). Reads were aligned using HISAT2 (Kim et al., 2015) with default parameters in paired-end mode to the X. laevis v9.1 genome (http://www.xenbase.org). Gene counts were obtained using featureCounts (Liao et al., 2014) on Xenbase primary gene models v1.8.3.2 in unstranded, paired-end mode using the gene_ID attribute and allowing multimappers (-p -s 0 -M). Raw counts were then normalized to transcripts per million. Expression values for L and S versions of each gene were summed.

To identify Ca2+-permeant channels, we extracted all genes annotated with Gene Ontology (GO) molecular function terms that contained the keywords “calcium” and “channel” (Dataset S1) using Xenbase GO term annotations. Because of incomplete annotation of the X. laevis genes, GO terms associated with Xenopus tropicalis genes were transferred to their X. laevis orthologues. Heatmaps were generated in R Studio-1.1.383 using the heatmap.2 command from the gplots package.

To estimate the number of IP3Rs in the egg, we combined the mass spectrometry–derived protein concentrations from Wühr et al. (2014) with the stoichiometry of the functional channel: four subunits of ITPRI for each functional channel (Ludtke et al., 2011; Murray et al., 2013). We then assumed that X. laevis eggs are spherical and calculated their volume based on their measured diameter of 1.4 mm (Wozniak et al., 2017).

Quantification and statistical analyses
Igor (WaveMetrics) and Excel (Microsoft) software were used for analysis of electrophysiology recordings. Data from whole-cell recordings experiments are displayed with Tukey box plot distributions, where the box contains the data between 25 and 75%, the central line denotes the median, and the whiskers extend out to 10 and 90%. Additionally, for each experimental condition, the mean values ± SEM are reported (Table 1). Depolarization rates were log10 transformed before statistical analysis. T tests (one tailed for depolarization rates and two tailed for resting and fertilization potentials) were used to determine differences between fertilization treatments. Data from embryonic development ass-

Online supplemental material
The following materials are included in the supplement: Dataset 1 lists GO terms used to identify Ca2+ channels; RNA-seq data are from Session et al. (2016), and proteomics data are from Wühr et al. (2014) of Ca2+ channels in X. laevis eggs. Fig. S1 shows RNA and protein expression of Ca2+ channels in X. laevis eggs.

Results
Fertilization signals a depolarization in X. laevis eggs
To characterize the fast block to polyspermy, we made whole-
cell recordings from X. laevis eggs during fertilization in control conditions (the MR/5 solution; see Materials and methods). Eggs with stable resting potentials were inseminated by pipetting the sperm suspension into the recording chamber directly above the egg. The eggs’ membrane potentials were recorded for up to 40 min (Fig. 1 A) or until the cortex contracted (Fig. 1 B), an early indicator of successful fertilization. Fig. 1 A depicts a typical fert-
ilization-evoked depolarization. A mean of 5.2 ± 0.5 min (n = 31) passed between sperm addition and the recorded depolarization of the fast block, which likely represents the time required for the sperm to penetrate the viscous jelly coat of the egg (Table 1; Glahn and Nuccitelli, 2003). Under control conditions, the mean egg resting potential was −17.9 ± 0.9 mV, and the fertilization potential was −2.6 ± 1.1 mV (n = 31; Fig. 1 C and Table 1). The mean rate of depolarization for eggs fertilized under the control conditions was 3.9 ± 0.8 mV/ms (n = 31; Fig. 1 D and Table 1).

Ca2+ entry into the egg is not required for the fast block in X. laevis
To identify the source of Ca2+ that signals the fast block, we first explored the hypothesis that fertilization may evoke Ca2+ entry from the extracellular environment through plasma mem-
brane-traversing Ca2+ channels. We interrogated existing pro-

Figure 2. Expression of Ca2+ channels in X. laevis eggs. Heatmaps of RNA (left) and protein (Prot; right) expression levels of Ca2+ channels whose transcript levels were >1 TPM. Transcript levels (shown as TPM) were obtained from Session et al. (2016). Protein concentrations were from Wühr et al. (2014) as determined by mass spectrometry (mass spec.)–based proteomics in log10 nanomol. Red arrows highlight plasma membrane localized Ca2+ channels found in eggs.
teomics and RNA-seq datasets and identified 49 Ca^{2+}-selective channels annotated in the *X. laevis* genome (Fig. S1 and Dataset S1). Out of the 18 transcripts that were expressed ≥1 transcript per million (TPM), proteomics detected 10 of these proteins in the *X. laevis* egg (Fig. 2; Wühr et al., 2014; Session et al., 2016). Of these 10 proteins, 4 were intracellular Ca^{2+} channels and transporters, including the IP_3_ receptor type 1 and the mitochondrial Ca^{2+} uniporter (ITPR1, MCU, TMCO1, and TPCC2; Sakuntabhai et al., 1999; Bobe et al., 2004; Zhang et al., 2005; Bertridge, 2009; Calcraff et al., 2009; Pitt et al., 2010; De Stefani et al., 2011; Wang et al., 2016; Sun et al., 2018). We also found four proteins that are not pore forming, although they are capable of regulating Ca^{2+} channel activity: the soluble Ca^{2+}-binding protein called frequinin (encoded by NCS1; Bourne et al., 2001; Nakamura et al., 2001), two guanine nucleotide exchange factors (DENCNDA and DENCDB), and the NAD synthase 1 (NADSYN1). The final two proteins were the transient receptor potential subfamily V, member 4 (TrpV4) channel, and polycystin-2 (PKD2); these were the only candidates that conduct Ca^{2+} entry from the extracellular environment into the egg. The proteomics and RNA-seq data reveal that fertilization could open a membrane Ca^{2+} channel to increase intracellular Ca^{2+} and signal the fast block.

Next, we determined whether Ca^{2+} entry is required for the fast block by recording from *X. laevis* eggs inseminated in the presence of broad-spectrum inhibitors known to inhibit most Ca^{2+}-permeant channels including TrpV4 and PKD2: Gd^{3+} (González-Perrett et al., 2001; Suzuki et al., 2003) or SK&F-96365 (Clapham, 2007; Ho et al., 2012). There were no significant differences between depolarizations recorded under control conditions (Fig. 1) and in solutions supplemented with 10 µM GdCl_3_ or 20 µM SK&F-96365 (Fig. 3, A and B; and Table 1). Like eggs fertilized under control conditions, the mean resting potential of eggs in Gd^{3+} was −17.0 ± 2.3 mV, and the fertilization potential was −2.6 ± 0.7 mV (n = 8; Fig. 3, C and D; and Table 1). The depolarization rate for eggs inseminated in Gd^{3+} was 10.6 ± 7.9 mV/ms (n = 8; Fig. 3 E and Table 1), compared with 3.9 ± 0.8 mV/ms in control conditions (n = 31; Fig. 1 C). Eggs recorded in SK&F-96365 had mean resting potentials of −13.6 ± 2.0 mV and depolarized to −6.4 ± 1.9 mV (n = 6; Fig. 3, C and D; and Table 1). The rate of depolarization of these eggs was 6.1 ± 3.7 mV/ms (Fig. 3 E and Table 1).

To further confirm that extracellular Ca^{2+} is not needed for the fast block, we quantified the incidence of polyspermy in eggs inseminated with Ca^{2+} channel blockers. Persistence of normal fertilization-evoked depolarizations and normal cleavage furrow formation in eggs inseminated with Ca^{2+} channel blockers demonstrates that Ca^{2+} entry from the extracellular environment is not required for the fast block.

The fast block requires IP_3_-evoked Ca^{2+} release from the ER

To explore whether Ca^{2+} release from an intracellular store activates the fast block, we first interrogated an IP_3_-mediated pathway. We reasoned that several minutes after fertilization, an increase of IP_3_ in the egg is known to initiate the slow block.

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**Figure 3. Fertilization-signaled depolarization does not require Ca^{2+} entry into *X. laevis* eggs. (A and B)** Representative fertilization recordings made in solutions with 10 µM GdCl_3_ (A) and 20 µM SK&F-96365 (B). Dashed lines denote 0 mV. (C–E) Tukey box plot distributions of the resting (C) and fertilization (D) potentials and depolarization rate (E) for indicated treatments (n = 8–12, recorded over 3 experiment days per treatment). In D and E, the gray lines denote the Tukey plot box distributions for recordings made in control conditions (in the MR/5 solution), where the solid line represents the median value, the dashed lines denote the data spread from 25 and 75%, and the whiskers reflect 10–90%. (F) Images of *X. laevis* embryos from monospermic (top) and polyspermic (bottom) fertilizations. (G) Proportion of polyspermic embryos out of total developed embryos in control, Gd^{3+}, and SK&F-96365 (n = 3–6, recorded over 3–6 experiment days per treatment, the mean values ± SEM are reported).
by signaling Ca²⁺ release from the ER to initiate cortical granule exocytosis (Sato et al., 2006). Additionally, injecting X. laevis eggs with an IP₃R antibody inhibits this Ca²⁺ wave and ceases development (Runft et al., 1999). Together, these studies indicate that IP₃ is necessary for early signaling events in X. laevis eggs. Furthermore, our own bioinformatic analysis substantiates the presence of the IP₃R (encoded by the ITPR1 gene) in X. laevis eggs (Fig. 2). Based on protein concentrations, we estimate that the X. laevis egg includes ∼5.3 × 10¹⁰ IP₃Rs. Although IP₃Rs are abundant in the X. laevis egg, it was unclear whether fertilization could evoke a fast-enough elevation of IP₃ to trigger the fast block. We therefore determined whether blocking the IP₃R altered the fertilization-signaled depolarization, as well as the incidence of polyspermy.

In the presence of the potent IP₃R inhibitor, Xestospongin C (Gafni et al., 1997; Kanki et al., 2001), fertilization failed to evoke any depolarization in five independent trials (Fig. 4 A), yet all five of these embryos developed cleavage furrows. The resting potential of eggs in 500 nM Xestospongin C was elevated compared with the control, −11.0 ± 1.4 mV in Xestospongin C (n = 5) versus −21.5 ± 4.2 mV in MR/5 with 2% DMSO (P < 0.05, t test; Fig. 4 B and Table 1). Others have shown that most eggs held at −11 mV are capable of fertilization and the initiation of embryonic development (Glahn and Nuccitelli, 2003). Because our Xestospongin C treatment included 2% DMSO, we controlled for these experiments by inseminating eggs in vehicle alone (2% DMSO in MR/5; Fig. 4 C and Table 1). We found that the biophysical properties of the fast block recorded from eggs inseminated in vehicle alone were not different from control recordings made in vehicle alone, including the mean depolarization rate in eggs inseminated in 2% DMSO (5.8 ± 3.9 mV/ms, n = 5), which was statistically indistinguishable from control depolarizations (P > 0.05, t test; Fig. 4, B, D, and E; and Table 1). Inhibition of the fertilization-signaled depolarization by Xestospongin C thereby demonstrates that the IP₃R mediate the depolarization of the egg.

In the presence of 2-APB (Maruyama et al., 1997), a less-potent IP₃R inhibitor, fertilization evoked slower depolarizations compared with recordings made under the control conditions (Fig. 4, E and F; and Table 1). Specifically, fertilization of eggs inseminated in 100 µM 2-APB depolarized at a rate of 0.6 ± 0.3 mV/ms (n = 8) compared with 3.9 ± 0.8 mV/ms (n = 31) in MR/5 (P < 0.05, t test). The depolarization rate is directly proportional to the number of TMEM16A channels opened by fertilization (Wozniak et al., 2018). Therefore, a slower depolarization rate in 2-APB reflects an attenuated Ca²⁺ release from the ER to ultimately open fewer TMEM16A channels. Based on rates measured in the presence and absence of 2-APB, we estimate that fertilization opened 6.5-fold fewer TMEM16A channels in the presence of the drug.

Insemination of eggs in either Xestospongin C or 2-APB led to a significant increase in the incidence of polyspermy compared with their controls (Fig. 3 G and Table 2). For example, 500 nM Xestospongin C led to 34 ± 6% polyspermy compared with 13 ± 4% in 2% DMSO (t test, P < 0.01; Fig. 3 G and Table 2). Overall, blockade of the IP₃R with either Xestospongin C or 2-APB diminished the fast block and increased the incidence of polyspermy. Together, these data demonstrate that an IP₃-evoked Ca²⁺ release from the ER is essential for the fast block.

The fast block requires PLC

IP₃ is generated de novo by the enzymatic activity of PLC, whereby the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) is cleaved into IP₃ and diacylglycerol. To
We found that eggs inseminated in U73122 had a significantly higher incidence of polyspermy than control (Fig. 5 F): 60 ± 11% (n = 3) polyspermy with U73122 versus 11 ± 7% (n = 3) in control conditions (ANOVA, P < 0.01). In contrast, the incidence of polyspermy in U73343 was the same as control (ANOVA, P > 0.05). Together these data suggest that the enzymatic activity of PLC, which generates IP₃, is required to initiate the fast block.

Discussion

Despite the widespread use of the fast polyspermy block by evolutionarily divergent species, the signaling pathways that underlie these fertilization-evoked depolarizations have remained elusive. Here we sought to uncover the source of Ca²⁺ that initiates this pathway in X. laevis. Our data demonstrate that the fast block is signaled by activation of PLC, to increase IP₃ and activate the ER-localized IP₃R (Fig. 6). Release of Ca²⁺ from the ER then opens TMEM16A channels to depolarize the egg as part of the fast polyspermy block (Fig. 6; Wozniak et al., 2018).

Previous studies showed that fertilization-evoked depolarization varies with respect to amplitude and shape, even when recorded under control conditions (Cross and Elinson, 1980; Grey et al., 1982; Webb and Nuccitelli, 1985). Our study further demonstrates that the rate of depolarization varies for each unique fertilization event. Because the depolarization rate is directly proportional to the number of TMEM16A channels that open (Wozniak et al., 2018), our data imply that different fertilization events lead to the opening of different numbers of channels. The variance in numbers of TMEM16A channels activated by response to fertilization may reflect varying amounts of Ca²⁺ released from the ER in different eggs. For example, if fertilization opens TMEM16A by a pathway that involves receptor activation and second-messenger signaling, variation may reveal that some sperm activate multiple receptors, whereas other sperm may activate only one receptor.

To uncover the source of Ca²⁺ that signals the fast block, we first explored whether Ca²⁺-permeant channels on the eggs’ plasma membranes were required for this fertilization-signaled depolarization. We have recently demonstrated that the jelly layer surrounding X. laevis eggs is enriched with 6.3 mM freely diffusing Ca²⁺ (Wozniak et al., 2017), thereby indicating that fertilization could signal Ca²⁺ entry even with changing environmental conditions. By mining existing proteomics and RNA-seq datasets (Wühr et al., 2014; Session et al., 2016), we uncovered that X. laevis eggs express two Ca²⁺-permeant channels, TrpV4 and PKD2 (Fig. 2). A possible role for either channel in the fast block was attractive because both are known to transduce physical stress into cationic currents (Delmas, 2005; Toft-Bertelsen et al., 2017), and sperm–egg fusion at fertilization theoretically exerts forces on the membrane of the egg that could trigger the fast block. We found, however, that fertilization evoked normal depolarizations in the presence of the broad-spectrum Ca²⁺ channel inhibitors Gd³⁺ or SK&F-96365 known to block both TrpV4 (Suzuki et al., 2003; Ho et al., 2012) and PKD2 (Ho et al., 2012; Fig. 3). These results reveal that Ca²⁺ entry into the egg is essential for the fast block, a surprising finding given that Ca²⁺ enters eggs from other external fertilizers at fertilization to ini-

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IP₃ induces fast polyspermy block in Xenopus eggs

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