



PLC ζ Induced Ca $^{2+}$ Oscillations in Mouse Eggs Involve a Positive Feedback Cycle of Ca $^{2+}$ Induced InsP₃ Formation From Cytoplasmic PIP₂

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Egg activation at fertilization in mammalian eggs is caused by a series of transient increases in the cytosolic free Ca $^{2+}$ concentration, referred to as Ca $^{2+}$ oscillations. It is widely accepted that these Ca $^{2+}$ oscillations are initiated by a sperm derived phospholipase C isoform, PLC ζ that hydrolyses its substrate PIP₂ to produce the Ca $^{2+}$ releasing messenger InsP₃. However, it is not clear whether PLC ζ induced InsP₃ formation is periodic or monotonic, and whether the PIP₂ source for generating InsP₃ from PLC ζ is in the plasma membrane or the cytoplasm. In this study we have uncaged InsP₃ at different points of the Ca $^{2+}$ oscillation cycle to show that PLC ζ causes Ca $^{2+}$ oscillations by a mechanism which requires Ca $^{2+}$ induced InsP₃ formation. In contrast, incubation in Sr $^{2+}$ media, which also induces Ca $^{2+}$ oscillations in mouse eggs, sensitizes InsP₃-induced Ca $^{2+}$ release. We also show that the cytosolic level Ca $^{2+}$ is a key factor in setting the frequency of Ca $^{2+}$ oscillations since low concentrations of the Ca $^{2+}$ pump inhibitor, thapsigargin, accelerates the frequency of PLC ζ induced Ca $^{2+}$ oscillations in eggs, even in Ca $^{2+}$ free media. Given that Ca $^{2+}$ induced InsP₃ formation causes a rapid wave during each Ca $^{2+}$ rise, we use a mathematical model to show that InsP₃ generation, and hence PLC ζ 's substrate PIP₂, has to be finely distributed throughout the egg cytoplasm. Evidence for PIP₂ distribution in vesicles throughout the egg cytoplasm is provided with a rhodamine-peptide probe, PBP10. The apparent level of PIP₂ in such vesicles could be reduced by incubating eggs in the drug propranolol which also reversibly inhibited PLC ζ induced, but not Sr $^{2+}$ induced, Ca $^{2+}$ oscillations. These data suggest that the cytosolic Ca $^{2+}$ level, rather than Ca $^{2+}$ store content, is a key variable in setting the pace of PLC ζ induced Ca $^{2+}$ oscillations in eggs, and they imply that InsP₃ oscillates in synchrony with Ca $^{2+}$ oscillations. Furthermore, they support the hypothesis that PLC ζ and sperm induced Ca $^{2+}$ oscillations in eggs requires the hydrolysis of PIP₂ from finely spaced cytoplasmic vesicles.

Keywords: Ca $^{2+}$ oscillations, phospholipase C, strontium, inositol trisphosphate, egg, phosphatidyl inositol bisphosphate

INTRODUCTION

The fertilization of a mammalian egg involves a series of low frequency Ca²⁺ oscillations that last for many hours. Such Ca²⁺ oscillations play the key role in egg activation and the subsequent development of the embryo (Stricker, 1999). The first Ca²⁺ increase takes ~10 s to travel as a wave across the egg from the point of sperm entry (Miyazaki et al., 1986; Deguchi et al., 2000). However, all the subsequent Ca²⁺ transients have a rising phase of about 1 s which is due to a fast Ca²⁺ wave (>50 μm/s) that crosses the egg from apparently random points in the egg cortex (Deguchi et al., 2000). Each Ca²⁺ increase during the oscillations is due to release from internal Ca²⁺ stores via inositol 1,4,5-trisphosphate receptors (IP3R) which are exclusively of type 1 IP3R in mammalian eggs (Miyazaki, 1988; Miyazaki et al., 1993). The sperm stimulates the Ca²⁺ oscillations via inositol 1,4,5-trisphosphate (InsP₃) production, and all the reproducible studies suggest that this is principally due to the introduction of a sperm specific phospholipase Czeta (PLCζ) into the egg after gamete fusion (Saunders et al., 2002). Injection of PLCζ protein or cRNA causes prolonged Ca²⁺ oscillations that mimic those seen at fertilization in eggs of mice, rat, humans, cows, and pigs (Cox et al., 2002; Saunders et al., 2002; Fujimoto et al., 2004; Kouchi et al., 2004; Kurokawa et al., 2005; Bedford-Guaus et al., 2008; Ito et al., 2008; Ross et al., 2008; Yoon et al., 2012; Sato et al., 2013). PLCζ is distinctive compared to most mammalian PLC isozymes in that it is stimulated by low levels of Ca²⁺ such that it is maximally sensitive to Ca²⁺ around the resting levels in eggs (Nomikos et al., 2005). PLCζ is expected to diffuse across the egg in about 10 min following sperm-egg fusion, hence the fast Ca²⁺ waves seen after the initial Ca²⁺ transient are propagated within a cytoplasm in which PLCζ has probably dispersed throughout the egg.

There are two classes of model to explain how InsP₃ causes Ca²⁺ oscillations in cells, both which have been proposed for fertilizing mammalian eggs (Dupont and Goldbeter, 1994; Politi et al., 2006). There are some models that propose Ca²⁺ dependent sensitization, and then de-sensitization, of the IP3R is necessary to generate each Ca²⁺ transient (Politi et al., 2006). This class of models supports the finding that mouse and hamster eggs can be stimulated to oscillate by sustained injection of InsP₃, or by injection of the IP3R agonist adenophostin (Swann et al., 1989; Brind et al., 2000; Jones and Nixon, 2000). On the other hand there are other models in which Ca²⁺ dependent production of InsP₃ generates each Ca²⁺ transient, and in which InsP₃ is predicted to oscillate alongside Ca²⁺ (Politi et al., 2006). This second class of model is supported by the detection of InsP₃ oscillations in mouse eggs injected with PLCζ, albeit at high levels of PLCζ (Shirakawa et al., 2006). However, it is not clear if any oscillatory changes in InsP₃ oscillations are necessary for generating Ca²⁺ increases. Either classes of model have to incorporate the observation that the Ca²⁺ oscillations have a dependence upon Ca²⁺ influx. So for example, if fertilizing hamster or mouse eggs are incubated in Ca²⁺ free media the oscillations run down and stop (Igusa and Miyazaki, 1983; Lawrence and Cuthbertson, 1995; McGuinness et al., 1996). It has

been suggested that the Ca²⁺ store content is critical in setting the timing of Ca²⁺ oscillations in mouse eggs. This is supported by evidence that the SERCA inhibitor thapsigargin can also be used to block sperm and PLCζ induced Ca²⁺ oscillations by depleting Ca²⁺ stores content (Kline and Kline, 1992b). However, changes in cytosolic Ca²⁺ may also play a role in the timing of oscillations since cytosolic Ca²⁺ can regulate both IP3Rs and PLCζ activity.

Sustained Ca²⁺ oscillations in mouse eggs can also be triggered by incubation in media containing Sr²⁺ instead of Ca²⁺ (Kline and Kline, 1992a; Bos-Mikich et al., 1995). Sr²⁺-induced Ca²⁺ oscillations resemble those seen at fertilization, and they are as effective as fertilization or PLCζ in triggering development to the blastocyst stage (Yu et al., 2008). The oscillations are dependent upon Sr²⁺ influx into the egg and the presence of functional IP3Rs (Zhang et al., 2005). However, it is not clear how Sr²⁺ causes Ca²⁺ oscillations. One study suggested that the effect of Sr²⁺ requires InsP₃ production (Zhang et al., 2005). However, unlike fertilization, there is no Sr²⁺ induced downregulation of IP3Rs and this suggests that Sr²⁺ does not cause any substantial InsP₃ generation (Jellerette et al., 2000). *In vitro* preparations of IP3Rs receptors can be stimulated to open by Sr²⁺ ions (Marshall and Taylor, 1994), so a direct effect of Sr²⁺ on IP3Rs is also likely, but any changes in InP₃ sensitivity in eggs have yet to be shown.

As well as its high sensitivity to Ca²⁺, another unusual characteristic of PLCζ is that it does not localize to the plasma membrane (Yu et al., 2012). The substrate for PLCζ, phosphatidylinositol 4,5-bisphosphate (PIP₂), can be detected in the plasma membrane of mouse eggs using the PH domain of PLCδ1 (Halet et al., 2002), but the depletion of such PIP₂ from the plasma membrane does not affect the generation of Ca²⁺ oscillations in response to PLCζ or fertilization (Yu et al., 2012). In contrast to somatic cells, mouse eggs have been shown to contain PIP₂ in intracellular vesicles (Yu et al., 2012). These vesicles were detected using PIP₂ antibodies and were found to be dispersed throughout the cytoplasm of mouse eggs (Yu et al., 2012). PLCζ can also be detected on small cytoplasmic vesicles using immunostaining (Yu et al., 2012). The significance of this type of intracellular localization of PLCζ and PIP₂ has not been made clear.

Here we report experiments that analyse the mechanism of PLCζ induced Ca²⁺ oscillations in mouse eggs. We use photo-release of caged InsP₃ to show that PLCζ causes Ca²⁺ oscillations via a positive feedback cycle of Ca²⁺ release and Ca²⁺ induced InsP₃ production. In contrast the Sr²⁺ induced Ca²⁺ oscillations in mouse eggs involve a sensitization of InsP₃ induced Ca²⁺ release. We go on to show that the cytosolic Ca²⁺ is more likely to be important for setting the pace of oscillations in eggs than Ca²⁺ store content. In addition, we present simulations to show that the restricted diffusion of InsP₃ in cytoplasm implies that the source of InsP₃ generation, PIP₂, needs to be dispersed through the egg interior to account for PLCζ induced rapid Ca²⁺ waves. Finally, we provide further evidence that PIP₂ is present on intracellular vesicles in eggs and that this is required for PLCζ and sperm induced Ca²⁺ oscillations in eggs.

MATERIALS AND METHODS

Handling and Microinjection of Mouse Eggs

MF1 mice between 6 and 8 weeks of age were injected with pregnant mare's serum gonadotrophin (PMSG, Intervet) followed by human chorionic gonadotrophin (hCG, Invervet) ~50 h later (Fowler and Edwards, 1957). Eggs were collected from these mice 15 h after HCG injection, from the dissected ovaries. All animal handling and procedures were carried out under a UK Home Office License and approved by the Animal Ethics Committee at Cardiff University. Once collected, the eggs were kept at 37°C in M2 media (Sigma Aldrich). All Ca²⁺ dyes and intracellular probes were introduced into the cytosol of the eggs using a high pressure microinjection system with the eggs maintained in M2 media throughout (Swann, 2013). For *in vitro* fertilization sperm was collected from the epididymis of F1 C57/CBA hybrid male mice. The sperm were isolated in T6 media containing 16 mg/ml bovine serum albumin (BSA, Sigma Aldrich) and left to capacitate for 2–3 h before adding to eggs (Yu et al., 2012).

Measurements and Analysis of Intracellular Ca²⁺ and InsP₃ Uncaging

In all experiments cytosolic Ca²⁺ was measured using fluorescent Ca²⁺ indicator Oregon Green BAPTA dextran (OGBD) (Life Technologies). OGDB was diluted in a KCl HEPES buffer (120 mM KCl, 20 mM HEPES at pH 7.4) so that the injection solution contained 0.33 or 0.5 mM OGDB. The OGDB mix was microinjected into eggs using high pressure pulses. In those eggs that were stimulated by adenophostin this was microinjected into eggs along with the OGDB. In this case instead of mixing the OGDB with KCL HEPES it was mixed with KCL HEPES containing 5 μM adenophostin in the same quantities. Where PLC ζ cRNA was used this was microinjected alongside OGDB in the same way at a concentration of 0.02 μg/μl. For imaging, eggs were then transferred to a glass-bottomed dish, containing HKSOM media, on an epifluorescence imaging system (Nikon TiU) attached to a cooled CCD camera as described previously (Swann, 2013). Ca²⁺ dynamics were measured using the time-lapse imaging mode of Micromanager software (<https://micromanager.org/>) where an image was captured every 10 s. Where IVF was performed, or drugs were later added to the eggs, the zona pellucidae were removed from the eggs using acid Tyrodes treatment prior to imaging. For those experiments that required InsP₃ stimulation, NPE-caged-InsP₃ (1 mM in the pipette) from ThermoFisher Scientific was microinjected prior to imaging at the same time as the injection of fluorescent dye (OGDB). In order to photo-release InsP₃ the eggs were exposed to an electronically gated UV LED light source (365 nm, Optoled Lite, Cairn Research Ltd) that was positioned just above the dish containing the eggs. The duration of the UV pulse was controlled by a time gated TTL pulse that was delivered in between two successive fluorescence acquisitions. All data measuring Ca²⁺ dynamics were recorded as .tif files using the Micromanger software on the epifluorescence system.

Media, Chemicals, and Drugs

M2 media was purchased from Sigma Aldrich as a working solution. HKSOM was made up to pH 7.4, in cell culture grade water as follows: 95 mM NaCl, 0.35 mM KH₂PO₄, 0.2 MgSO₄, 2.5 mM KCl, 4 mM NaHCO₃, 20 mM HEPES, 0.01 mM EDTA, 0.2 mM Na Pyruvate, 1 mM L-glutamine, 0.2 mM glucose, 10 mM Na Lactate 1.7 mM CaCl₂, 0.063 g/l Benzylpenicillin, and 10 mg/l phenol red. Ca²⁺ free media was made in the same way as HKSOM however CaCl₂ was not added and the media was supplemented with 100 μM EGTA. Sr²⁺ containing media was made in the same way as HKSOM however, instead of adding 1.7 mM CaCl₂, 10 mM SrCl₂ was added instead.

All drugs and chemicals used, unless otherwise mentioned, were purchased from Sigma Aldrich. Propranolol was used at a working concentration of 300 μM in HKSOM media. A stock of 300 mM propranolol was made up in DMSO which was then diluted 1:100 in HKSOM media. Then 100 μl of this solution was pipetted into the imaging dish containing 900 μl of standard HKSOM. Propranolol was removed by washing out this media and replacing it with fresh HKSOM media using a perfusion system that passed 10 ml of clean HKSOM through the dish containing the eggs to ensure sufficient wash out. In a similar way a stock of 5 mM thapsigargin in DMSO was diluted 1:1,000 to a concentration of 5 μM in HKSOM and then 100 μl of this thapsigargin solution was added to the imaging dish containing 900 μl of HKSOM to give a working concentration of 500 nM of thapsigargin.

Confocal Imaging

In those eggs that were microinjected with PBP10, a solution of 1 mM PBP10 (Tocris Biosciences, UK) was made up in KCl HEPES and ~4–10 pl of this solution was microinjected into each egg. Following PBP10 microinjection, eggs were imaged on a Leica SP2 Confocal (Leica, Wetzler, Germany) microscope using a Helium-Neon laser (543 nm) at 30% intensity. Eggs were imaged in M2 media using a x63 oil objective and a pinhole aperture of 91 nm. Images were acquired with a line averaging of 8 and a resolution of 1,058 × 1,058 pixels. For each egg a single z-stack image of (1 μm depth) was captured of an equatorial slice through the egg. All images were exported as .tif files and analyzed using Image J (<https://imagej.nih.gov/ij/>).

Data Analysis

Quantitative data measuring the Ca²⁺ dynamics of the eggs on the widefield imaging system was extracted from .tif stacks using Image J (<https://imagej.nih.gov/ij/>). Background fluorescence was first subtracted from the egg fluorescence value. These fluorescence values were then normalized by dividing each fluorescence value in the egg by the baseline fluorescence value at the start of the imaging run to provide a relative change in fluorescence (F/F₀) that could be plotted against time. These traces were produced and analyzed using SigmaPlot 12. The Confocal images were also analyzed using Image J software. PIP₂ positive vesicle size and distribution was calculated using the particle analysis function on Image J and a nearest neighbor distance (Nnd) plugin in Image J. A bandpass filter function was applied to the images (large objects were filtered down to

40 pixels and small ones enlarged to 3 pixels). The threshold was altered to between 2 and 5% so only the fluorescence of the vesicles inside the image of the egg were included in the analysis. The particle analysis function was applied and configured so it recorded area, integrated intensity and coordinates for each fluorescent vesicle in the egg. These areas were used to work out the radius and diameter of the vesicles. The coordinates were fed into a nearest distance neighbor plugin (https://icme.hpc.msstate.edu/mediawiki/index.php/Nearest_Neighbor_Distances_Calculation_with_ImageJ) to give the mean distance between the vesicles. The total fluorescence of the vesicles was calculated by adding all the integrated intensity readings for a single egg which was carried out using the measure tool in ImageJ and background fluorescence values were subtracted. Statistical analysis was carried out using SigmaPlot 12. If not stated otherwise the data is presented as the mean and standard errors of the mean. Shapiro-Wilk tests for normality and tests for equal variances were conducted prior to carrying out group comparison tests. If the data passed both these tests a Student's *T-test* was conducted. If the data failed either or both of these tests a Mann-Whitney *U-test* was conducted instead.

Mathematical Method of Ca²⁺ Waves

The model and associated parameter values are based on the work of (Politi et al., 2006; Theodoridou et al., 2013). The reaction-diffusion equations define the interactions between free cytosolic calcium, *u*; stored calcium, *v*; and IP₃, *p*,

$$\frac{du}{dt} = d\nabla^2 u + A - D \frac{u^{ed}}{u^{ed} + u_d^{ed}} \left(1 - \frac{p^{es}}{p^{es} + p_s^{es}} \right) + K(u, v, p), \quad (1)$$

$$\frac{dv}{dt} = d\nabla^2 v - K(u, v, p)S(x, y, L_0), \quad (2)$$

$$\frac{dp}{dt} = d\nabla^2 p + \epsilon + PLC \frac{u^{ep}}{u^{ep} + u_p^{ep}} S(x, y, L) - rp, \quad (3)$$

$$K(u, v, p) = -B \frac{u^{eb}}{u^{eb} + u_b^{eb}} + C \frac{v^{ec}}{v^{ec} + v_c^{ec}} \frac{p^{epc}}{p^{epc} + p_c^{epc}} \frac{u^{epa}}{u^{epa} + u_{pa}^{epa}} \left(1 - \frac{u^{epi}}{u^{epi} + u_{pi}^{epi}} \right) - Ev. \quad (4)$$

$$S(x, y, L) = \begin{cases} 1 & \text{if } \left(\frac{x}{L} - \lfloor \frac{x}{L} \rfloor \right) < \frac{L_{on}}{L} \text{ and } \left(\frac{y}{L} - \lfloor \frac{y}{L} \rfloor \right) < \frac{L_{on}}{L}, \\ 0 & \text{Otherwise.} \end{cases} \quad (5)$$

The equations represent interactions in which free Ca²⁺ acts as a self-inhibitor but, along with InsP₃ and stored Ca²⁺, stimulates the release of stored Ca²⁺, creating a system that can produce oscillations in the concentrations of calcium and InsP₃. Critically, all species are able to diffuse with the same diffusion coefficient, *d*.

The actions of the stored Ca²⁺ and the InsP₃ only occur in discrete regions. This spatial discreteness is controlled by the repeating function *S*(*x*, *y*, *L*). Essentially, the function *S*(*x*, *y*, *L*)

creates a regular grid of squares of size *L_{on}* × *L_{on}* in which the specified kinetics are active. We are then able to alter the wavelength, or separation distance, *L*, between these active regions.

The equations were simulated using a finite element Runge-Kutta method on a two-dimensional disk of diameter 70 μm, which was discretised into 6,550 elements. The 2D assumption is considered valid because any dilution effects of going to three dimensions are offset equally by an increase in the third dimension production. The two-dimensional simulations can be thought of a single slice through a cell and it offers speed, clarity and insight. Finally, the boundary was specified to have a zero-flux condition, meaning that no substances were able to leak out of the domain. This is a simplification considered valid since it is known that PLCζ induced Ca²⁺ spikes can be generated in mouse eggs where no membrane Ca²⁺ fluxes occur (Miao et al., 2012). The equations are accompanied by the parameter values specified in Table 1, where all unit dimensions are chosen to make *u*, *v*, and *p* have units of μMol, space is in μm and time is in seconds. The initial conditions for all populations were at steady state except for a small perturbation of a two-dimensional Gaussian profile at the point (20,20), in the free Ca²⁺ population.

TABLE 1 | Parameter values for Equations (1)–(5).

Parameter	Value	Definition
<i>A</i>	0.25	Calcium source
<i>B</i>	200	Strength of calcium induced calcium degradation
<i>C</i>	3,125	Calcium release depending on all forms of calcium and IP ₃
<i>D</i>	7.5	Strength of IP ₃ blocking calcium degradation
<i>E</i>	0.00125	Calcium leakage
<i>PLC</i>	100	Strength of calcium induced IP ₃ release
<i>ε</i>	0.001	IP ₃ source
<i>r</i>	10	IP ₃ degradation
<i>d</i>	10	Diffusion rate
<i>u_d</i>	0.5	Calcium degradation sensitivity to calcium
<i>ed</i>	2	Hill coefficient
<i>p_s</i>	0.1	Calcium degradation sensitivity to IP ₃
<i>es</i>	3	Hill coefficient
<i>u_p</i>	0.025	IP ₃ production sensitivity to calcium
<i>ep</i>	4	Hill coefficient
<i>u_b</i>	2.25	Calcium degradation sensitivity to calcium
<i>eb</i>	2	Hill coefficient
<i>v_c</i>	9	Calcium release sensitivity to stored calcium
<i>ec</i>	2	Hill coefficient
<i>u_{pa}</i>	0.45	Calcium release sensitivity to cytosolic calcium
<i>e_{pa}</i>	4	Hill coefficient
<i>u_{pi}</i>	1	Calcium release sensitivity to cytosolic calcium
<i>e_{pi}</i>	5	Hill coefficient
<i>p_c</i>	0.1	Calcium release sensitivity to IP ₃
<i>e_{pc}</i>	2	Hill coefficient
<i>L₀</i>	1.5	Calcium store spacing

All unit dimensions have been chosen to make *u*, *v*, and *p* have units of μMol, space is in μm and time is in seconds.

RESULTS

PLC ζ and Sr²⁺ Trigger Ca²⁺ Oscillations in Eggs via Different Mechanisms

We investigated the mechanism generating Ca²⁺ oscillations by using photo-release of caged InsP₃ that was microinjected into mouse eggs. In initial experiments we uncaged InsP₃ in unfertilized (control) mouse eggs that were not undergoing any Ca²⁺ oscillations. **Figure 1A** shows that UV pulses of light from 50 ms through to 2 s generated Ca²⁺ increases with the amplitudes that were larger with longer duration pulses. With the protocol we used there was adequate amounts of caged InsP₃ for multiple releases of InsP₃ even with longer duration pulses of UV light as illustrated by **Figure 1B** which shows that 3 s pulses could generate repeated large rises in Ca²⁺ in control eggs. We then tested the effects of triggering such pulses during Ca²⁺ oscillations induced by either Sr²⁺ media or by PLC ζ injection. **Figure 1C** shows that when a 100 ms pulse was used in eggs injected with PLC ζ the uncaging of InsP₃ caused no

Ca²⁺ increase. In contrast, **Figure 1D** shows Ca²⁺ oscillations occurring in response to Sr²⁺ media and in such eggs there was a rapid and large Ca²⁺ transient every time a pulse of just 100 ms was used to uncage InsP₃. Since the response to 100 ms pulses of UV were minimal in control eggs (**Figure 1A**) these data show that Sr²⁺ media sensitizes eggs to InsP₃ induced Ca²⁺ release and that, in contrast, IP3R are not sensitized to InsP₃ by PLC ζ injection.

The two classes of model for Ca²⁺ oscillations, those that involve the dynamic properties of IP3Rs and those that involve InsP₃ production oscillations, can be distinguished in a definitive manner by examining the response to a sudden pulse of InsP₃ (Sneyd et al., 2006). Models that are dependent upon IP3R kinetics alone respond to a pulse of InsP₃ by showing a transient increase in the frequency of Ca²⁺ oscillations (Sneyd et al., 2006). In contrast, models that depend on Ca²⁺ induced InsP₃ production, and imply InsP₃ oscillations, respond to a sudden increase in InsP₃ by showing an interruption of the oscillations which leads to a resetting of the phase of oscillations

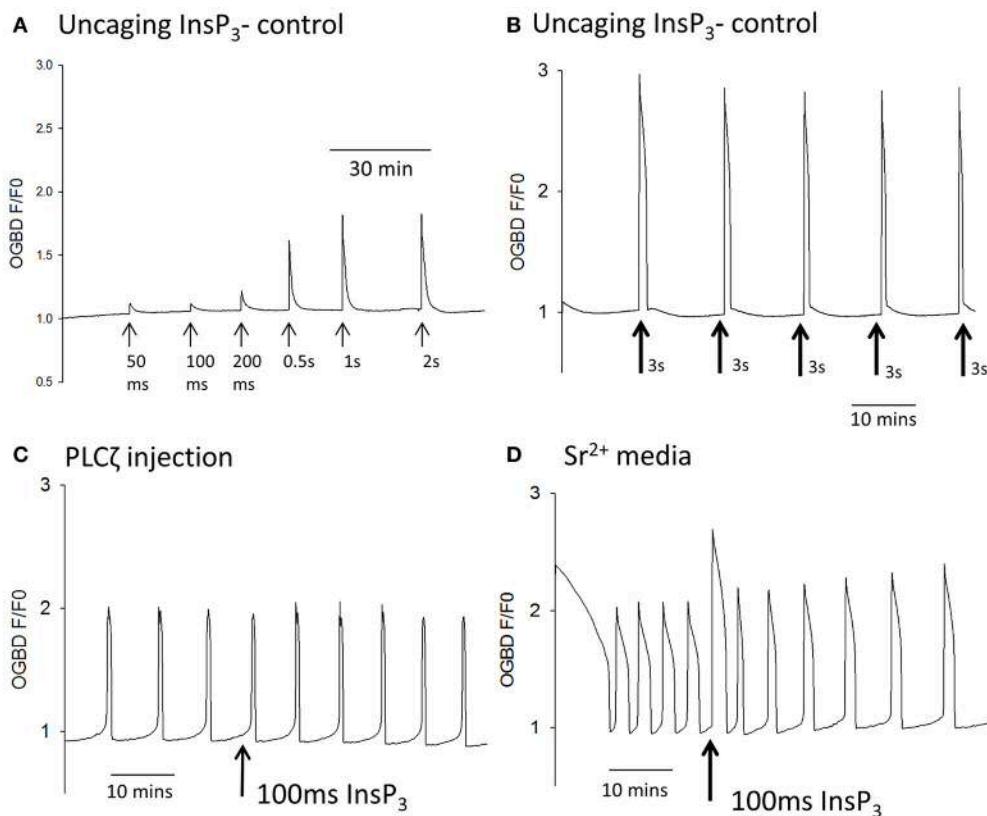


FIGURE 1 | Ca²⁺ oscillations and uncaging pulses of InsP₃. In **(A)** an example trace is shown of Ca²⁺ increases (as measured by OGBD fluorescence) in an egg in response to different amounts of InsP₃. Eggs were injected with caged InsP₃ and exposed to varying durations of UV light pulse (from 50 ms to 2 s) to photo-release the InsP₃ (trace typical of $n = 21$ eggs). In this and all other traces shown, the pulses were applied at points indicated by the arrows. In **(B)** an example trace is shown of changes in cytosolic Ca²⁺ in an egg in response to the “uncaging” of caged InsP₃ using long duration UV pulses of 3 s. Arrows indicate where pulses of UV light were applied (typical of $n = 7$ eggs). In **(C)** an example trace is shown of changes in cytosolic Ca²⁺ in an egg stimulated following the microinjection of mouse derived PLC ζ cRNA (0.02 μ g/ μ l) and caged InsP₃. The arrow indicates where a 100 ms pulse of UV light was applied ($n = 14$ eggs). In all 14/14 such recordings there was no sudden increase in Ca²⁺ even when the pulse was applied during the pacemaker rising phase of Ca²⁺. In **(D)** an example trace is shown with changes in cytosolic Ca²⁺ in an egg stimulated by media containing 10 mM Sr²⁺. The arrow indicates where a 100 ms pulse of UV light was applied to uncage InsP₃ ($n = 32$ eggs). In all 32/32 cases there was a rapid Ca²⁺ increase that started with the very next OGBD fluorescence measurement after the UV pulse (<10 s).

(Sneyd et al., 2006). We tested the effect of using large uncaging pulses of InsP₃ on Sr²⁺ induced, or PLCζ induced, Ca²⁺ oscillations in mouse eggs. **Figure 2A** shows that during Sr²⁺ induced oscillations a 3 s uncaging pulse of InsP₃ caused a large increase in Ca²⁺ followed by a significant increase in the frequency of Ca²⁺ oscillations. In contrast, with PLCζ induced Ca²⁺ oscillations, **Figure 2B** shows that the same 3 s uncaging pulse of InsP₃ did not cause any increase in frequency, but interrupted the periodicity of oscillations leading to a delay before the next Ca²⁺ increase. To confirm that this phenomenon was phase resetting, we plotted the shift in phase (PS) caused by uncaging of InsP₃ against the time delay (dt) of the InsP₃ pulse from the subsequent Ca²⁺ spike (see **Figure 2C**). Each of these values was divided by the time period T in order to take into account the different frequency of Ca²⁺ oscillations in each egg. With phase resetting this plot should give a line from 1 to 1 on each axis, and **Figure 2D** shows that the data from 23 PLCζ injected eggs exposed to uncaging pulses of InsP₃ fit closely on such a line. These data clearly show that a pulse of InsP₃ causes phase resetting of Ca²⁺ oscillations in mouse eggs, which is completely different from that seen with Sr²⁺ induced oscillations. Hence, overall the data suggest

that PLCζ and Sr²⁺ media trigger Ca²⁺ oscillations in mouse eggs via fundamentally different mechanisms. Sr²⁺ stimulates IP3Rs to make them effectively more sensitive to InsP₃, and that PLCζ induced Ca²⁺ oscillations involve Ca²⁺ stimulated InsP₃ production where InsP₃ acts as a dynamic variable that should oscillate in synchrony with Ca²⁺ oscillations.

Cytosolic Ca²⁺ vs. Ca²⁺ Stores and the Frequency of Ca²⁺ Oscillations

Since Ca²⁺ release and InsP₃ formation are predicted to form part of a positive feedback loop we decided to re-investigate some observation previously made on Ca²⁺ oscillations in eggs. One finding made in hamster and mouse eggs is that both sperm (and PLCζ)-triggered Ca²⁺ oscillations “run down” and can cease entirely in Ca²⁺ free media (Igusa and Miyazaki, 1983; Lawrence and Cuthbertson, 1995). This phenomena has been explained in terms of Ca²⁺ store depletion but the level of cytosolic Ca²⁺ and its effect on InsP₃ production could also be important. We re-examined the role of Ca²⁺ stores and resting Ca²⁺ using the SERCA inhibitor thapsigargin. Previous studies used high concentrations (>10 μM) of thapsigargin to completely block Ca²⁺ oscillations in eggs (Kline and Kline,

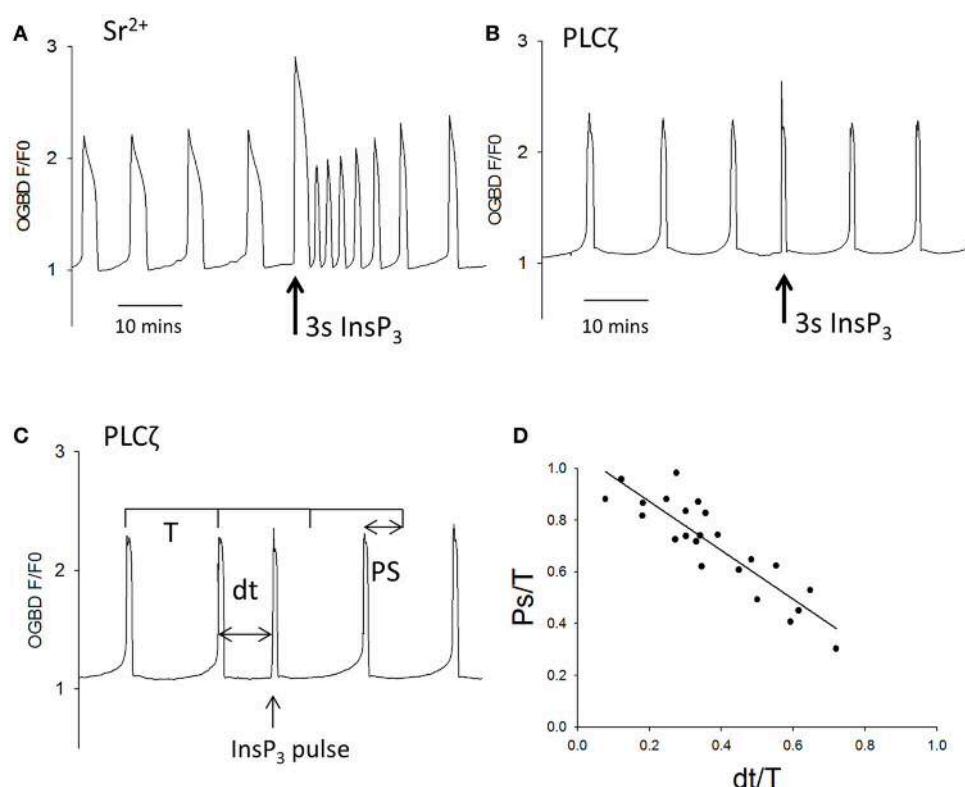


FIGURE 2 | The effect of large pulses of InsP₃ on PLCζ or Sr²⁺ triggered Ca²⁺ oscillations. **(A)** shows an example of the way eggs responded a large uncaging pulse of InsP₃ (3 s UV light at the arrow) by an increase in the frequency of Sr²⁺ triggered Ca²⁺ oscillations ($n = 20$ eggs). There was a significant increase in frequency of Ca²⁺ spikes from 3.45 (± 0.27 sem) in 20 min to 5.05 (± 0.35 sem) in 20 min ($p < 0.001$). In **(B)** a similar experiment is shown but with PLCζ induced Ca²⁺ oscillations. The sample trace in **(B)** shows that a 3 s uncaging pulse of InsP₃ (at the arrow) caused an immediate Ca²⁺ increase but no increase in frequency ($n = 23$ eggs). We analyzed the ability of such pulses to reset the phase of oscillations by measuring then phase shift (PS) and comparing it to time delay (dt) at which the InsP₃ pulse was applied. **(C)** Illustrates how these values were measured on an actual sample trace. Each value was divided by the time-period (T) for the oscillations in order to normalize the values between different eggs. **(D)** Shows a plot of these values for all 23 eggs tested.

1992b). To investigate the role of Ca²⁺ store content we used much lower concentrations of thapsigargin which caused only a small elevation of cytosolic Ca²⁺. Figures 3A,B show that the

addition of 500 nM thapsigargin to mouse eggs caused a small and prolonged increase in resting cytosolic Ca²⁺ in normal media and Ca²⁺ free media, which is consistent with a slight inhibition

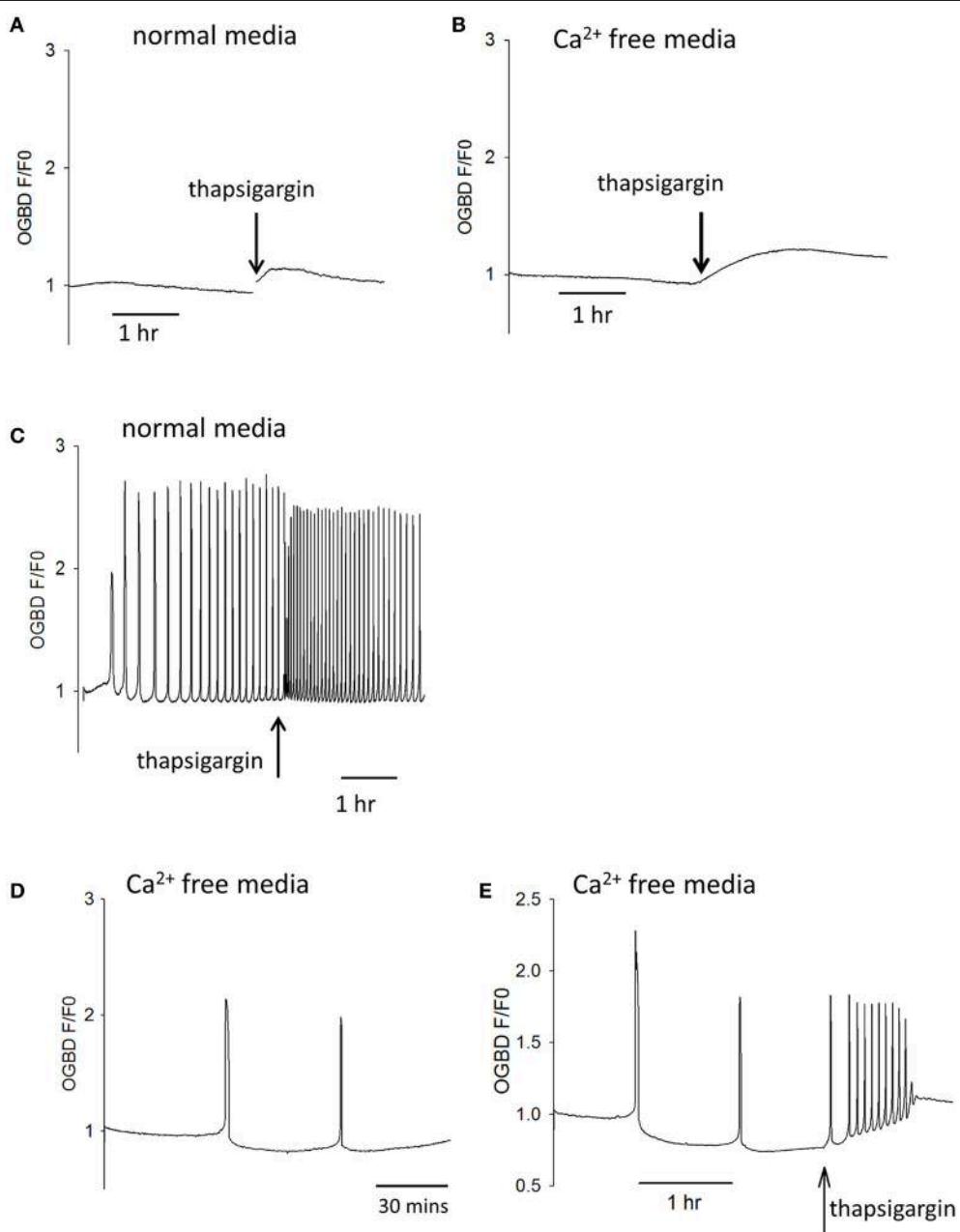


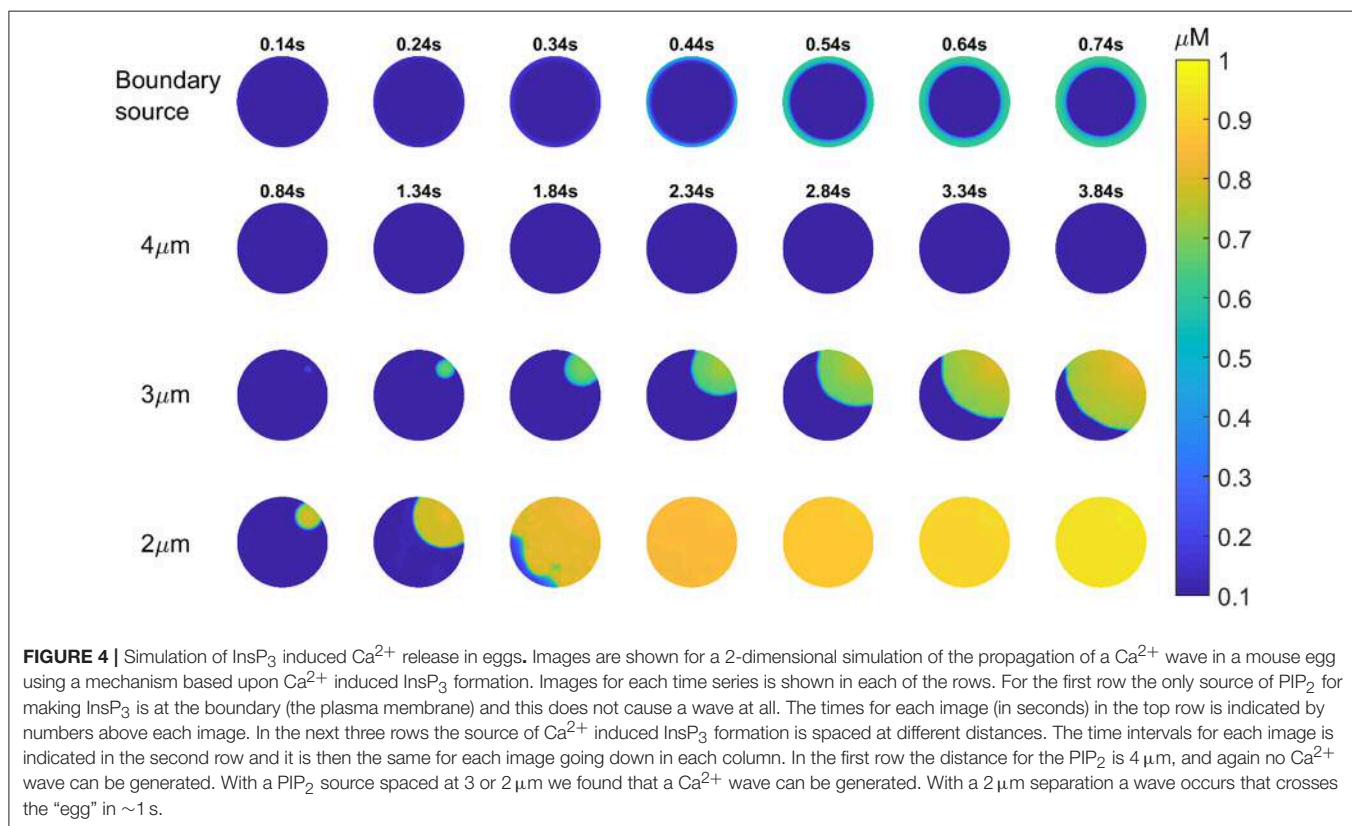
FIGURE 3 | Cytosolic Ca²⁺ and the frequency of Ca²⁺ oscillations. In (A) an example is shown of a trace showing changes in cytosolic Ca²⁺ in an egg incubated in normal HKSOM media following the addition of 500 nM thapsigargin (typical of $n = 12$ eggs). The increase in Ca²⁺ was from 0.936 ± 0.013 SEM to 1.07 ± 0.019 SEM which is a significant ($P < 0.01$). In (B) a similar example is shown of the addition 500 nM thapsigargin to an egg in Ca²⁺ free media (containing 100 μM EGTA), which was typical of $n = 12$ eggs. The increase in basal Ca²⁺ was from 0.908 ± 0.0134 SEM up to 1.29 ± 0.0168 SEM which was significant ($P < 0.001$). In (C) an example of one of 6 eggs is shown where the same low concentration of thapsigargin increased the frequency of Ca²⁺ oscillations by 1.72-fold (± 0.07 SEM). In (D) a trace is shown from an egg that was injected with PLC ζ RNA and then placed in Ca²⁺ free media. The mean number of Ca²⁺ spikes in such experiments was 1.56 ($n = 18$ eggs, ± 0.31 SEM) Ca²⁺ spikes in 10,000 s (2 h 47 min) h. The Ca²⁺ levels decreased to 0.84 ± 0.029 SEM which was significantly less than the starting level ($P < 0.001$). In (E) is shown an example of an egg that had been injected with PLC ζ RNA and then placed in Ca²⁺ free media as in (C). However, in these experiments 500 nM thapsigargin was added after >2 h. In 16/17 such treated eggs there was an increase in the frequency of Ca²⁺ oscillations. There were an average of 1.77 spikes (± 0.18 SEM) before adding thapsigargin but a mean of 7.11 spikes (± 1.3 SEM) after thapsigargin addition. The resting Ca²⁺ level increased from 0.84 ± 0.029 SEM, before adding thapsigargin to 1.076 ± 0.017 SEM in eggs where it stabilized. This is a significant increase in Ca²⁺ concentration ($P < 0.001$).

of SERCA pumps. When the same concentration of thapsigargin was added to eggs undergoing Ca²⁺ oscillations in response to PLC ζ there was a marked acceleration of Ca²⁺ oscillations, and a reduction in the amplitude of Ca²⁺ spikes (**Figure 3C**). Similar to previous reports, we found that the pattern of PLC ζ induced Ca²⁺ oscillations show a run down in Ca²⁺ free media (containing EGTA). We noted that this was associated with a decline in the fluorescence of OGBD, suggesting that resting Ca²⁺ levels were also undergoing a decline (**Figure 3D**). When low concentrations of thapsigargin (500 nM) were added to PLC ζ injected eggs in Ca²⁺ free media there was a restoration of Ca²⁺ oscillations (**Figure 3E**). It is noteworthy that in **Figure 3E** the eggs were in Ca²⁺ free media and yet the addition of thapsigargin, which would cause further Ca²⁺ store depletion, actually leads to a restoration of Ca²⁺ oscillations. Nevertheless, the restoration of Ca²⁺ oscillations was associated with a rise in the “basal” Ca²⁺ level (**Figure 3E**). These data are consistent with the idea that cytosolic Ca²⁺ plays a key role in triggering each Ca²⁺ rise, and that Ca²⁺ stores are not significantly depleted in mouse eggs by incubation in Ca²⁺ free media.

PLC ζ Induced Ca²⁺ Oscillations and Intracellular PIP₂

Previous studies of fertilizing mouse and hamster eggs show that most Ca²⁺ waves cross the egg in about 1 s, and propagate through the cytoplasm at speeds in excess of 50 $\mu\text{m}/\text{s}$. This matches the rising phase of (all but the initial) Ca²⁺ transients in mouse eggs which is ~ 1 s after fertilization or after PLC ζ protein

injection (Deguchi et al., 2000). Since data in **Figure 2** implies that the upstroke of each Ca²⁺ rise involves an InsP₃ and Ca²⁺ positive feedback loop, then it is necessary for both molecules to be sufficiently diffusible. The Ca²⁺ stores (the endoplasmic reticulum) are spread across the egg. However, this may not be the case with PIP₂ that is the precursor to InsP₃. In most cells PIP₂ is in the plasma membrane, and if this is used in Ca²⁺ waves in eggs then InsP₃ diffusion range might constrain the ability to generate fast Ca²⁺ waves. Recently, the diffusion coefficient of InsP₃ in intact cells has been shown to be $<10 \mu\text{m}^2/\text{s}$ which means that InsP₃ may only diffuse $<5 \mu\text{m}$ in 1 s (Dickinson et al., 2016). We have previously presented models of Ca²⁺ oscillations based upon Ca²⁺ induced InsP₃ formation and InsP₃ induced opening of Ca²⁺ release channels (Theodoridou et al., 2013). We have now simulated the Ca²⁺ waves in mouse eggs using a similar set of equations in a two-dimensional model of the Ca²⁺ wave. **Figure 4** shows that with the source of Ca²⁺ stimulated InsP₃ production at the periphery (plasma membrane) it is not possible to generate a Ca²⁺ wave through the egg cytoplasm, and only a concentric pattern of Ca²⁺ release occurs. We previously presented evidence for PIP₂ being present in intracellular vesicles spread throughout the cytoplasm in mouse eggs (Yu et al., 2012). These could provide a source of InsP₃ that might carry a Ca²⁺ wave through the cytoplasm if they are sufficiently dispersed. In **Figure 4** we show simulations based upon Ca²⁺ induced InsP₃ generation where the PIP₂ is dispersed on vesicles at different distances apart (from 2 to 4 μm). Our simulations show that when the PIP₂ vesicles are within 2 or 3 μm of each other a



rapid Ca²⁺ can be generated, but that once the PIP₂ is more than 3 μm the Ca²⁺ increase fails to occur. These results suggests that PIP₂ needs to be present on vesicles spaced <3 μm apart in the cytoplasm in order to propagate a rapid Ca²⁺ wave of the type seen in fertilizing and PLC ζ injected eggs.

Previous evidence for the existence of PIP₂ within the cytoplasm of eggs came from studies using antibodies to PIP₂ (Yu et al., 2012). Gelsolin is a protein that has been shown to bind to PIP₂, and contains a short peptide sequence responsible for PIP₂ binding (Cunningham et al., 2001). We injected mouse eggs with PBP-10, which is a probe in which rhodamine is coupled to a gelsolin peptide that binds PIP₂. **Figure 5A** shows a mouse egg injected with PBP-10. After >1 h the fluorescence of PBP-10

could be predominantly seen in many small vesicles throughout the egg cytoplasm, with the occasional larger aggregate. This supports the hypothesis that PIP₂ is localized in vesicles within mouse eggs (Yu et al., 2012). Further examination of these vesicles using particle analysis indicates that they are distributed throughout the whole egg cytoplasm. Interestingly, following nearest neighbor analysis, we found that these vesicles were ~2 μm apart (**Figures 5A,D**). This suggests that these PIP₂ containing vesicles are within the correct distance predicted to produce the rapid rising phase of 1 s for each wave as predicted by our mathematical modeling.

We have previously sought to modify the level of PIP₂ in mouse eggs using various phosphatases, but without success.

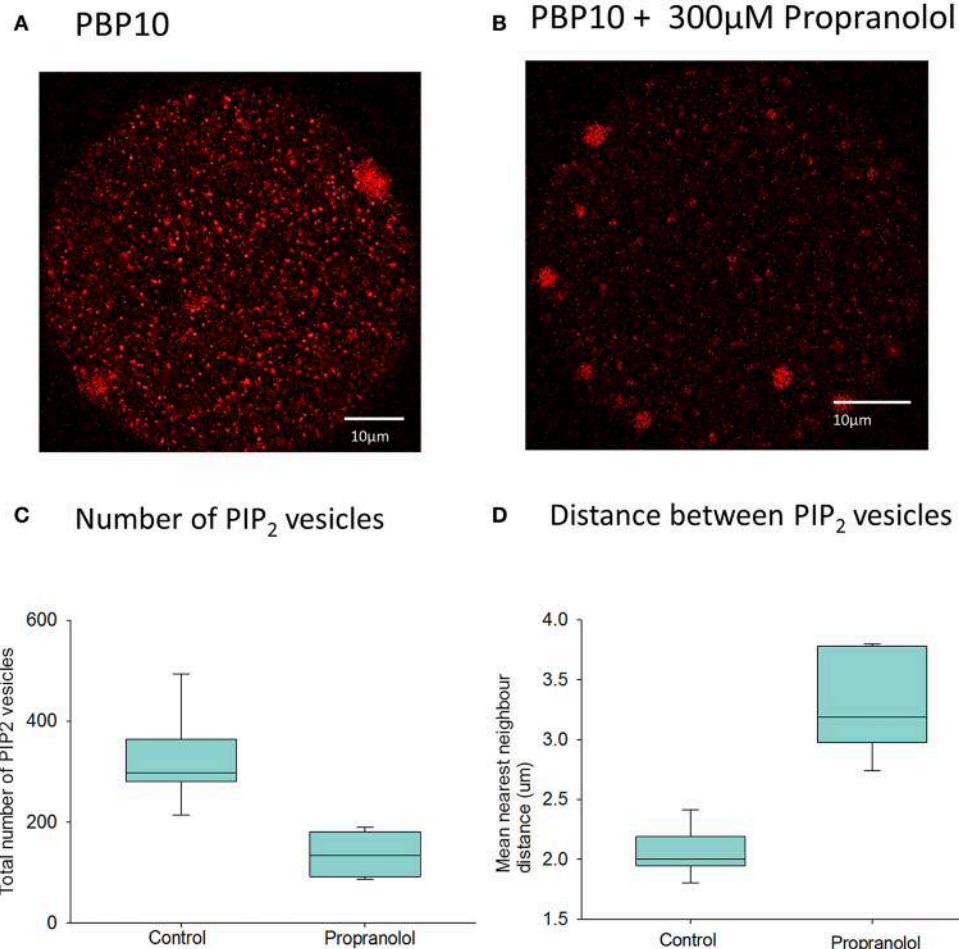


FIGURE 5 | PIP₂ distribution in mouse eggs using PBP10. In **(A)** an example is shown of the distribution of fluorescence of PBP10 in a mouse egg 1.5 h after injection of PBP10 ($n = 21$). Scale bars are 10 μm. **(A)** nearest neighbor analysis indicated that the mean vesicle distance for all 21 control eggs is 2.2 μm. In **(B)** an example is shown of an egg injected with PBP10 where and incubated in media with 300 μM propranolol ($n = 13$). In **(C)** particle analysis ($n = 14$ eggs) indicates that the mean vesicle diameter is 0.89 μm and the mean number of vesicles present per egg is 298.9. **(C)** Shows a plot of the total number of PIP₂ positive vesicles present in eggs following injection of PBP10 using particle analysis. Results are shown for both eggs incubated in standard M2 media (control) (mean number of vesicles = 324, $n = 7$) and those incubated in M2 containing 300 μM propranolol during imaging (mean number of vesicles = 131, $n = 7$). There is a significant reduction in the number of PBP10 vesicles following propranolol treatment compared to control media ($p = < 0.001$, Student's *T*-test). **(D)** shows a plot of the mean nearest neighbor distances of PIP₂ positive vesicles present in eggs. The results are shown for parallel groups of eggs incubated in standard M2 media (control) (mean distance = 2.0 μm, $n = 7$) and for those incubated in M2 containing 300 μM propranolol during imaging, (mean distance = 3.3 μm, $n = 7$). A Mann–Whitney *U*-test showed a significant increase in the distance between the PBP10 vesicles following propranolol treatment compared to control media ($p = < 0.001$).

Internal membranes in somatic cells do not in general contain much PIP₂, but one organelle where PIP₂ and DAG have been reported in some cells is the Golgi apparatus. In mature mammalian eggs, like mitotic cells, the Golgi is fragmented into small vesicles (Moreno et al., 2002; Axelsson and Warren, 2004). It has been shown that propranolol blocks DAG synthesis in Golgi membranes and leads to a loss of Golgi structure (Asp et al., 2009). We applied propranolol to mouse eggs injected with PBP10 and found a marked loss of staining (**Figure 5B**). Further particle analysis showed that the mean number of these PIP₂ vesicles was significantly reduced following the addition of propranolol (**Figure 5C**). Furthermore, the distance of these vesicles from each other was significantly increased in those eggs treated with propranolol (**Figure 5D**). The overall total fluorescence of the vesicles was seen to reduce by approximately half from a mean of 5.77×10^4 RFU ($n = 7$) in control eggs to a mean of 2.93×10^4 RFU ($n = 7$) in those eggs treated with propranolol. This difference was significant following a Student's *T*-test ($p = 0.006$). This implies that propranolol is affecting PIP₂ levels in cytoplasmic vesicles.

Since propranolol appears to reduce PIP₂ inside eggs, we investigated the effect of propranolol on Ca²⁺ oscillations. **Figure 6A** shows that propranolol addition to eggs undergoing Ca²⁺ oscillations in response to fertilization by IVF were rapidly blocked. **Figure 6B** shows the same effect of propranolol on those eggs stimulated by PLC ζ cRNA. The inhibition by propranolol was associated with a slight decline in Ca²⁺ levels and the inhibition was reversed upon removal of propranolol (**Figure 6C**). However, whilst it blocked sperm and PLC ζ induced responses, propranolol did not block Ca²⁺ oscillations induced in eggs by Sr²⁺ media, or by injection of the IP3R agonist adenophostin (**Figures 6D,E**). These data show that the inhibitory effects of propranolol are both reversible and specific to PLC ζ and sperm induced Ca²⁺ oscillations. They support the proposal that PIP₂ in vesicles in the cytoplasm of mouse eggs is important for the generation of PLC ζ induced Ca²⁺ oscillations.

DISCUSSION

The Ca²⁺ oscillations seen in mammalian eggs at fertilization have distinct characteristics compared with those seen in somatic cell types (Dupont and Goldbeter, 1994; Politi et al., 2006). The oscillations at fertilization are low frequency, and long lasting, but they have a very rapid rising phase that occurs throughout the whole cytoplasm of a very large cell, in less than a second. Considerable evidence suggests that PLC ζ is the primary stimulus for these Ca²⁺ oscillations (Saunders et al., 2002). The current data shows that PLC ζ induced Ca²⁺ oscillations are driven by Ca²⁺ induced InsP₃ formation. In contrast, we show that Sr²⁺ media sensitizes eggs to InsP₃ induced Ca²⁺ release. Hence, there are at least two different mechanisms for generating Ca²⁺ oscillations in mouse eggs. Our data also implies that the substrate of PLC ζ , PIP₂, needs to be localized in a finely distributed source within the egg in order to generate fast Ca²⁺ wave, and we present evidence

that such vesicular PIP₂ is required for PLC ζ induced Ca²⁺ oscillations.

There are two fundamentally different classes of models for InsP₃ induced Ca²⁺ oscillations in cells. One relies on the properties of InsP₃ receptor and implies that stimulation involves an elevated but monotonic or constant elevation of InsP₃ levels. The other involves a positive feedback model of InsP₃ induced Ca²⁺ release and Ca²⁺ induced InsP₃ formation. It is possible to determine which one of these two model types applies by studying the Ca²⁺ responses after triggering a large pulsed release of InsP₃ (Sneyd et al., 2006). The IP3R based models respond to a pulse of InsP₃ by temporarily increasing the frequency of Ca²⁺ oscillations, whereas the Ca²⁺-induced InsP₃ formation models show an interruption in the series of Ca²⁺ transients with a resetting of the phase of the oscillations (Sneyd et al., 2006). We previously presented preliminary evidence for an interruption in the series transients with sperm or PLC ζ induced Ca²⁺ oscillations responding to a pulse of InsP₃ (Swann and Yu, 2008). We now show that the response of PLC ζ induced Ca²⁺ oscillations to a sudden large pulse of InsP₃ is clearly characterized by a resetting of the phase of oscillations. This means that InsP₃ has to be a dynamic variable in the oscillation cycle and that it will undergo oscillations in close phase with the oscillations in Ca²⁺. Small oscillations in InsP₃ have been recorded previously in response to high frequency Ca²⁺ oscillations achieved with high concentrations of PLC ζ (Shirakawa et al., 2006). The sensitivity of such indicators may be limited since we can now assert that InsP₃ oscillations should occur with all the PLC ζ induced Ca²⁺ oscillations and, most significantly, that increased InsP₃ production plays a causal role in generating each Ca²⁺ rise. We have also shown here that Sr²⁺ works via an entirely different mechanism in mouse eggs. The increase in frequency of Ca²⁺ oscillations caused by uncaging InsP₃ indicates that Sr²⁺ induced oscillations rely on the properties of the IP3R. This is supported by the finding that Sr²⁺ media sensitized mouse eggs to InsP₃ pulses, which is consistent with the idea that Sr²⁺ stimulates the opening of InsP₃ receptor. These data overall show that mouse eggs have more than one mechanism for generating Ca²⁺ oscillations and that in some cases Ca²⁺ oscillations can appear to be similar in form, but be generated by different mechanisms.

It is well-established that Ca²⁺ free media leads to a reduction or abolishment of Ca²⁺ oscillations in response to fertilization or PLC ζ injection in mammalian eggs (Igusa and Miyazaki, 1983; Lawrence and Cuthbertson, 1995). It has been assumed that this reflects the loss or some reduction of Ca²⁺ in the endoplasmic reticulum (Kline and Kline, 1992b). However, our data suggest that a reduction in resting, or interspike, cytosolic Ca²⁺ levels also occurs during incubation in Ca²⁺ free media. The reduction in cytosolic Ca²⁺ is apparent with the Ca²⁺ dye we used because it is dextran linked and hence is only within the cytosolic compartment, and because the K_d for OGBD and Ca²⁺ is around 250 nm. The reduction in resting Ca²⁺ level appears to cause the inhibition of Ca²⁺ oscillations, rather than a loss of Ca²⁺ store content, because low concentrations of thapsigargin, which will only reduce Ca²⁺ stores content further, actually restores Ca²⁺ oscillations in Ca²⁺ free media.

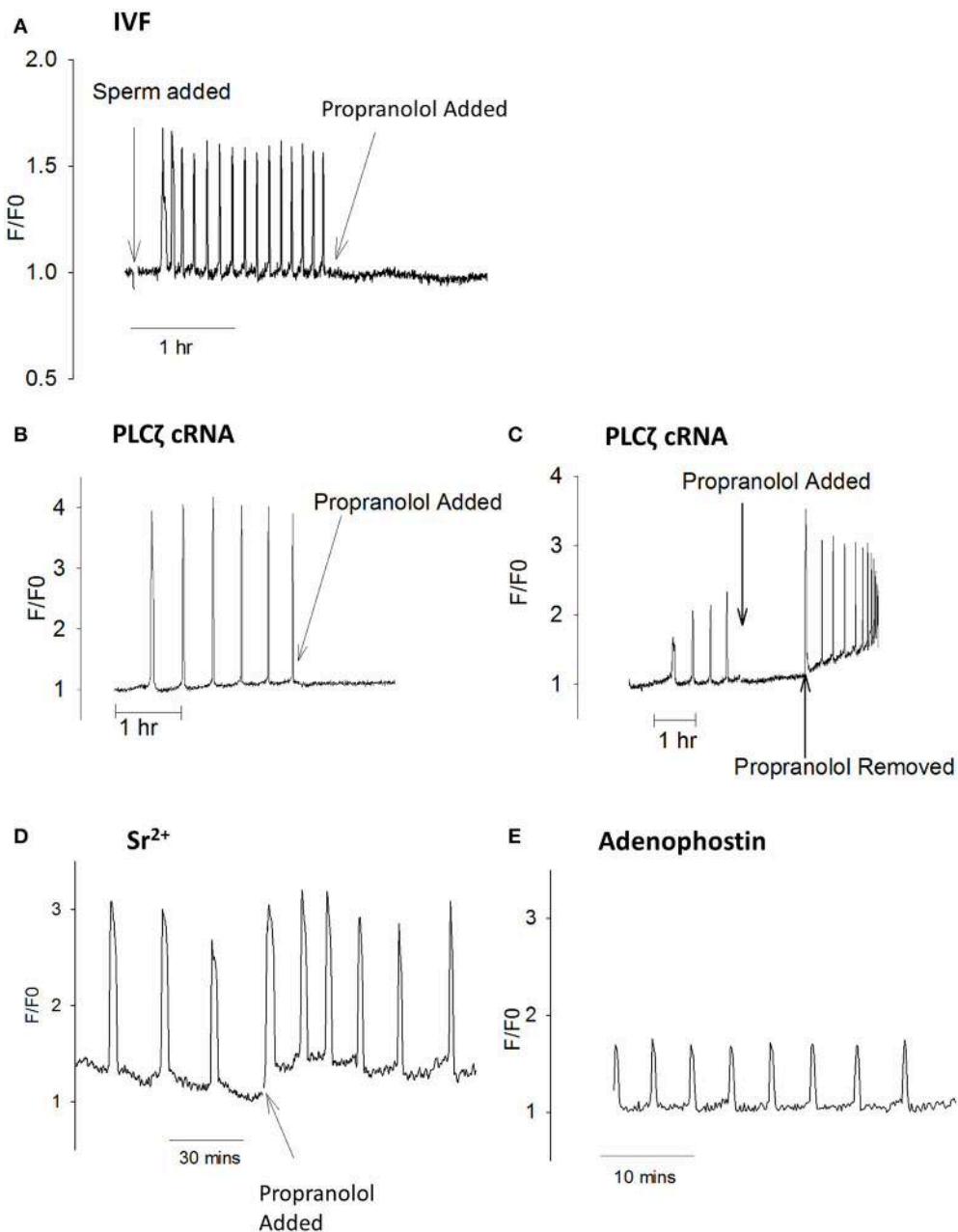


FIGURE 6 | Ca²⁺ oscillations blocked by propranolol. In (A) an example is shown of a mouse egg undergoing Ca²⁺ oscillations at fertilization where the addition of 300 μM propranolol inhibited subsequent oscillations ($n = 13$ eggs). Before addition of propranolol the mean frequency was 12.2 ± 1.14 spikes/h with all eggs oscillating. After adding propranolol there were 0.8 ± 0.23 spikes/h (a significant difference from before propranolol, $p = < 0.0001$). 6/13 eggs stopped oscillating immediately, 4/13 eggs had one Ca²⁺ spike, and 3/13 had 2 spikes in an hour. (B) shows PLCζ cRNA (pipette concentration = 0.02 μg/μl) induced Ca²⁺ oscillations inhibited by propranolol ($n = 21$ eggs). Before propranolol all eggs oscillated with 4.3 ± 0.46 spikes/h. After addition of propranolol there were 0.95 ± 0.25 spikes/h (a significant difference $p < 0.0001$). With propranolol, 10/21 eggs stopped Ca²⁺ oscillations, 6/21 showed a single spike, and 5/21 had >1 Ca²⁺ spike. In (C) an example is shown of an egg where PLCζ induced Ca²⁺ oscillations were blocked by the addition of propranolol but then oscillations were restored when propranolol was washed out (typical of $n = 8$ eggs). Before propranolol, all eggs oscillated with 6.7 ± 1.3 spikes/h. After propranolol this decreased to 1.33 ± 0.29 spikes/h, with all oscillations stopping after 2 spikes. When propranolol was removed there were 10 ± 0.55 spikes in 30 min. Adding propranolol and then removing it both caused significant changes in the number of Ca²⁺ spikes ($p < 0.001$). (D) shows an example of an egg undergoing Ca²⁺ oscillations in response to Sr²⁺ media where propranolol was subsequently added ($n = 10$ eggs). Before propranolol all eggs oscillated with 4.1 ± 0.29 spikes/h. After adding propranolol all eggs continues to oscillate with 3.9 ± 0.66 Ca²⁺ spikes/h (not significantly different). In (E) an example is shown of an egg undergoing Ca²⁺ oscillations in response to microinjection of 5 μM adenophostin in media that contained 300 μM propranolol from the start of the experiment ($n = 8$ eggs). In propranolol there were 5.5 ± 0.51 Ca²⁺ spikes/30 min, compared with 10.4 ± 0.71 spikes/30 min ($n = 11$) for eggs in media with HKSOM. This is significantly different (unpaired *t*-test, $p = < 0.0001$).

The restoration of such Ca²⁺ oscillations by thapsigargin in our experiments was clearly associated with a rise in the basal Ca²⁺ level. PLC ζ induced Ca²⁺ oscillations eventually stopped in Ca²⁺ free media with thapsigargin and this could be because Ca²⁺ stores eventually became depleted. However, the earlier rise in cytosolic Ca²⁺ seems to be a stimulatory factor because low concentrations of thapsigargin, which raise basal Ca²⁺, could also increase the frequency Ca²⁺ oscillations in normal media. This was associated a reduction in the amplitude of Ca²⁺ spikes, presumably because Ca²⁺ store content is reduced. Low concentrations of thapsigargin have also previously been found to stimulate Ca²⁺ oscillations in immature mouse oocytes (Igusa and Miyazaki, 1983; Lawrence and Cuthbertson, 1995). Hence, these data together imply that cytosolic Ca²⁺ level, rather than Ca²⁺ store content is the more significant factor setting the frequency and occurrence of physiological Ca²⁺ oscillations.

These data are consistent with recent studies measuring free Ca²⁺ inside the endoplasmic reticulum in mouse eggs (Wakai et al., 2013). It was shown that a reduction in ER Ca²⁺ occurs following each Ca²⁺ spike, but that there is no correlation between when a Ca²⁺ transient is initiated and the level of Ca²⁺ in the ER (Wakai et al., 2013). Whilst it is obvious that some Ca²⁺ store refilling will occur in the intervals between Ca²⁺ spikes, it is not likely that this sets of the pace of the low frequency Ca²⁺ oscillations characteristic of mammalian eggs. We suggest that the pacemaker that determines when the next Ca²⁺ transient occurs after PLC ζ injection is more likely to be the rise in cytosolic Ca²⁺. A gradual rise in cytosolic Ca²⁺ between spikes is evident in the PLC ζ induced Ca²⁺ oscillations in all the traces in this paper. This gradual Ca²⁺ increase could promote a gradual rise in InsP₃ that will eventually lead to a positive feedback loop and a regenerative Ca²⁺ wave.

Although the Ca²⁺ oscillations triggered by fertilization in mammalian eggs are of low frequency, each of the waves of Ca²⁺ release that causes the upstroke of a Ca²⁺ increase crosses the egg remarkably quickly. Previous analysis of the wave dynamics of Ca²⁺ release in mammalian eggs have suggested that the rising phase of each Ca²⁺ oscillation is ~1 s. This correlates with the speed of the Ca²⁺ wave that crosses the egg at a speed of >50 $\mu\text{m}/\text{s}$. This is significant because the diffusion coefficient of InsP₃ in intact cells has been estimated to be no more than 10 $\mu\text{m}^2/\text{s}$ (Dickinson et al., 2016). In models where InsP₃ is elevated at a constant level during Ca²⁺ oscillations the restricted diffusion of InsP₃ is not an issue because it will reach a steady state concentration across the egg. However, our data shows that Ca²⁺ and InsP₃ act together in a positive feedback loop to cause each propagating Ca²⁺ wave. In this case the diffusion of InsP₃ could be a rate limiting step. If all the InsP₃ is generated in the plasma membrane then our simulations show that a Ca²⁺ induced InsP₃ production model cannot generate Ca²⁺ waves through the egg cytoplasm. If we simulate the InsP₃ production from discrete sites within the egg cytoplasm then rapid Ca²⁺ waves of some type can be generated, but full waves can only be seen when the sites of InsP₃ generation are within 3 μm of each other. This suggests that in order to explain both the fast Ca²⁺ waves and the basic mechanism of sperm or PLC ζ induced oscillations in mammalian eggs, the PIP₂ substrate has to be dispersed in

sites throughout the egg cytoplasm. This conclusion is similar to that previously suggested for ascidian oocyte at fertilization which also show rapid Ca²⁺ waves and oscillations (Dupont and Dumollard, 2004).

We previously reported evidence for a vesicular source of PIP₂ in mouse eggs using immunostaining (Yu et al., 2012). The vesicular staining with PIP₂ antibodies closely mimics the distribution of PLC ζ also probed with antibodies (Yu et al., 2012). We now report a similar pattern of vesicular staining using another probe (PBP10) which based upon the PIP₂ binding region of gelsolin (Cunningham et al., 2001). This probe has the advantage that it is microinjected into eggs that can then be imaged whilst still alive and so does not require the fixation and permeabilization procedures associated with immunostaining. It gives a very different pattern of staining from another commonly used probe for PIP₂ which is the GFP-PH domain which localizes predominantly to the plasma membrane in mouse eggs (Halet et al., 2002). However, the PH domain of PLC $\delta 1$ that is used for the localization of PIP₂ in such a probe may also bind cholesterol so may be influenced by factors other than PIP₂ (Rissanen et al., 2017). It is entirely possible that PBP10 is also influenced by factors other than PIP₂, but it is noteworthy that the PBP10 staining gives a vesicular localization pattern that closely resembles that seen with the PIP₂ antibodies. The fact that two very different methods for localization PIP₂ in eggs, immunostaining with a monoclonal antibody and a fluorescently tagged peptide, show such a distinctive and similar pattern of localization provides good evidence that PIP₂ is indeed localized within vesicles in the cytoplasm of mouse eggs. Using the live cell probe, PBP10, we were able to estimate that the apparently PIP₂ containing vesicles we see in eggs are within about 2 μm of each other. This distance closely correlates with the estimate of how close PIP₂ vesicles need to be in order to propagate a Ca²⁺ wave across the egg within ~1 s. Hence, our data provide a coherent view of PLC ζ induced Ca²⁺ release in eggs in which Ca²⁺ induced InsP₃ formation from closely spaced vesicles containing PIP₂ accounts for the upstroke of each Ca²⁺ rise.

The precise nature of the PIP₂ containing vesicles that appear to exist in mouse eggs is unclear. We have tested a number of antibodies and other probes for specific organelles in eggs and found that many either localize to the endoplasmic reticulum or else show only a limited overlap in staining with the PIP₂ or PLC ζ positive vesicles. The identification of PBP10 positive vesicles is further complicated by our finding that its pattern of localization does not persist after fixation and membrane permeabilization (Sanders and Swann, unpublished). In somatic cells, non-plasma membrane PIP₂ has been found in the Golgi apparatus (De Matteis et al., 2005). Mature mouse egg are unusual compared with somatic cells in that they are arrested in meiosis, which is similar to the mitotic phase of the cell cycle. During mitosis the Golgi fragments to form small vesicles known as the Golgi haze (Axelsson and Warren, 2004), and the Golgi in mouse eggs has been shown to be fragmented into small vesicles (Moreno et al., 2002). The structure of the Golgi and its associated vesicles is maintained by the presence of diacyglycerol (DAG) (Asp et al., 2009). The drug propranolol disrupts Golgi resident proteins and lipids by inhibiting DAG production and as a result, it

also disrupts Golgi-ER trafficking (Asp et al., 2009). Interestingly propranolol was found to block Ca²⁺ oscillations triggered by PLC ζ and fertilization. This effect was specific in that the same concentration of propranolol did not effect oscillations when added to other Ca²⁺ releasing agents such as Sr²⁺ media which causes a pattern of oscillations most similar to fertilization. The small effect on adenophostin induced Ca²⁺ oscillations is unlikely to be sufficient to explain the effects of propranolol because it was only a 2-fold decrease in oscillations compared the cessation of oscillations after propranolol in most eggs that were fertilized or injected with PLC ζ . It is also noteworthy that the Ca²⁺ levels remained low in propranolol treated eggs, and that its effects were reversible. In mouse eggs we found that propranolol also decreased the number of the PIP₂ containing vesicles and the mean distance between vesicles, therefore presumably, the availability of the vesicular PIP₂ to propagate a Ca²⁺ wave. This effect could be because propranolol disrupts the structure of the vesicles or because trafficking between the Golgi and the ER is inhibited. Whatever the actual mechanism, the loss of PIP₂ after treatment with propranolol supports our hypothesis that these vesicles are required for generating Ca²⁺ oscillations in

eggs in response to sperm or PLC ζ . Since there is evidence for intracellular PIP₂ on organelles in frog and sea urchin eggs, which also show Ca²⁺ waves at fertilization, it is attractive to speculate that intracellular PIP₂ is an important feature that allows eggs to generate the Ca²⁺ signal needed for egg activation.

AUTHOR CONTRIBUTIONS

JS: Performed some of the Ca²⁺ measurements and the PIP₂ imaging experiments, analyzed data, and co-wrote the manuscript; BA and AM: Performed Ca²⁺ measurement experiments on eggs and analyzed data; TW: Produced and analyzed the mathematical simulation; KS: Conceived the study, directed experiments and co-wrote the manuscript. All authors approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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