PLCζ induced Ca\(^{2+}\) oscillations in mouse eggs involve a positive feedback cycle of Ca\(^{2+}\) induced InsP\(_3\) formation from cytoplasmic PIP\(_2\).
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ABSTRACT

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, PLCζ that hydrolys its substrate PIP\(_2\) to produce the Ca\(^{2+}\) releasing messenger InsP\(_3\). However, it is not clear whether PLCζ induced InsP\(_3\) formation is periodic or monotonic, and whether the PIP\(_2\) source for generating InsP\(_3\) from PLCζ is in the plasma membrane or the cytoplasm. In this study we have uncaged InsP\(_3\) at different points of the Ca\(^{2+}\) oscillation cycle to show that PLCζ causes Ca\(^{2+}\) oscillations by a mechanism which requires Ca\(^{2+}\) induced InsP\(_3\) formation. In contrast, incubation in Sr\(^{2+}\) media, which also induces Ca\(^{2+}\) oscillations in mouse eggs, sensitizes InsP\(_3\)-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 PLCζ–induced Ca\(^{2+}\) oscillations in eggs, even in Ca\(^{2+}\) free media. Given that Ca\(^{2+}\) induced InsP\(_3\) formation causes a rapid wave during each Ca\(^{2+}\) rise, we use a mathematical model to show that InsP\(_3\) generation, and hence PLCζ’s substate PIP\(_2\), has to be finely distributed throughout the egg cytoplasm. Evidence for PIP\(_2\) distribution in vesicles throughout the egg cytoplasm is provided with a rhodamine-peptide probe, PBP10. The apparent level of PIP\(_2\) in such vesicles could be reduced by incubating eggs in the drug propranolol which also reversibly inhibited PLCζ 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 PLCζ induced Ca\(^{2+}\) oscillations in eggs, and they imply that InsP\(_3\) oscillates in synchrony with Ca\(^{2+}\) oscillations. Furthermore, they support the hypothesis that PLCζ and sperm induced Ca\(^{2+}\) oscillations in eggs requires the hydrolysis of PIP\(_2\) from finely spaced cytoplasmic vesicles.
INTRODUCTION

The fertilization of a mammalian egg involves a series of low frequency Ca\textsuperscript{2+} oscillations that last for many hours. Such Ca\textsuperscript{2+} oscillations play the key role in egg activation and the subsequent development of the embryo (Stricker, 1999). The first Ca\textsuperscript{2+} increase takes approximately 10 seconds to travel as a wave across the egg from the point of sperm entry (Deguchi et al., 2000; Miyazaki et al., 1986). However, all the subsequent Ca\textsuperscript{2+} transients have a rising phase of about 1 second which is due to a fast Ca\textsuperscript{2+} wave (>50 \( \mu \text{m/sec} \)) that crosses the egg from apparently random points in the egg cortex (Deguchi et al., 2000).

Each Ca\textsuperscript{2+} increase during the oscillations is due to release from internal Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} oscillations via inositol 1,4,5-trisphosphate (Ins\textsubscript{P}3) production, and all the reproducible studies suggest that this is principally due to the introduction of a sperm specific phospholipase Czeta (PLC\textsubscript{ζ}) into the egg after gamete fusion (Saunders et al., 2002).

Injection of PLC\textsubscript{ζ} protein or cRNA causes prolonged Ca\textsuperscript{2+} oscillations that mimic those seen at fertilization in eggs of mice, rat, humans, cows and pigs (Bedford-Guauas et al., 2008; Cox et al., 2002; Fujimoto et al., 2004; Ito et al., 2008; Kouchi et al., 2004; Kurokawa et al., 2005; Ross et al., 2008; Sato et al., 2013; Saunders et al., 2002; Yoon et al., 2012). PLC\textsubscript{ζ} is distinctive compared to most mammalian PLC isozymes in that it is stimulated by low levels of Ca\textsuperscript{2+} such that it is maximally sensitive to Ca\textsuperscript{2+} around the resting levels in eggs (Nomikos et al., 2005). PLC\textsubscript{ζ} is expected to diffuse across the egg in about 10 mins following sperm-egg fusion, hence the fast Ca\textsuperscript{2+} waves seen after the initial Ca\textsuperscript{2+} transient are propagated within a cytoplasm in which PLC\textsubscript{ζ} has probably dispersed throughout the egg.

There are two classes of model to explain how Ins\textsubscript{P}3 causes Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} dependent sensitization, and then de-sensitization, of the IP3R is necessary to generate each Ca\textsuperscript{2+} 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 Ins\textsubscript{P}3, or by injection of the IP3R agonist adenophostin (Brind et al., 2000; Jones and Nixon, 2000; Swann et al., 1989). On the other hand there are other models in which Ca\textsuperscript{2+} dependent production of Ins\textsubscript{P}3 generates each Ca\textsuperscript{2+} transient, and in which Ins\textsubscript{P}3 is predicted to oscillate alongside Ca\textsuperscript{2+} (Politi et al., 2006). This
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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 Ca\(^{2+}\) increases. Either classes of model have to incorporate the observation that the Ca\(^{2+}\) oscillations have a dependence upon Ca\(^{2+}\) influx. So for example, if fertilizing hamster or mouse eggs are incubated in Ca\(^{2+}\) 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 Ca\(^{2+}\) store content is critical in setting the timing of Ca\(^{2+}\) oscillations in mouse eggs. This is supported by evidence that the SERCA inhibitor thapsigargin can also be used to block sperm and PLCζ induced Ca\(^{2+}\) oscillations by depleting Ca\(^{2+}\) stores content (Kline and Kline, 1992b). However, changes in cytosolic Ca\(^{2+}\) may also play a role in the timing of oscillations since cytosolic Ca\(^{2+}\) can regulate both IP3Rs and PLCζ activity.

Sustained Ca\(^{2+}\) oscillations in mouse eggs can also be triggered by incubation in media containing Sr\(^{2+}\) instead of Ca\(^{2+}\) (Bos-Mikich et al., 1995; Kline and Kline, 1992a). Sr\(^{2+}\)-induced Ca\(^{2+}\) 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 Sr\(^{2+}\) influx into the egg and the presence of functional IP3Rs (Zhang et al., 2005). However, it is not clear how Sr\(^{2+}\) causes Ca\(^{2+}\) oscillations. One study suggested that the effect of Sr\(^{2+}\) requires InsP₃ production (Zhang et al., 2005). However, unlike fertilization, there is no Sr\(^{2+}\) induced downregulation of IP3Rs and this suggests that Sr\(^{2+}\) does not cause any substantial InsP₃ generation (Jellerette et al., 2000). In vitro preparations of IP3Rs receptors can be stimulated to open by Sr\(^{2+}\) ions (Marshall and Taylor, 1994), so a direct effect of Sr\(^{2+}\) 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 Ca\(^{2+}\), 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 Ca\(^{2+}\) 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) approximately 50 hours later (Fowler and Edwards, 1957). Eggs were collected from these mice 15 hours after HCG injection, from the dissected ovaries. All animal handling and procedures were carried out under a UK Home Office Licence 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, 2013b). 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 hours 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+ indictor Oregon Green BAPTA dextran (OGBD) (Life Technologies). OGBD was diluted in a KCl HEPES buffer...
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(120 mM KCl, 20 mM HEPES at pH 7.4) so that the injection solution contained 0.33 or 0.5 mM OGBD. The OGBD 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 OGBD. In this case instead of mixing the OGBD with KCL HEPES it was mixed with KCL HEPES containing 5μM adenophostin in the same quantities. Where PLCζ cRNA was used this was microinjected alongside OGBD 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, 2013a). Ca\textsuperscript{2+} dynamics were measured using the time-lapse imaging mode of Micromanager software (https://micro-manager.org/) where an image was captured every 10 seconds. Where IVF was performed, or drugs were later added to the eggs, the zona pellucidas were removed from the eggs using acid Tyrodes treatment prior to imaging. For those experiments that required InsP3 stimulation, NPE-caged-InsP3 (1mM in the pipette) from ThermoFisher Scienific was microinjected prior to imaging at the same time as the injection of fluorescent dye (OGBD). In order to photo-release InsP3 the eggs were exposed to an electronically gated UV LED light source (365nm, 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 Ca\textsuperscript{2+} 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 KH\textsubscript{2}PO\textsubscript{4}, 0.2 MgSO\textsubscript{4}, 2.5 mM KCl, 4mM NaHCO\textsubscript{3}, 20 mM HEPES, 0.01 mM EDTA, 0.2 mM Na Pyruvate, 1 mM L-glutamine, 0.2 mM glucose, 10mM Na Lactate 1.7 mM CaCl\textsubscript{2}, 0.063 g/l Benzylpenicllin and 10 mg/l phenol red. Ca\textsuperscript{2+} free media was made in the same way as HKSOM however CaCl\textsubscript{2} was not added and the media was supplemented with 100μM EGTA. Sr\textsuperscript{2+} containing media was made in the same way as HKSOM however, instead of adding 1.7mM CaCl\textsubscript{2}, 10 mM SrCl\textsubscript{2} 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 1:1000 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 1mM PBP10 (Tocris Biosciences, UK) was made up in KCl HEPES and approximately 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 1058 x 1058 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 analysed using Image J (https://imagej.nih.gov/ij/).

Data analysis

Quantitative data measuring the Ca\textsuperscript{2+} 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 normalised 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 \((F/F_0)\) that could be plotted against time. These traces were produced and analysed using SigmaPlot 12. The Confocal images were also analysed using Image J software. PIP\textsubscript{2} positive vesicle size and distribution was calculated using the particle analysis function on Image J and a nearest neighbour 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...
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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 neighbour 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 Ca\(^{2+}\) 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, \(p\),

\[
\frac{du}{dt} = d \nabla^2 u + A - D \frac{u_{ed}}{u_{ed} + u_{ad}} \left(1 - \frac{p_{es}}{p_{es} + p_{es}}\right) + K(u, v, p),
\]

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

\[
\frac{dp}{dt} = d \nabla^2 p + \epsilon + PLC \frac{u_{ep}}{u_{ep} + u_{p}} S(x, y, L) - rp,
\]

\[
K(u, v, p) = -B \frac{u_{eb}}{u_{eb} + u_{ub}} + C \frac{u_{ce}}{u_{ce} + u_{ec}} \frac{u_{pc}}{u_{pc} + u_{cp}} \frac{u_{pa}}{u_{pa} + u_{epa}} \left(1 - \frac{u_{epi}}{u_{epi} + u_{epi}}\right) + Ev.
\]

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

The equations represent interactions in which free Ca\(^{2+}\) acts as a self-inhibitor but, along with InsP\(_3\) and stored Ca\(^{2+}\), stimulates the release of stored Ca\(^{2+}\), creating a system that can produce oscillations in the concentrations of calcium and InsP\(_3\). Critically, all species are able to diffuse with the same diffusion coefficient, \(d\).

The actions of the stored Ca\(^{2+}\) and the InsP\(_3\) 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 \(L_{on} \times L_{on}\) 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 $70 \mu m$, which was discretised into 6550 elements. The 2D assumption is considered valid because any dilution effects of going to three dimensions are off set 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 PLC$\zeta$ induced Ca$^{2+}$ spikes can be generated in mouse eggs where no membrane Ca$^{2+}$ 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 $\mu$Mol, space is in $\mu m$ 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 Ca$^{2+}$ population.

RESULTS

PLC$\zeta$ and Sr$^{2+}$ trigger Ca$^{2+}$ oscillations in eggs via different mechanisms.

We investigated the mechanism generating Ca$^{2+}$ oscillations by using photo-release of caged InsP$_3$ that was microinjected into mouse eggs. In initial experiments we uncaged InsP$_3$ in unfertilized (control) mouse eggs that were not undergoing any Ca$^{2+}$ oscillations. Fig.1A shows that UV pulses of light from 50msec through to 2 sec generated Ca$^{2+}$ increases with the amplitudes that were larger with longer duration pulses. With the protocol we used there was adequate amounts of caged InsP$_3$ for multiple releases of InsP$_3$ even with longer duration pulses of UV light as illustrated by Fig1B which shows that 3 sec pulses could generate repeated large rises in Ca$^{2+}$ in control eggs. We then tested the effects of triggering such pulses during Ca$^{2+}$ oscillations induced by either Sr$^{2+}$ media or by PLC$\zeta$ injection. Fig 1C shows that when a 100msec pulse was used in eggs injected with PLC$\zeta$ the uncaging of InsP$_3$ caused no Ca$^{2+}$ increase. In contrast, Fig 1D shows Ca$^{2+}$ oscillations occurring in response to Sr$^{2+}$ media and in such eggs there was a rapid and large Ca$^{2+}$ transient every time a pulse of just 100msec was used to uncage InsP$_3$. Since the response to 100msec pulses of
UV were minimal in control eggs (Fig 1A) these data show that Sr\(^{2+}\) media sensitizes eggs to
insP\(_3\) induced Ca\(^{2+}\) release and that, in contrast, IP3R are not sensitized to InsP\(_3\) by PLC\(\zeta\)
 injection.

The two classes of model for Ca\(^{2+}\) oscillations, those that involve the dynamic properties of
IP3Rs and those that involve InsP\(_3\) production oscillations, can be distinguished in a
definitive manner by examining the response to a sudden pulse of InsP\(_3\) (Sneyd et al., 2006).
Models that are dependent upon IP3R kinetics alone respond to a pulse of InsP\(_3\) by showing a
transient increase in the frequency of Ca\(^{2+}\) oscillations (Sneyd et al., 2006). In contrast,
models that depend on Ca\(^{2+}\) induced InsP\(_3\) production, and imply InsP\(_3\) oscillations, respond
to a sudden increase in InsP\(_3\) by showing an interruption of the oscillations which leads to a
resetting of the phase of oscillations (Sneyd et al., 2006). We tested the effect of using large
uncaging pulses of InsP\(_3\) on Sr\(^{2+}\) induced, or PLC\(\zeta\) induced, Ca\(^{2+}\) oscillations in mouse eggs.
Fig 2A shows that during Sr\(^{2+}\) induced oscillations a 3sec uncaging pulse of InsP\(_3\) caused a
large increase in Ca\(^{2+}\) followed by a significant increase in the frequency of Ca\(^{2+}\) oscillations.
In contrast, with PLC\(\zeta\) induced Ca\(^{2+}\) oscillations, Fig 2B shows that the same 3 sec uncaging
pulse of InsP\(_3\) did not cause any increase in frequency, but interrupted the periodicity of
oscillations leading to a delay before the next Ca\(^{2+}\) increase. To confirm that this
phenomenon was phase resetting, we plotted the shift in phase (PS) caused by uncaging of
InsP\(_3\) against the time delay (dt) of the InsP\(_3\) pulse from the subsequent Ca\(^{2+}\) spike (see Fig
2C). Each of these values was divided by the time period T in order to take into account the
different frequency of Ca\(^{2+}\) oscillations in each egg. With phase resetting this plot should give
a line from 1 to 1 on each axis, and Fig 2D shows that the data from 23 PLC\(\zeta\) injected eggs
exposed to uncaging pulses of InsP\(_3\) fit closely on such a line. These data clearly show that a
pulse of InsP\(_3\) causes phase resetting of Ca\(^{2+}\) oscillations in mouse eggs, which is completely
different from that seen with Sr\(^{2+}\) induced oscillations. Hence, overall the data suggest that
PLC\(\zeta\) and Sr\(^{2+}\) media trigger Ca\(^{2+}\) oscillations in mouse eggs via fundamentally different
mechanisms. Sr\(^{2+}\) stimulates IP3Rs to make them effectively more sensitive to InsP\(_3\), and that
PLC\(\zeta\) induced Ca\(^{2+}\) oscillations involve Ca\(^{2+}\) stimulated InsP\(_3\) production where InsP\(_3\) acts as
a dynamic variable that should oscillate in synchrony with Ca\(^{2+}\) oscillations.

Cytosolic Ca\(^{2+}\) versus Ca\(^{2+}\) stores and the frequency of Ca\(^{2+}\) oscillations.
Since Ca\(^{2+}\) release and InsP\(_3\) formation are predicted to form part of a positive feedback loop
we decided to re-investigate some observation previously made on Ca\(^{2+}\) oscillations in eggs.
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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 (>10μM) of thapsigargin to completely block Ca2+ oscillations in eggs (Kline and Kline, 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+. Fig 3A and B show that the addition of 500nM 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 of SERCA pumps. When the same concentration of thapsigargin was added to eggs undergoing Ca2+ oscillations in response to PLCζ there was a marked acceleration of Ca2+ oscillations, and a reduction in the amplitude of Ca2+ spikes (Fig 3C). Similar to previous reports, we found that the pattern of PLCζ 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 (Fig 3D). When low concentrations of thapsigargin (500nM) were added to PLCζ injected eggs in Ca2+ free media there was a restoration of Ca2+ oscillations (Fig 3E). It is noteworthy that in Fig 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 (Fig 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.

**PLCζ 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 second, and propagate through the cytoplasm at speeds in excess of 50μm/sec. This matches the rising phase of (all but the initial) Ca2+ transients in mouse eggs which is ~1 sec after fertilization or after PLCζ protein injection (Deguchi et al., 2000). Since data in Fig 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
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329 PIP\(_2\) that is the precursor to InsP\(_3\). In most cells PIP\(_2\) is in the plasma membrane, and if this is
330 used in Ca\(^{2+}\) waves in eggs then InsP\(_3\) diffusion range might constrain the ability to generate
331 fast Ca\(^{2+}\) waves. Recently, the diffusion coefficient of InsP\(_3\) in intact cells has been shown to
332 be less than 10 \(\mu\)m\(^2\)/sec which means that InsP\(_3\) may only diffuse less than 5\(\mu\)m in 1 second
333 ( Dickinson et al., 2016). We have previously presented models of Ca\(^{2+}\) oscillations based
334 upon Ca\(^{2+}\) induced InsP\(_3\) formation and InsP\(_3\) induced opening of Ca\(^{2+}\) release channels
335 (Theodoridou et al., 2013). We have now simulated the Ca\(^{2+}\) waves in mouse eggs using a
336 similar set of equations in a two-dimensional model of the Ca\(^{2+}\) wave. Fig. 4 shows that with
337 the source of Ca\(^{2+}\) stimulated InsP\(_3\) production at the periphery (plasma membrane) it is not
338 possible to generate a Ca\(^{2+}\) wave through the egg cytoplasm, and only a concentric pattern of
339 Ca\(^{2+}\) release occurs. We previously presented evidence for PIP\(_2\) being present in intracellular
340 vesicles spread throughout the cytoplasm in mouse eggs (Yu et al., 2012). These could
341 provide a source of InsP\(_3\) that might carry a Ca\(^{2+}\) wave through the cytoplasm if they are
342 sufficiently dispersed. In Fig 4 we show simulations based upon Ca\(^{2+}\) induced InsP\(_3\)
343 generation where the PIP\(_2\) is dispersed on vesicles at different distances apart (from 2 to 4
344 \(\mu\)m). Our simulations show that when the PIP\(_2\) vesicles are within 2 or 3\(\mu\)m of each other a
345 rapid Ca\(^{2+}\) can be generated, but that once the PIP\(_2\) is more than 3\(\mu\)m the Ca\(^{2+}\) increase fails
346 to occur. These results suggests that PIP\(_2\) needs to be present on vesicles spaced less than 3
347 \(\mu\)m apart in the cytoplasm in order to propagate a rapid Ca\(^{2+}\) wave of the type seen in
348 fertilizing and PLCζ injected eggs.

349 Previous evidence for the existence of PIP\(_2\) within the cytoplasm of eggs came from studies
350 using antibodies to PIP\(_2\) (Yu et al., 2012). Gelsolin is a protein that has been shown to bind
351 to PIP\(_2\), and contains a short peptide sequence responsible for PIP\(_2\) binding (Cunningham et
352 al., 2001). We injected mouse eggs with PBP-10, which is a probe in which rhodamine is
353 coupled to a gelsolin peptide that binds PIP\(_2\). Fig 5A shows a mouse egg injected with PBP-
354 10. After > 1 hour the fluorescence of PBP-10 could be predominantly seen in many small
355 vesicles throughout the egg cytoplasm, with the occasional larger aggregate. This supports
356 the hypothesis that PIP\(_2\) is localized in vesicles within mouse eggs (Yu et al., 2012). Further
357 examination of these vesicles using particle analysis indicates that they are distributed
358 throughout the whole egg cytoplasm. Interestingly, following nearest neighbour analysis, we
359 found that these vesicles were approximately 2\(\mu\)m apart (Fig 5A and D). This suggests that
360 these PIP\(_2\) containing vesicles are within the correct distance predicted to produce the rapid
361 rising phase of 1 sec for each wave as predicted by our mathematical modelling.
PLCζ induced Ca\(^{2+}\) oscillations in mouse eggs

We have previously sought to modify the level of PIP\(_2\) in mouse eggs using various phosphatases, but without success. Internal membranes in somatic cells do not in general contain much PIP\(_2\), but one organelle where PIP\(_2\) 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 (Axelsson and Warren, 2004; Moreno et al., 2002). 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 (Fig 5B). Further particle analysis showed that the mean number of these PIP\(_2\) vesicles was significantly reduced following the addition of propranolol (Fig 5C). Furthermore, the distance of these vesicles from each other was significantly increased in those eggs treated with propranolol (Fig 5D). The overall total fluorescence of the vesicles was seen to reduce by approximately half from a mean of 5.77x. 10\(^4\) RFU (n=7) in control eggs to a mean of 2.93x10\(^4\) 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 PIP\(_2\) levels in cytoplasmic vesicles.

Since propranolol appears to reduce PIP\(_2\) inside eggs, we investigated the effect of propranolol on Ca\(^{2+}\) oscillations. Fig. 6A shows that propranolol addition to eggs undergoing Ca\(^{2+}\) oscillations in response to fertilization by IVF were rapidly blocked. Fig 6B shows the same effect of propranolol on those eggs stimulated by PLCζ cRNA. The inhibition by propranolol was associated with a slight decline in Ca\(^{2+}\) levels and the inhibition was reversed upon removal of propranolol (Fig 6C). However, whilst it blocked sperm and PLCζ induced responses, propranolol did not block Ca\(^{2+}\) oscillations induced in eggs by Sr\(^{2+}\) media, or by injection of the IP3R agonist adenophostin (Fig.6D and Fig 6E). These data show that the inhibitory effects of propranolol are both reversible and specific to PLCζ and sperm induced Ca\(^{2+}\) oscillations. They support the proposal that PIP\(_2\) in vesicles in the cytoplasm of mouse eggs is important for the generation of PLCζ induced Ca\(^{2+}\) oscillations.

**DISCUSSION**

The Ca\(^{2+}\) oscillations seen in mammalian eggs at fertilisation 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 PLCζ is the primary stimulus for
these Ca\(^{2+}\) oscillations (Saunders et al., 2002). The current data shows that PLC\(\zeta\) induced Ca\(^{2+}\) oscillations are driven by Ca\(^{2+}\) induced InsP\(_3\) formation. In contrast, we show that Sr\(^{2+}\) media sensitizes eggs to InsP\(_3\) induced Ca\(^{2+}\) release. Hence, there are at least two different mechanisms for generating Ca\(^{2+}\) oscillations in mouse eggs. Our data also implies that the substrate of PLC\(\zeta\), PIP\(_2\), needs to be localized in a finely distributed source within the egg in order to generate fast Ca\(^{2+}\) wave, and we present evidence that such vesicular PIP\(_2\) is required for PLC\(\zeta\) induced Ca\(^{2+}\) oscillations.

There are two fundamentally different classes of models for InsP\(_3\) induced Ca\(^{2+}\) oscillations in cells. One relies on the properties of InsP\(_3\) receptor and implies that stimulation involves an elevated but monotonic or constant elevation of InsP\(_3\) levels. The other involves a positive feedback model of InsP\(_3\) induced Ca\(^{2+}\) release and Ca\(^{2+}\) induced InsP\(_3\) formation. It is possible to determine which one of these two model types applies by studying the Ca\(^{2+}\) responses after triggering a large pulsed release of InsP\(_3\) (Sneyd et al., 2006). The IP3R based models respond to a pulse of InsP\(_3\) by temporarily increasing the frequency of Ca\(^{2+}\) oscillations, whereas the Ca\(^{2+}\)-induced InsP\(_3\) formation models show an interruption in the series of Ca\(^{2+}\) 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\(\zeta\) –induced Ca\(^{2+}\) oscillations responding to a pulse of InsP\(_3\) (Swann and Yu, 2008). We now show that the response of PLC\(\zeta\) induced Ca\(^{2+}\) oscillations to a sudden large pulse of InsP\(_3\) is clearly characterized by a resetting of the phase of oscillations. This means that InsP\(_3\) has to be a dynamic variable in the oscillation cycle and that it will undergo oscillations in close phase with the oscillations in Ca\(^{2+}\). Small oscillations in InsP\(_3\) have been recorded previously in response to high frequency Ca\(^{2+}\) oscillations achieved with high concentrations of PLC\(\zeta\) (Shirakawa et al., 2006). The sensitivity of such indicators may be limited since we can now assert that InsP\(_3\) oscillations should occur with all the PLC\(\zeta\) induced Ca\(^{2+}\) oscillations and, most significantly, that increased InsP\(_3\) production plays a causal role in generating each Ca\(^{2+}\) rise. We have also shown here that Sr\(^{2+}\) works via an entirely different mechanism in mouse eggs. The increase in frequency of Ca\(^{2+}\) oscillations caused by uncaging InsP\(_3\) indicates that Sr\(^{2+}\) induced oscillations rely on the properties of the IP3R. This is supported by the finding that Sr\(^{2+}\) media sensitized mouse eggs to InsP\(_3\) pulses, which is consistent with the idea that Sr\(^{2+}\) stimulates the opening of InsP\(_3\) receptor. These data overall show that mouse eggs have more than one mechanism for generating Ca\(^{2+}\) oscillations and...
that in some cases Ca\(^{2+}\) oscillations can appear to be similar in form, but be generated by different mechanisms.

It is well established that Ca\(^{2+}\) free media leads to a reduction or abolishment of Ca\(^{2+}\) oscillations in response to fertilization or PLC\(\zeta\) 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 Ca\(^{2+}\) in the endoplasmic reticulum (Kline and Kline, 1992b). However, our data suggest that a reduction in resting, or interspike, cytosolic Ca\(^{2+}\) levels also occurs during incubation in Ca\(^{2+}\) free media. The reduction in cytosolic Ca\(^{2+}\) is apparent with the Ca\(^{2+}\) dye we used because it is dextran linked and hence is only within the cytosolic compartment, and because the K\(_d\) for OGBD and Ca\(^{2+}\) is around 250nm. The reduction in resting Ca\(^{2+}\) level appears to cause the inhibition of Ca\(^{2+}\) oscillations, rather than a loss of Ca\(^{2+}\) stores content, because low concentrations of thapsigargin, which will only reduce Ca\(^{2+}\) stores content further, actually restores Ca\(^{2+}\) oscillations in Ca\(^{2+}\) free media. The restoration of such Ca\(^{2+}\) oscillations by thapsigargin in our experiments was clearly associated with a rise in the basal Ca\(^{2+}\) level. PLC\(\zeta\) induced Ca\(^{2+}\) oscillations eventually stopped in Ca\(^{2+}\) free media with thapsigargin and this could be because Ca\(^{2+}\) stores eventually became depleted. However, the earlier rise in cytosolic Ca\(^{2+}\) seems to be a stimulatory factor because low concentrations of thapsigargin, which raise basal Ca\(^{2+}\), could also increase the frequency Ca\(^{2+}\) oscillations in normal media. This was associated a reduction in the amplitude of Ca\(^{2+}\) spikes, presumably because Ca\(^{2+}\) store content is reduced. Low concentrations of thapsigargin have also previously been found to stimulate Ca\(^{2+}\) oscillations in immature mouse oocytes (Igusa and Miyazaki, 1983; Lawrence and Cuthbertson, 1995). Hence, these data together imply that cytosolic Ca\(^{2+}\) level, rather than Ca\(^{2+}\) store content is the more significant factor setting the frequency and occurrence of physiological Ca\(^{2+}\) oscillations.

These data are consistent with recent studies measuring free Ca\(^{2+}\) inside the endoplasmic reticulum in mouse eggs (Wakai et al., 2013). It was shown that a reduction in ER Ca\(^{2+}\) occurs following each Ca\(^{2+}\) spike, but that there is no correlation between when a Ca\(^{2+}\) transient is initiated and the level of Ca\(^{2+}\) in the ER (Wakai et al., 2013). Whilst it is obvious that some Ca\(^{2+}\) store refilling will occur in the intervals between Ca\(^{2+}\) spikes, it is not likely that this sets of the pace of the low frequency Ca\(^{2+}\) oscillations characteristic of mammalian eggs. We suggest that the pacemaker that determines when the next Ca\(^{2+}\) transient occurs after PLC\(\zeta\) injection is more likely to be the rise in cytosolic Ca\(^{2+}\). A gradual rise in cytosolic
Ca\textsuperscript{2+} between spikes is evident in the PLC\textgreek{z} induced Ca\textsuperscript{2+} oscillations in all the traces in this paper. This gradual Ca\textsuperscript{2+} increase could promote a gradual rise in InsP\textgreek{3} that will eventually lead to a positive feedback loop and a regenerative Ca\textsuperscript{2+} wave.

Although the Ca\textsuperscript{2+} oscillations triggered by fertilization in mammalian eggs are of low frequency, each of the waves of Ca\textsuperscript{2+} release that causes the upstroke of a Ca\textsuperscript{2+} increase crosses the egg remarkably quickly. Previous analysis of the wave dynamics of Ca\textsuperscript{2+} release in mammalian eggs have suggested that the rising phase of each Ca\textsuperscript{2+} oscillation is approximately 1 sec. This correlates with the speed of the Ca\textsuperscript{2+} wave that crosses the egg at a speed of \textgreek{>} 50\textmu m/s. This is significant because the diffusion coefficient of InsP\textgreek{3} in intact cells has been estimated to be no more that 10\textmu m\textsuperscript{2}/sec (Dickinson et al., 2016). In models where InsP\textgreek{3} is elevated at a constant level during Ca\textsuperscript{2+} oscillations the restricted diffusion of InsP\textgreek{3} is not an issue because it will reach a steady state concentration across the egg. However, our data shows that Ca\textsuperscript{2+} and InsP\textgreek{3} act together in a positive feedback loop to cause each propagating Ca\textsuperscript{2+} wave. In this case the diffusion of InsP\textgreek{3} could be a rate limiting step. If all the InsP\textgreek{3} is generated in the plasma membrane then our simulations show that a Ca\textsuperscript{2+} induced InsP\textgreek{3} production model cannot generate Ca\textsuperscript{2+} waves through the egg cytoplasm. If we simulate the InsP\textgreek{3} production from discrete sites within the egg cytoplasm then rapid Ca\textsuperscript{2+} waves of some type can be generated, but full waves can only be seen when the sites of InsP\textgreek{3} generation are within 3\textmu m of each other. This suggests that in order to explain both the fast Ca\textsuperscript{2+} waves and the basic mechanism of sperm or PLC\textgreek{z} induced oscillations in mammalian eggs, the PIP\textgreek{2} 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 Ca\textsuperscript{2+} waves and oscillations (Dupont and Dumollard, 2004).

We previously reported evidence for a vesicular source of PIP\textgreek{2} in mouse eggs using immunostaining (Yu et al., 2012). The vesicular staining with PIP\textgreek{2} antibodies closely mimics the distribution of PLC\textgreek{z} 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 PIP\textgreek{2} 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 PIP\textgreek{2} which is the GFP-PH domain which localizes predominantly to the plasma membrane in mouse eggs (Halet et
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However, the PH domain of PLCδ1 that is used for the localization of PIP\(_2\) in such a probe may also bind cholesterol so may be influenced by factors other than PIP\(_2\) (Rissanen et al., 2017). It is entirely possible that PBP10 is also influenced by factors other than PIP\(_2\), but it is noteworthy that the PBP10 staining gives a vesicular localization pattern that closely resembles that seen with the PIP\(_2\) antibodies. The fact that two very different methods for localization PIP\(_2\) 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 PIP\(_2\) is indeed localized within vesicles in the cytoplasm in of mouse eggs.

Using the live cell probe, PBP10, we were able to estimate that the apparently PIP\(_2\) containing vesicles we see in eggs are within about 2\(\mu\)m of each other. This distance closely correlates with the estimate of how close PIP2 vesicles need to be in order to propagate a Ca\(^{2+}\) wave across the egg within ~1 sec. Hence, our data provide a coherent view of PLCζ induced Ca\(^{2+}\) release in eggs in which Ca\(^{2+}\) induced InsP\(_3\) formation from closely spaced vesicles containing PIP\(_2\) accounts for the upstroke of each Ca\(^{2+}\) rise.

The precise nature of the PIP\(_2\) 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 PIP\(_2\) or PLCζ 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 PIP\(_2\) 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 diacylglycerol (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 Ca\(^{2+}\) oscillations triggered by PLCζ and fertilisation. This effect was specific in that the same concentration of propranolol did not effect oscillations when added to other Ca\(^{2+}\) releasing agents such as Sr\(^{2+}\) media which causes a pattern of oscillations most similar to fertilization. The small effect on adenosphostin induced Ca\(^{2+}\) oscillations is unlikely to be
sufficient to explain the effects of propranolol because it was only a two fold decrease in oscillations compared the cessation of oscillations after propranolol in most eggs that were fertilized or injected with PLCζ. 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 PLCζ. 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, analysed data, and co-wrote the manuscript. BA and AM performed Ca²⁺ measurement experiments on eggs and analysed data. TW produced and analysed 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

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could have taken as a conflict of interest.
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PLCζ induced Ca^{2+} oscillations in mouse eggs


FIGURE LEGENDS

FIGURE 1. Ca$^{2+}$ oscillations and uncaging pulses of InsP$_3$. In A an example trace is shown of Ca$^{2+}$ increases (as measured by OGBD fluorescence) in an egg in response to different amounts of InsP$_3$. Eggs were injected with caged InsP$_3$ and exposed to varying durations of UV light pulse (from 50msec to 2sec) to photo-release the InsP$_3$ (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 Ca$^{2+}$ in an egg in response to the “uncaging” of caged IP3 using long duration UV pulses of 3 second. 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 Ca$^{2+}$ in an egg stimulated following the microinjection of mouse derived PLC$\zeta$ cRNA (0.02 $\mu$g/$\mu$l) and caged InsP$_3$. The arrow indicates where a 100ms pulse of UV light was applied (n=14 eggs). In all 14/14 such recordings there was no sudden increase in Ca$^{2+}$ even when the pulse was applied during the pacemaker rising phase of Ca$^{2+}$. In D an example trace is shown with changes in cytosolic Ca$^{2+}$ in an egg stimulated by media containing 10mM Sr$^{2+}$. The arrow indicates where a 100ms pulse of UV light was applied to uncage InsP$_3$ (n=32 eggs). In all 32/32 cases there was a rapid Ca$^{2+}$ increase that started with the very next OGBD fluorescence measurement after the UV pulse (<10sec).

FIGURE 2. The effect of large pulses of InsP$_3$ on PLC$\zeta$ or Sr$^{2+}$ triggered Ca$^{2+}$ oscillations. A shows an example of the way eggs responded a large uncaging pulse of InsP$_3$ (3 sec UV light at the arrow) by an increase in the frequency of Sr$^{2+}$ triggered Ca$^{2+}$ oscillations (n= 20 eggs). There was a significant increase in frequency of Ca$^{2+}$ spikes from 3.45 (+/-0.27 sem) in 20 mins to 5.05 (+/-0.35 sem) in 20 mins ($p<0.001$). In B a similar experiment is shown but with PLC$\zeta$ induced Ca$^{2+}$ oscillations. The sample trace in B shows that a 3 sec uncaging pulse of InsP$_3$ (at the arrow) caused an immediate Ca$^{2+}$ increase but no increase in frequency (n=23 eggs). We analysed 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 InsP$_3$ pulse was applied. Part 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. Part D shows a plot of these values for all 23 eggs tested.
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**FIGURE 3. Cytosolic Ca\(^{2+}\) and the frequency of Ca\(^{2+}\) oscillations.** In A an example is shown of a trace showing changes in cytosolic Ca\(^{2+}\) in an egg incubated in normal HKSOM media following the addition of 500nM thapsigargin (typical of n=12 eggs). The increase in Ca\(^{2+}\) was from 0.936 +/- 0.013 SEM to 1.07 +/- 0.0197 SEM which is a significant (P<0.01) . In B a similar example is shown of the addition 500nM thapsigargin to an egg in Ca\(^{2+}\) free media (containing 100μM EGTA), which was typical of n=12 eggs. The increase in basal Ca\(^{2+}\) 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 Ca\(^{2+}\) oscillations by 1.72 fold (+/-0.07 SEM). In D a trace is shown from an egg that was injected with PLCζ RNA and then placed in Ca\(^{2+}\) free media. The mean number of Ca\(^{2+}\) spikes in such experiments was 1.56 (n=18 eggs, +/-0.31 SEM) Ca\(^{2+}\) spikes in 10,000 sec (2hr 47 min) hours. The Ca\(^{2+}\) 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 PLCζ RNA and then placed in Ca\(^{2+}\) free media as in C. However, in these experiments 500nM thapsigarin was added after > 2 hours. In 16/17 such treated eggs there was an increase in the frequency of Ca\(^{2+}\) oscillations. There were an average of 1.77 spikes (+/-0.18 SEM) before adding thapsigarin but a mean of 7.11 spikes (+/-1.3 SEM) after thapsigarin addition. The resting Ca\(^{2+}\) 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 Ca\(^{2+}\) concentration (P<0.001).

**FIGURE 4. Simulation of InsP\(_3\) induced Ca\(^{2+}\) release in eggs.** Images are shown for a 2-dimensional simulation of the propagation of a Ca\(^{2+}\) wave in a mouse egg using a mechanism based upon Ca\(^{2+}\) induced InsP\(_3\) formation. Images for each time series is shown in each of the rows. For the first row the only source of PIP\(_2\) for making InsP\(_3\) is at the boundary (the plasma membrane) and this does not cause a wave at all. The times for each image (in seconds) in the top row is indicted by numbers above each image. In the next three rows the source of Ca\(^{2+}\) induced InsP\(_3\) formation is spaced at different distances. The time intervals for each image is indicated in the second row and it is then the same for each image going down in each column. In the first row the distance for the PIP\(_2\) is 4 μm, and again no Ca\(^{2+}\) wave can be generated. With a PIP\(_2\) source spaced at 3 or 2 μm we found that a Ca\(^{2+}\) wave can be generated. With a 2 μm separation a wave occurs that crosses the ‘egg’ in ~1 second.
**FIGURE 5. PIP\textsubscript{2} 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 hours after injection of PBP10 (n=21). Scale bars are 10\(\mu\)m. A nearest neighbour analysis indicated that the mean vesicle distance for all 21 control eggs is 2.2\(\mu\)m. In B an example is shown of an egg injected with PBP10 where and incubated in media 300 \(\mu\)M propranolol (n=13). In C particle analysis (n=14 eggs) indicates that the mean vesicle diameter is 0.89 \(\mu\)m and the mean number of vesicles present per egg is 298.9. C shows a plot of the total number of PIP\textsubscript{2} 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 \(\mu\)M 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 neighbour distances of PIP\textsubscript{2} positive vesicles present in eggs. The results are shown for parallel groups of eggs incubated in standard M2 media (control) (mean distance = 2.0 \(\mu\)m, n=7) and for those incubated in M2 containing 300 \(\mu\)M propranolol during imaging, (mean distance = 3.3 \(\mu\)m, 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).

**Fig.6. Ca\textsuperscript{2+} oscillations blocked by propranolol.** In A an example is shown of a mouse egg undergoing Ca\textsuperscript{2+} oscillations at fertilization where the addition of 300\(\mu\)M propranolol inhibited subsequent oscillations (n= 13 eggs). Before addition of propranolol the mean frequency was 12.2 +/-1.14 spikes/hour with all eggs oscillating. After adding propranolol there were 0.8 +/-0.23 spikes /hour (a significant difference from before propranolol, p=<0.0001). 6/13 eggs stopped oscillating immediately, 4/13 eggs had one Ca\textsuperscript{2+} spike and 3/13 has 2 spikes in an hour. B shows PLC\(\zeta\) cRNA (pipette concentration= 0.02 \(\mu\)g/\(\mu\)l) induced Ca\textsuperscript{2+} oscillations inhibited by propranolol (n=21 eggs). Before propranolol all eggs oscillated with 4.3 +/- 0.46 spikes / hour. After addition of propranolol there were 0.95 +/-0.25 spikes / hour (a significant difference p<0.0001). With propranolol, 10/21 eggs stopped Ca\textsuperscript{2+} oscillations, 6/21 showed a single spike and 5/21 had > 1 Ca\textsuperscript{2+} spike. In C an example is shown of an egg where PLC\(\zeta\) induced Ca\textsuperscript{2+} 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 / hour. After propranolol this decreased to 1.33 +/-0.29 spikes / hour, with all oscillations stopping after 2 spikes. When
propranolol was removed there were 10 +/-0.55 spikes in 30 mins. Adding propranolol and then removing it both caused significant changes in the number of Ca^{2+} spikes (p<0.001). D shows an example of an egg undergoing Ca^{2+} oscillations in response to Sr^{2+} media where propranolol was subsequently added (n=10 eggs). Before propranolol all eggs oscillated with 4.1 +/- 0.29 spikes / hour. After adding propranolol all eggs continues to oscillate with 3.9 +/-0.66 Ca^{2+} spikes/hour (not significantly different). In E an example is shown of an egg undergoing Ca^{2+} oscillations in response to microinjection of 5µM adenophostin in media that contained 300 µM propranolol form the start of the experiment (n=8 eggs). In propranolol there were 5.5 +/- 0.51 Ca^{2+} spikes 30 mins, compared with 10.4 +/- 0.71 spikes / 30 mins (n=11) for eggs in media with HKSOM. This is significantly different (unpaired t test, p= <0.0001).
Table 1. Parameter values for equations (1)-(5). All unit dimensions have been chosen to make $u$, $v$, and $p$ have units of $\mu$Mol, space is in $\mu$m and time is in seconds.

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<th>Definition</th>
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<td>$eb$</td>
<td>2</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$v_c$</td>
<td>9</td>
<td>Calcium release sensitivity to stored calcium</td>
</tr>
<tr>
<td>$ec$</td>
<td>2</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$u_{pa}$</td>
<td>0.45</td>
<td>Calcium release sensitivity to cytosolic calcium</td>
</tr>
<tr>
<td>$epa$</td>
<td>4</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$u_{pi}$</td>
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<td>Calcium release sensitivity to cytosolic calcium</td>
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<tr>
<td>$epi$</td>
<td>5</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$p_c$</td>
<td>0.1</td>
<td>Calcium release sensitivity to IP$_3$</td>
</tr>
<tr>
<td>$epc$</td>
<td>2</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>$\lambda_0$</td>
<td>1.5</td>
<td>Calcium store spacing</td>
</tr>
</tbody>
</table>
FIGURE 1

A Uncaging InsP$_3$- control

B Uncaging InsP$_3$- control

C PLCζ injection

D Sr$^{2+}$ media
FIGURE 2

A. $\text{Sr}^{2+}$

B. PLCζ

C. PLCζ

D. $\text{Ps}/T$ vs. $dt/T$

- $3s \text{InsP}_3$
- $10 \text{ mins}$
- $\text{OGBD} F/F_0$
- $\text{InsP}_3 \text{ pulse}$

In review
In review
FIGURE 5

A  PBP10

B  PBP10 + 300μM Propranolol

C  Number of PIP$_2$ vesicles

D  Distance between PIP$_2$ vesicles

In review