The dynamics of plasma membrane PtdIns(4,5)P_2 at fertilization of mouse eggs

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Summary

A series of intracellular Ca^{2+} oscillations are responsible for triggering egg activation and cortical granule exocytosis at fertilization in mammals. These Ca^{2+} oscillations are generated by an increase in inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which results from the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. Using confocal imaging to simultaneously monitor Ca^{2+} and plasma membrane PtdIns(4,5)P_2 in single living mouse eggs we have sought to establish the relationship between the kinetics of PtdIns(4,5)P_2 metabolism and the Ca^{2+} oscillations at fertilization. We report that there is no detectable net loss of plasma membrane PtdIns(4,5)P_2 either during the latent period or during the subsequent Ca^{2+} oscillations. When phosphatidylinositol 4-kinase is inhibited with micromolar wortmannin a limited decrease in plasma membrane PtdIns(4,5)P_2 is detected in half the eggs studied. Although we were unable to detect a widespread loss of PtdIns(4,5)P_2, we found that fertilization triggers a net increase in plasma membrane PtdIns(4,5)P_2 that is localized to the vegetal cortex. The fertilization-induced increase in PtdIns(4,5)P_2 follows the increase in Ca^{2+}, is blocked by Ca^{2+} buffers and can be mimicked, albeit with slower kinetics, by photoreleasing Ins(1,4,5)P_3. Inhibition of Ca^{2+}-dependent exocytosis of cortical granules, without interfering with Ca^{2+} transients, inhibits the PtdIns(4,5)P_2 increase. The increase appears to be due to de novo synthesis since it is inhibited by micromolar wortmannin. Finally, there is no increase in PtdIns(4,5)P_2 in immature oocytes that are not competent to extrude cortical granules. These studies suggest that fertilization does not deplete plasma membrane PtdIns(4,5)P_2 and that one of the pathways for increasing PtdIns(4,5)P_2 at fertilization is invoked by exocytosis of cortical granules.

Key words: Phosphatidylinositol 4,5-bisphosphate, Oocyte, GFP

Introduction

At fertilization in mammals, the sperm triggers a series of low-frequency cytosolic Ca^{2+} oscillations that continue for several hours (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992). This Ca^{2+} signal is responsible for the exocytosis of cortical granules, resumption of meiosis and activation of development (Kline and Kline, 1992; Miyazaki et al., 1993; Xu et al., 1994; Abbott and Ducibella, 2001). In mammals, the main intracellular Ca^{2+} channel involved in Ca^{2+} release at fertilization is the type I Ins(1,4,5)P_3-receptor [Ins(1,4,5)P_3R]. It is present far in excess of the other isoforms (Jellerette et al., 2000; Brind et al., 2000) and injection of functionally inhibitory antibodies or downregulation of the receptor during maturation, both lead to an inhibition of fertilization-induced Ca^{2+} transients (Miyazaki et al., 1993; Brind et al., 2000). Thus, Ca^{2+} release and egg activation at fertilization is triggered by Ins(1,4,5)P_3 production.

Ins(1,4,5)P_3 is generated via phospholipase C (PLC)-mediated hydrolysis of PtdIns(4,5)P_2 (Berridge, 1993). The mechanism of PLC activation at fertilization is an active area of research and two main models are emerging in which either sperm-derived PLCs (mammals) or egg-derived PLCs (sea urchins/starfish) are activated (for reviews, see Stricker, 1999; Jaffe et al., 2001; Carroll, 2001). In mammals, PLC activation and the resultant PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3 production must persist for several hours to support the generation of long-lasting Ca^{2+} oscillations. Studies in somatic cells suggest that the dynamics of PtdIns(4,5)P_2 hydrolysis and Ins(1,4,5)P_3 production that are required to bring about Ca^{2+} oscillations involve a pulsatile generation of Ins(1,4,5)P_3 that initiates each Ca^{2+} spike (Meyer and Stryer, 1988; Harootunian et al., 1991; Hirose et al., 1999; Nash et al., 2001) or a persistent low level of activation that sensitizes the Ins(1,4,5)P_3R to Ca^{2+}-induced Ca^{2+} release (Wakui et al., 1989; Missiaen et al., 1991; Berridge, 1993). In mouse eggs, the finding that non-hydrolysable Ins(1,4,5)P_3 analogues (Sato et al., 1998) and low persistent levels of Ins(1,4,5)P_3 (Jones and Nixon, 2000) can cause repetitive Ca^{2+} oscillations provides some support for the latter of these two models.

To date, Ins(1,4,5)P_3 and PtdIns(4,5)P_2 have been measured at fertilization in large populations of sea urchin and *Xenopus* eggs using biochemical mass assays. These studies have revealed that both Ins(1,4,5)P_3 and its precursor PtdIns(4,5)P_2 increase at the time of fertilization (Turner et al., 1984; Ciapa et al., 1992; Stith et al., 1993; Stith et al., 1994; Snow et al., 1996). This apparently counterintuitive result is explained by the increase in PtdIns(4,5)P_2 being a small difference in a very large increase in the turnover of polyphosphoinositides (PPI)

(Ciapa et al., 1992). Although these biochemical studies provide insightful snapshots into the metabolism of PPI at fertilization in sea urchins, it is not known precisely when PtdIns(4,5)P_2 changes start in relation to the Ca^{2+} wave or whether a similar large increase in PPI turnover is necessary to drive long-lasting Ca^{2+} oscillations in mammals.

In addition to PtdIns(4,5)P_2 providing a source of Ins(1,4,5)P_3 for the release of Ca^{2+}, recent evidence suggests that PtdIns(4,5)P_2 is also required during exocytosis. In this role PtdIns(4,5)P_2 acts as a signaling molecule, rather than as a substrate for an enzyme, that can interact directly with target proteins containing PtdIns(4,5)P_2-binding motifs. Acting in this mode, it has been proposed that PtdIns(4,5)P_2 is involved in a number of steps in Ca^{2+}-dependent exocytosis, including vesicle docking, priming, fusion, actin remodeling and subsequent endocytosis (Martin, 1998; Martin, 2001; Cremona and De Camilli, 2001). Presumably, PtdIns(4,5)P_2 also plays a role in the Ca^{2+}-dependent exocytosis of cortical granules that is stimulated at fertilization (Xu et al., 1994; Abbott and Ducibella, 2001). As such, PtdIns(4,5)P_2 is required to play multiple roles at fertilization. First, it is required for hydrolysis in order to supply Ins(1,4,5)P_3 and, as a consequence of the Ins(1,4,5)P_3-induced Ca^{2+} release, it is required in a signaling role to participate in the exocytosis of cortical granules. Clearly a tight regulation of PtdIns(4,5)P_2 is required at fertilization if it is to perform these dual, and competing roles.

Recently, it has become possible to monitor PtdIns(4,5)P_2 in living cells using a GFP-fusion protein (Stauffer et al., 1998; Varnai and Balla, 1998). The indicator consists of the pleckstrin homology (PH) domain of PLCδ1 coupled to GFP (PH-GFP). The PH domain has a high affinity for PtdIns(4,5)P_2 and localises to the plasma membrane, consistent with the known distribution of PtdIns(4,5)P_2 in mammalian cells (Lemmon et al., 1995; Kavran et al., 1998; Balla et al., 2000). The fusion protein provides a dynamic measure of PtdIns(4,5)P_2 since activation of PLC and hydrolysis of PtdIns(4,5)P_2 leads to a redistribution of PH-GFP from the plasma membrane to the cytosol (Stauffer et al., 1998; Varnai and Balla, 1998; van der Wal et al., 2001). In some cell types, high levels of Ins(1,4,5)P_3, which also has a high affinity for PH-GFP, can compete with PtdIns(4,5)P_2 for PH-GFP (Hirose et al., 1999; Nash et al., 2001). As such there is some controversy as to whether PH-GFP reports PtdIns(4,5)P_2 or Ins(1,4,5)P_3 (van der Wal et al., 2001). In this study, we use PH-GFP to report plasma membrane PtdIns(4,5)P_2 in mouse eggs at the time of fertilization. We found no evidence for significant loss of plasma membrane PtdIns(4,5)P_2, rather a net increase that is dependent on exocytosis of cortical granules.

Materials and Methods

Gametes collection and fertilization

Immature germinal vesicle-stage (GV) oocytes and mature metaphase II-arrested (MII) oocytes were recovered from hormone-primed MF1 mice and stored in M2 medium (Fulton and Whittingham, 1978) as previously described (Brind et al., 2000). Sperm from the epididymis of proven MF1 mice was released in 1 ml of T6 (Quinn et al., 1982) and allowed to disperse for 20 minutes before diluting in a total of 5 ml of the same medium for capacitation. For in vitro fertilization, the zona pellucida was removed by a short incubation in acidic Tyrode's at 37°C. Zona-free oocytes were fertilized on the stage of the confocal microscope by adding 20 μl of the sperm suspension into the incubation chamber. All experiments were conducted in M2 at 37°C.

DNA construct and cRNA microinjection

The chimera encoding the PH domain of PLCδ1 fused to enhanced green fluorescent protein (PH-GFP) was constructed as described previously (Varnai and Balla, 1998), and cloned into pcDNA3.1, which contains a T7 primer enabling cRNA synthesis. Plasmids were linearised after the 3′UTR with *Sma*I and in vitro transcription was performed with T7 polymerase (Promega) according to the manufacturer's protocol. The cRNA was purified by RNeasy column (Qiagen) and polyadenylated as described previously (Subramanian and Meyer, 1997). The polyadenylated cRNA was recovered by RNeasy column in Rnase-free water, aliquoted and stored at –80°C. cRNA was diluted in injection buffer (120 mM KCl, 20 mM Hepes, pH 7.4) to a final concentration of 0.1-0.2 μg/μl, and microinjected (estimated 2-5% of egg volume) using pressure-injection. Imaging was started 2-3 hours after cRNA injection. Some oocytes were injected with a cRNA encoding the same fusion-protein, carrying a point mutation (R40L) in the PH domain on one critical basic residue involved in PtdIns(4,5)P_2 binding (Varnai and Balla, 1998).

Manipulation and treatment of oocytes

For $[Ca^{2+}]_i$ measurements, oocytes were incubated in M2 containing 10 μM fura-red-AM (Molecular Probes) at 37°C for 15 minutes. In wortmannin experiments, oocytes were preincubated with 30 μM wortmannin (Calbiochem) for 15 minutes. This rather high concentration was used in order to fully block type III phosphatidylinositol 4-kinase (PtdIns 4-kinase) activity in vivo using a short incubation time, and considering that oocytes have a relatively low surface area/volume ratio compared with somatic cells. For BAPTA experiments, oocytes were preincubated with 1 or 10 μM BAPTA-AM (Molecular Probes) at 37°C for 15 minutes. Since 10 μM BAPTA-AM abolished Ca^{2+} oscillations, fertilization was confirmed at the end of the experiments by staining chromatin with Hoechst 33342 (see below). For jasplakinolide experiments, oocytes were preincubated with 100 nM Jasplakinolide (Molecular Probes) for 30-60 minutes. During the recordings, Jasplakinolide was continuously present in the experimental chamber at the optimal final concentration of 100 nM (Terada et al., 2000). For caged-Ins(1,4,5)P_3 experiments, oocytes expressing PH-GFP were further injected with NPE-caged Ins(1,4,5)P_3 (Molecular Probes; 1 mM pipette concentration) 30 minutes prior to the imaging (see below). Injections were 2-5% of egg volume (based on cytoplasmic displacement) giving an estimated final concentration in the range of 20-50 μM. Some oocytes expressing PH-GFP were also injected with the catalytically active light chain of Botulinum neurotoxin A (BoNT/A-LC, 5 μM in the pipette). The final concentration of BoNT/A in the eggs is 100-250 nM, based on the injection volume described above. BoNT/A-LC was kindly provided by G. Schiavo (Imperial Cancer Research Fund, London, UK).

Confocal imaging and Ins(1,4,5)P_3 uncaging

Zona-free oocytes were transferred to an experimental chamber seated in a heating stage and were observed with a Zeiss LSM-510 laser-scanning microscope (Carl Zeiss Inc.) with a 20× (0.75 NA) objective. In fertilization experiments, confocal images were taken at 7 or 10 second intervals, as soon as one sperm remained bound to the oocyte. Transmitted light images, GFP and fura-red fluorescence images were acquired through the equator of the oocyte, using the 488 nm line of an argon laser. GFP fluorescence from the PH-GFP fusion protein was recorded through a BP505-530 emission filter. Fura-red fluorescence and GFP were monitored in the same confocal slice, using a

LP650 emission filter. For caged-Ins(1,4,5)P_3 experiments, oocytes were scanned simultaneously with the 488 nm line of the Argon laser and the 364 nm line of a UV laser (1.5 mW). The intensity of UV illumination was adjusted by setting the acusto-optical tunable filter (AOTF) to 0.5 or 5% excitation, in order to photolyse low and high amounts of caged-Ins(1,4,5)P_3, respectively. For all experiments, the 488 nm laser power and pin-hole size were kept the same (confocal slices were 3.5 μm thick). For DNA staining, oocytes were incubated with 1 μg/ml Hoechst 33342 for 15 minutes and images of the chromosomes were acquired with UV laser scanning.

Confocal data analysis

Confocal images were analyzed using Metamorph (Universal Imaging). Plasma membrane and cytoplasmic GFP fluorescence were measured by drawing regions around the egg's perimeter and in the cytoplasm, respectively, and expressed as arbitrary units. Fura-red fluorescence was measured in a cytoplasmic circular region. The same regions were used for each individual frame of an image series. Calculation of the ratio of membrane to cytosolic GFP fluorescence and graphs displaying GFP or Fura-red fluorescence along time, were obtained with Microsoft Excel 2000. Since Fura-red fluorescence decreases when $[Ca^{2+}]_i$ rises, changes in $[Ca^{2+}]_i$ were expressed as the negative value of the change in Fura-red fluorescence relative to baseline fluorescence (ΔF/F), to obtain positive values for measurements of amplitude and rate of rise.

Assay for exocytosis of cortical granules

Cortical granule exocytosis was detected on living eggs by the fluorescent lectin-staining method (Lee et al., 1988). Zona-free oocytes were fertilized in M2 by incubation with sperm for 15 minutes at 37°C, then washed and left in M2 for 20 minutes. Fertilized eggs were incubated with FITC- or TRITC-conjugated *Lens culinaris* agglutinin (FITC/TRITC-LCA) for 3 minutes. Eggs were then washed three times in M2 with gentle pipetting, and the pattern of FITC/TRITC-LCA staining was observed by fluorescence microscopy using a fluorescein or rhodamine filter set and a 40× objective. Images were acquired with a CCD camera (Princeton) and analyzed with Metafluor (Universal Imaging). Eggs displaying the typical punctate staining pattern on their surface were considered to have released their cortical granules.

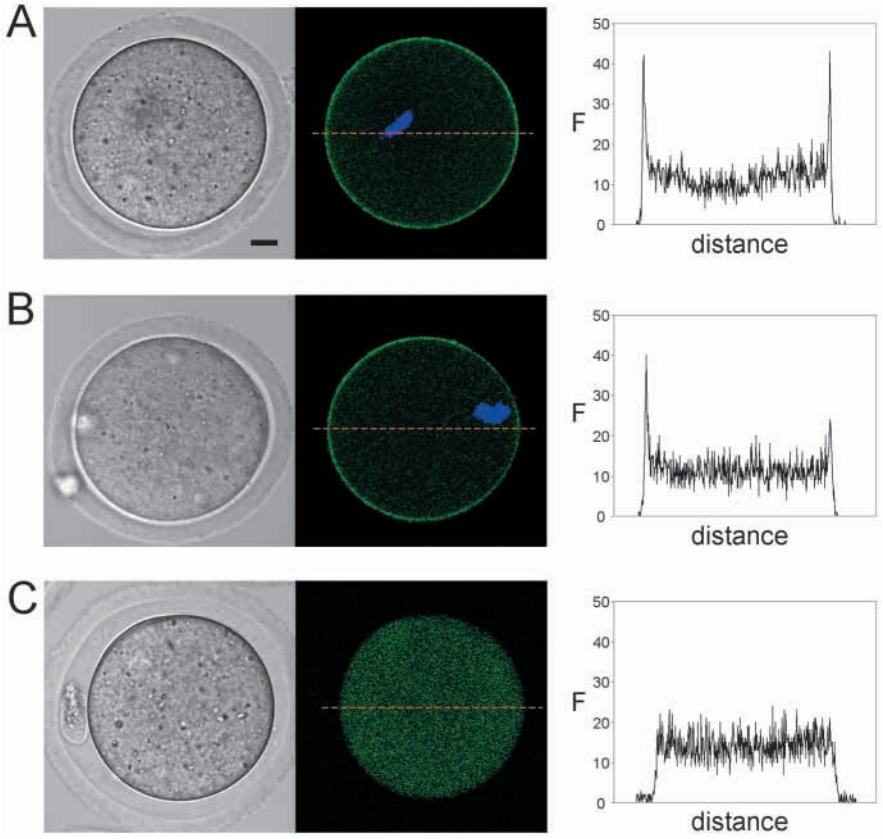

Fig. 1. PH-GFP binds to plasma membrane PtdIns(4,5)P_2 in mouse oocytes. Confocal images of GFP fluorescence in the equatorial plane of mouse MII oocytes, acquired 2-3 hours after injection with the cRNA encoding PH-GFP (A,B) or the mutant PH(R40L)-GFP, which does not bind to PtdIns(4,5)P_2 (C). Corresponding line intensity profiles are displayed on the right, with GFP fluorescence expressed as relative fluorescence units (F) and bright field images shown on the left. (A) MII oocyte with the animal pole out of focus (fluorescence from the chromosomes, stained with Hoechst 33342, was captured in a plane above and superimposed). (B) MII oocyte with the animal pole in the focal plane. Note the higher fluorescence intensity associated with the plasma membrane in the vegetal pole. (C) MII oocyte expressing PH(R40L)-GFP. For each condition, similar patterns of staining were obtained in at least 20 oocytes. Bar, 10 μm.

Results

Expression of PH-GFP in mouse eggs

To monitor PtdIns(4,5)P_2 in mouse eggs we expressed a fusion protein of the pleckstrin homology (PH) domain of PLCδ1 and green fluorescent protein (PH-GFP). PH-GFP has been used previously to monitor the dynamics of PtdIns(4,5)P_2 or Ins(1,4,5)P_3 (Stauffer et al., 1998; Varnai and Balla, 1998; Hirose et al., 1999; Balla et al., 2000; van der Wal et al., 2001; Nash et al., 2001). PH-GFP exhibited a strong accumulation in the plasma membrane (PM), whereas cytoplasmic fluorescence (C) was low and homogeneous (Fig. 1A). A chimera carrying a point mutation preventing PtdIns(4,5)P_2 recognition by the PH domain (PH(R40L)-GFP) did not localize to the plasma membrane (Fig. 1C), which suggests that the cortical accumulation of PH-GFP reflects binding to plasma membrane PtdIns(4,5)P_2. In addition, plasma membrane staining was enriched in the vegetal pole of the oocytes (Fig. 1B). This may result from a specific localization of the PtdIns(4,5)P_2 synthesis machinery and/or the presence of microvilli, which increase the plasma membrane area (Lee et al., 1988).

We next sought to verify that PH-GFP did not interfere with the early events of fertilization. Monitoring $[Ca^{2+}]_i$ in PH-GFP-expressing eggs revealed that the fusion protein had no effect on the ability of the sperm to generate the typical series of Ca^{2+} transients (Fig. 2). In addition, exocytosis, as monitored by lectin staining, was similar in eggs expressing PH-GFP [87% labeled (13/15)] and in non-expressing controls [85% labeled (23/27)]. Thus, Ca^{2+} release and exocytosis proceeds normally in the presence of PH-GFP.

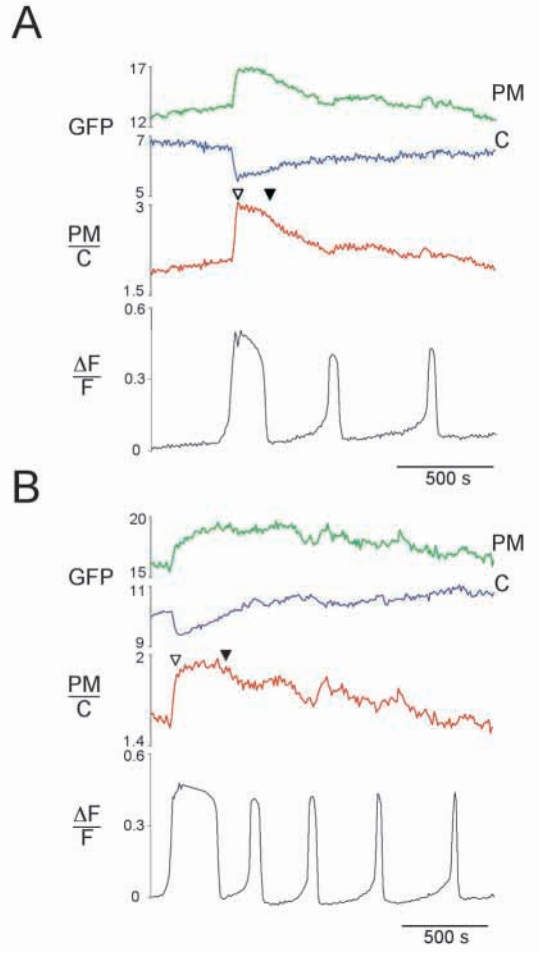

Fig. 2. Plasma membrane PtdIns(4,5)P_2 increases at fertilization. MII oocytes were fertilized on the stage of the microscope and GFP fluorescence (in arbitrary units; PM, plasma membrane; C, cytoplasm) and Fura-red fluorescence (ΔF/F) were continuously recorded at a rate of 1 frame every 7 seconds (A) or 10 seconds (B). Note the translocation of PH-GFP from the cytoplasm to the plasma membrane. The increase in PM/C ratio indicates an increase in plasma membrane PtdIns(4,5)P_2. The white and black arrowheads indicate the position for measurement of the peak change in PM/C and value of PM/C at the end of the first Ca^{2+} transient, respectively. This data is displayed in Table 1 as the ΔPM/C(peak) and ΔPM/C(end) and used to compare the different properties of the increase in PtdIns(4,5)P_2.

Plasma membrane PtdIns(4,5)P_2 increases at fertilization

To examine PtdIns(4,5)P_2 dynamics at fertilization we monitored $[Ca^{2+}]_i$ and PtdIns(4,5)P_2 simultaneously. At fertilization in mice there is a latent period of 1-3 minutes between the time of sperm-egg fusion and the initiation of the first Ca^{2+} transient (Lawrence et al., 1997; Jones et al., 1998). Close examination of PtdIns(4,5)P_2 during the latent period did not reveal any detectable change in plasma membrane PtdIns(4,5)P_2 (Fig. 1). The first change in PtdIns(4,5)P_2 was stimulated within one minute of the first Ca^{2+} transient and took the form of an increase in plasma membrane PH-GFP fluorescence and a concomitant decrease in the cytoplasm (Fig. 2A). This increase in the PM/C ratio reflects the recruitment of PH-GFP to the plasma membrane, which indicates an increase in PtdIns(4,5)P_2. The recruitment of PH-GFP peaked 30 seconds after the first peak of $[Ca^{2+}]_i$ and was sustained while $[Ca^{2+}]_i$ remained elevated, before slowly returning to baseline (Fig. 2A; Table 1). There was often a second or third PtdIns(4,5)P_2 increase of a smaller amplitude associated with subsequent Ca^{2+} transients. In some eggs with high frequency Ca^{2+} oscillations, the increase in PtdIns(4,5)P_2 was sustained for up to 10 minutes (Fig. 2B). Therefore, the increase in PtdIns(4,5)P_2 showed a complex temporal pattern that was dependent on a short-lived mechanism that could be influenced by the frequency of Ca^{2+} oscillations.

To examine the spatial organization of the increase in PtdIns(4,5)P_2, confocal scans along the animal-vegetal axis were collected during fertilization and analyzed to determine the relative changes in PtdIns(4,5)P_2 in the plasma membrane of both poles. The fluorescence was lower in the animal pole at rest but there was no detectable increase at fertilization (Fig. 3A). By contrast, in the vegetal pole, a reliable increase in PtdIns(4,5)P_2 was stimulated (Fig. 3A,B). The increase was reasonably uniform throughout the confocal slice of the vegetal hemisphere suggesting that it was not due to a highly localized increase at the site of sperm-egg fusion (Fig. 3B). Thus,

Table 1. Amplitude and kinetic parameters of the rises in $[Ca^{2+}]_i$ and PtdIns(4,5)P_2 at fertilization

	$[Ca^{2+}]_i$ (Fura-red)		PtdIns(4,5)P_2 (PH-GFP)				
	ΔF/F	Rate ×10^3	ΔPM/C (peak)	Rate ×10^3	ΔPM/C (end)	Delay (starts)	Delay (peaks)
Control (*n*=9)	0.55±0.03	12.3±0.8	0.24±0.02	8.9±2.5	0.27±0.06	58±6	31±9
Wortmannin (*n*=8)	0.54±0.04	13.4±1.3	0.09±0.03†	2.1±1.4*	0.02±0.03†	80±10	26±3
BAPTA-AM (*n*=15)	0.53±0.02	8.9±1.0*	0.15±0.02*	1.9±0.4†	0.04±0.02†	113±12†	ND
Caged-InsP_3 (*n*=8)	0.51±0.03	41.3±8.4†	0.19±0.03	2.3±0.2*	0.18±0.03	20±8†	86±13†
Jasplakinolide (*n*=6)	0.52±0.05	11.0±1.1	0.15±0.04	7.1±2.2	0.01±0.01†	77±6‡	6±3*,‡
BoNT/A (*n*=9)	0.57±0.02	14.0±1.3	ND	3.0±0.6*	0.28±0.03	72±11	ND

Changes in Fura-red and GFP fluorescence were measured for each experimental condition. Parameters are given for the first Ca^{2+} transient and the first rise in PtdIns(4,5)P_2 triggered at fertilization. The amplitude (in arbitrary units) and maximal rate of rise (in arbitrary units.s^{-1}) were measured for the first peak in $[Ca^{2+}]$ (ΔF/F) and the first peak in PM/C [ΔPM/C (peak)]. The amplitude of the change in PM/C was also measured after termination of the first Ca^{2+} transient (as indicated in Fig. 2) in order to give an estimation of the duration of the rise in PtdIns(4,5)P_2. Delay (starts/peaks) indicates the delay, in seconds, separating the onset or first peak of the rise in PtdIns(4,5)P_2 from the onset or first peak of the rise in $[Ca^{2+}]_i$, respectively. Caged-InsP_3 data refer to experiments conducted with 0.5% UV intensity. Data are mean±s.e.m. ND, not determined.

*P<0.05.

†P<0.01.

‡n=5.

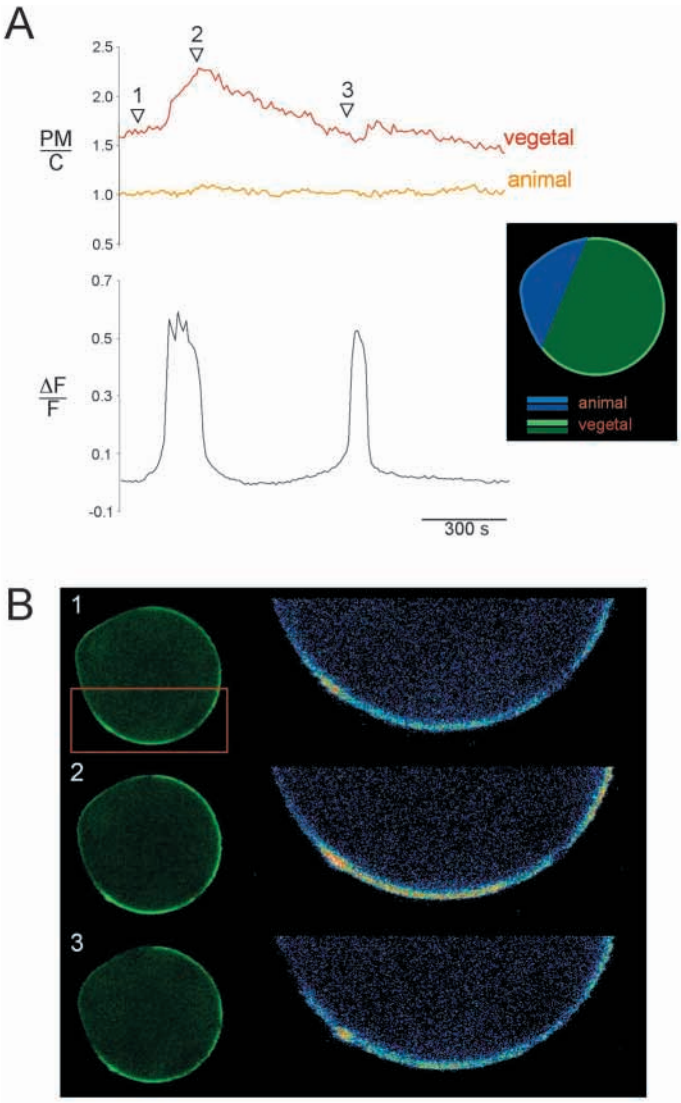

Fig. 3. The increase in PtdIns(4,5)P_2 is polarized to the vegetal pole. (A) Changes in $[Ca^{2+}]_i$ and PH-GFP distribution at fertilization in an egg in which the confocal slice was taken through both the animal and vegetal poles. Drawing on right illustrates the regions selected for measurements of PM and C fluorescence in the animal (dark/light blue) and vegetal (dark/light green) poles. Changes in PM/C in the vegetal and animal poles are illustrated by a red or orange trace, respectively. For abbreviations, see Fig. 2 legend. (B) GFP images corresponding to selective time points (arrowheads numbered 1-3 in A) for the experiment described in A. The lower region of the egg (red box) was expanded and displayed in pseudocolor to show more clearly the changes in fluorescence intensity.

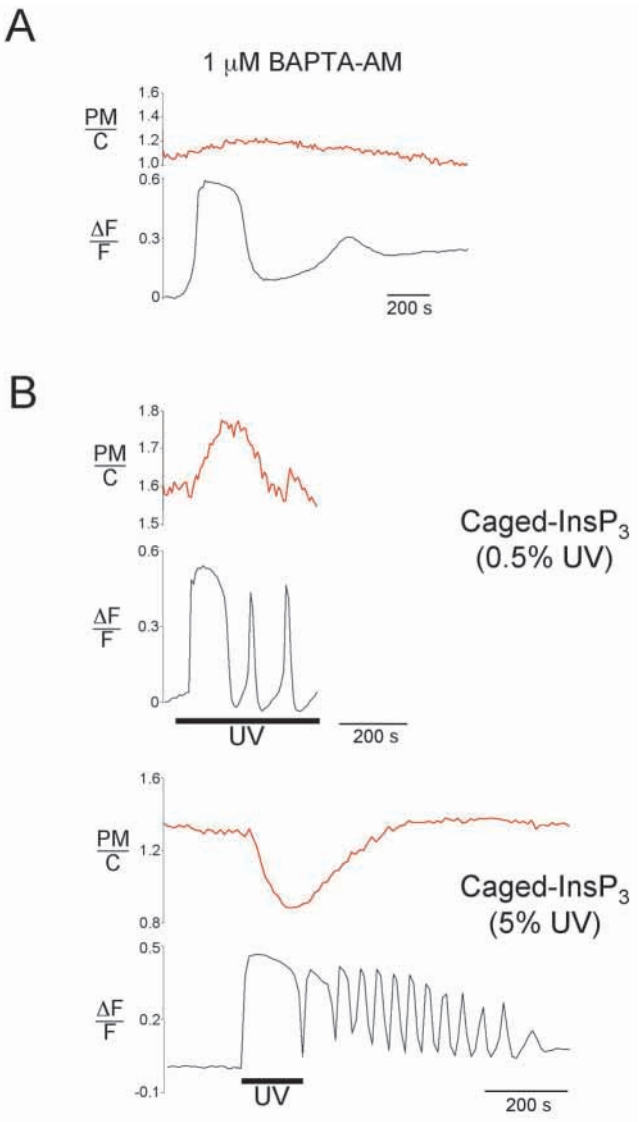

Fig. 4. PtdIns(4,5)P_2 synthesis at fertilization is Ca^{2+}-dependent. (A) Changes in $[Ca^{2+}]_i$ and PH-GFP distribution recorded at fertilization in an egg pretreated with 1 μM BAPTA-AM. Similar results were obtained in 15 eggs. (B) Upper panel, changes in $[Ca^{2+}]_i$ and PH-GFP distribution elicited by uncaging Ins(1,4,5)P_3 using a low UV power (0.5% excitation-indicated by the horizontal bar). Similar results were obtained in eight eggs. Lower panel, changes in $[Ca^{2+}]_i$ and PH-GFP distribution elicited by uncaging Ins(1,4,5)P_3 using a tenfold higher UV power (5% excitation-indicated by the horizontal bar). Similar results were obtained in five eggs. For abbreviations, see Fig. 2 legend.

the increase in PtdIns(4,5)P_2 at fertilization is spatially and temporally organized.

An increase in $[Ca^{2+}]_i$ is necessary and sufficient for the increase in PtdIns(4,5)P_2

The close association between the increases in $[Ca^{2+}]_i$ and PtdIns(4,5)P_2 raises the question whether Ca^{2+} is necessary and sufficient for the stimulation of PtdIns(4,5)P_2 synthesis. To investigate whether an increase in $[Ca^{2+}]_i$ is necessary, we used BAPTA to buffer changes in $[Ca^{2+}]_i$. In eggs preincubated with 10 μM BAPTA-AM for 15 minutes, fertilization-induced increases in $[Ca^{2+}]_i$ and PtdIns(4,5)P_2 were completely abolished (not shown; n=10). Further, slowing the kinetics of Ca^{2+} release by using lower concentrations of BAPTA-AM (1 μM, 15 minutes) strongly attenuated the increase in PtdIns(4,5)P_2 (Fig. 4A; Table 1). These experiments suggest a requirement for rapid changes in $[Ca^{2+}]_i$ to induce the increase in PtdIns(4,5)P_2 at fertilization.

To examine whether the Ca^{2+} increase is sufficient to

stimulate PtdIns(4,5)P_2 synthesis, we generated Ca^{2+} transients in unfertilized MII oocytes by UV-uncaging of Ins(1,4,5)P_3. Uncaging low levels of Ins(1,4,5)P_3 continuously by using a UV laser attenuated to 0.5% of maximum power, generated Ca^{2+} transients similar to those seen at fertilization (Fig. 4B, upper panel). During the first Ins(1,4,5)P_3-induced Ca^{2+} transient, translocation of PH-GFP to the plasma membrane was observed, although at a significantly slower rate than seen at fertilization (Fig. 4B, upper panel; Table 1). The ability to drive an increase in PtdIns(4,5)P_2 by triggering Ins(1,4,5)P_3-induced Ca^{2+} release suggests a Ca^{2+}-dependent process is controlling the increase in PtdIns(4,5)P_2 at fertilization. The slower rate of rise indicates that fertilization provides a more effective stimulus for PtdIns(4,5)P_2 synthesis, perhaps by invoking additional signaling pathways.

PH-GFP reports PtdIns(4,5)P_2 rather than Ins(1,4,5)P_3 in mouse eggs

PH-GFP has a high affinity for Ins(1,4,5)P_3 and has previously been used as an Ins(1,4,5)P_3 reporter (Lemmon et al., 1995; Hirose et al., 1999; Nash et al., 2001). However, the increase in plasma membrane PH-GFP fluorescence detected at fertilization suggests that PH-GFP is reporting PtdIns(4,5)P_2 and not being influenced by fertilization-induced increases in Ins(1,4,5)P_3. To determine whether high levels of Ins(1,4,5)P_3 may compete with PtdIns(4,5)P_2 for binding PH-GFP, we released excess Ins(1,4,5)P_3 using a tenfold higher UV laser intensity (attenuated to 5% of maximum power). The results show that excess Ins(1,4,5)P_3 causes reversible translocation of PH-GFP from the plasma membrane to the cytoplasm (Fig. 4B, lower panel). This suggests that PH-GFP can report changes in Ins(1,4,5)P_3 but, in mouse eggs, with a relatively low surface area-to-volume ratio, the concentration of Ins(1,4,5)P_3 generated at fertilization is not sufficient to displace PH-GFP from the plasma membrane. This data, consistent with a previous report (van der Wal et al., 2001), further validates our use of this probe as an indicator of plasma membrane PtdIns(4,5)P_2.

The increase in PtdIns(4,5)P_2 is a result of exocytosis

We have demonstrated that Ca^{2+} is necessary and sufficient for the increase in PtdIns(4,5)P_2, but how Ca^{2+} stimulates this increase is not known. A number of observations point to an association with cortical granule exocytosis (CGE). First, the Ca^{2+} requirement for both processes is similar; second, cortical granules are located in the vegetal pole, which is consistent with polarized PtdIns(4,5)P_2 synthesis; and third, the transient nature of the PtdIns(4,5)P_2 increase is consistent with the kinetics of exocytosis in mammalian eggs (Kline and Stewart-Savage, 1994). The relationship between PtdIns(4,5)P_2 synthesis and exocytosis was investigated using actin filament-targeting drugs known to inhibit CGE (Terada et al., 2000; Abbott and Ducibella, 2001). In preliminary experiments, we noticed that the fertilization-associated rise in PtdIns(4,5)P_2 was inhibited by cytochalasin B (n=5; not shown). The possibility that actin filament disassembly by cytochalasin B may interfere with localization of some elements of the PtdIns(4,5)P_2 synthesis pathway prompted us to investigate the effects of the actin filaments-stabilizing reagent jasplakinolide. Jasplakinolide has recently been shown to inhibit exocytosis in fertilized mouse eggs (Terada et al., 2000), a finding we confirmed by monitoring CGE with lectin staining (Fig. 5D). Exocytosis was found to occur in only 15% (6/39) of fertilized eggs treated with jasplakinolide (100 nM), compared with 85% of fertilized control eggs. In eggs treated with jasplakinolide (100 nM) normal Ca^{2+} oscillations were generated; however, the duration of the rise in PtdIns(4,5)P_2 was reduced to a small short-lived transient that finished well before the first Ca^{2+} transient returned to baseline (Table 1; Fig. 5A). Changes in PH-GFP localization were never observed for the subsequent Ca^{2+} transients, and one egg displayed no change in PH-GFP (not shown).

To interfere more specifically with exocytosis, eggs were injected with the catalytically active light chain of Botulinum neurotoxin A (BoNT/A-LC; 5 μM). This toxin cleaves SNAP-25, which is a plasma membrane protein essential for Ca^{2+}-dependent exocytosis (Xu et al., 1998) (for a review, see Schiavo et al., 2000). In secretory cells, cleavage of SNAP-25 by BoNT/A slows the kinetics of the Ca^{2+}-triggered exocytic burst (Xu et al., 1998; Schiavo et al., 2000). BoNT/A also cleaves SNAP-25 and inhibits exocytosis in mouse eggs (Ikebuchi et al., 1998). In our hands exocytosis was detected in 47% (16/34) of fertilized eggs previously injected with BoNT/A-LC compared with 85% of controls, which indicates a partial inhibition of exocytosis. To examine the effect of this inhibition on PtdIns(4,5)P_2 synthesis, BoNT/A-LC was injected into eggs expressing PH-GFP. At fertilization, Ca^{2+} transients were similar to controls but the rate of rise of PtdIns(4,5)P_2 was threefold slower than controls and no obvious peak was detectable (Table 1; Fig. 5B). Together, these data show that interfering with exocytosis prevents the full development of the rise in plasma membrane PtdIns(4,5)P_2 (c.f. Fig. 5C).

The increase in PtdIns(4,5)P_2 is not stimulated at fertilization of immature oocytes

At fertilization of immature oocytes it is known that Ca^{2+} oscillations are attenuated (Fujiwara et al., 1993; Mehlmann and Kline, 1994) (for a review, see Carroll et al., 1996) and that they do not have the capacity to undergo Ca^{2+}-activated exocytosis (Abbott and Ducibella, 2001). Therefore, it was of interest to examine the PtdIns(4,5)P_2 dynamics in immature oocytes. PH-GFP fluorescence was distributed evenly around the plasma membrane (Fig. 6A). At fertilization, Ca^{2+} transients in immature oocytes were smaller and did not persist for as long as those seen in mature eggs (Fig. 6B). During these increases in $[Ca^{2+}]_i$, there were no detectable changes in the distribution of PH-GFP (Fig. 6B). This finding is consistent with exocytosis being required for the increase in PtdIns(4,5)P_2, although other developmental events may also be required.

Micromolar wortmannin inhibits the PtdIns(4,5)P_2 increase but not exocytosis

The increase in PH-GFP staining at fertilization may be a result PtdIns(4,5)P_2 synthesis or an increase in PtdIns(4,5)P_2 available for binding to PH-GFP. To investigate the possibility that exocytosis increases PtdIns(4,5)P_2 synthesis we have pre-

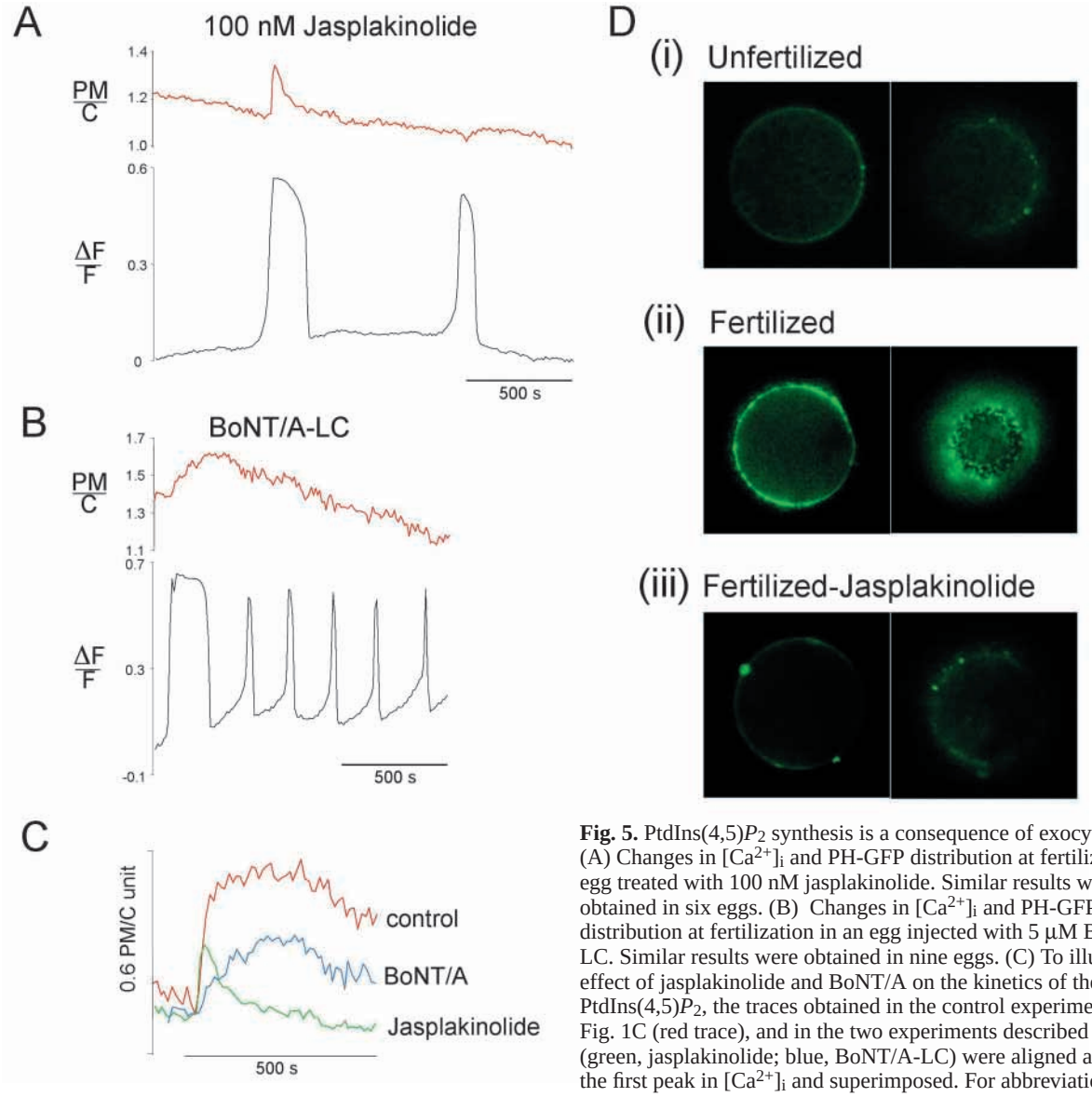

Fig. 5. PtdIns(4,5)P_2 synthesis is a consequence of exocytosis. (A) Changes in $[Ca^{2+}]_i$ and PH-GFP distribution at fertilization in an egg treated with 100 nM jasplakinolide. Similar results were obtained in six eggs. (B) Changes in $[Ca^{2+}]_i$ and PH-GFP distribution at fertilization in an egg injected with 5 μM BoNT/A-LC. Similar results were obtained in nine eggs. (C) To illustrate the effect of jasplakinolide and BoNT/A on the kinetics of the rise in PtdIns(4,5)P_2, the traces obtained in the control experiment shown in Fig. 1C (red trace), and in the two experiments described above (green, jasplakinolide; blue, BoNT/A-LC) were aligned according to the first peak in $[Ca^{2+}]_i$ and superimposed. For abbreviations, see Fig. 2 legend. (D) FITC-LCA labelling on (i) unfertilized MII oocyte; (ii) control fertilized egg; and (iii) jasplakinolide (100 nM)-treated fertilized egg. For each condition, the equatorial plane (left) and another focal plane close to the surface (right) of the same cell are displayed. Note the incomplete ring of fluorescence in the equatorial planes, illustrating the cortical granule-free domain in the animal pole. Exocytosis is indicated by an increased fluorescence in the equatorial plane and by a punctate fluorescence in the section close to the surface. Similar patterns of staining were observed in at least 20 cells, for each condition.

treated the oocytes with micromolar wortmannin (30 μM, 15 minutes), which inhibits the type III PtdIns 4-kinase (Downing et al., 1996). Treatment with wortmannin abolished the translocation of PH-GFP to the plasma membrane in all eggs examined (Fig. 7A). In four out of eight eggs, small downward inflections in the PM/C ratio were evident, suggesting that a limited hydrolysis of PtdIns(4,5)P_2 was revealed when synthesis was inhibited (Fig. 7A; Table 1). These effects were not due to inhibition of PtdIns 3-kinase, as a low concentration of wortmannin (100 nM) had no effect on PtdIns(4,5)P_2 synthesis (n=3, not shown).

Finally, since PtdIns(4,5)P_2 has been suggested to be necessary for exocytosis (Martin, 1998; Martin, 2001; Holz et al., 2000; Cremona and De Camilli, 2001), we inhibited the PtdIns(4,5)P_2 increase with wortmannin and examined the effect on exocytosis using lectin staining. The data show that wortmannin-treated eggs and controls fertilized in the absence of wortmannin display a similar staining pattern. Thus wortmannin had no effect on the exocytosis of cortical granules [80% eggs labeled (37/46); Fig. 7B] suggesting that the increase in PtdIns(4,5)P_2 seen at fertilization is not necessary for the fusion of cortical granules.

Discussion

Using a fluorescent fusion protein that binds selectively to PtdIns(4,5)P_2, we have investigated PtdIns(4,5)P_2 distribution and dynamics at fertilization in mouse eggs. We found that PH-GFP labeled plasma membrane PtdIns(4,5)P_2. Fertilization did not lead to a detectable loss of PtdIns(4,5)P_2 but rather a Ca^{2+}-

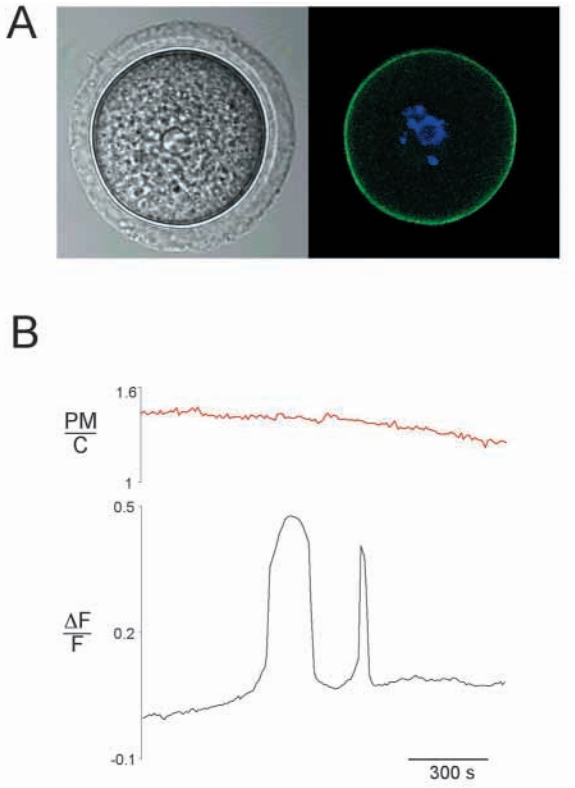

Fig. 6. Fertilization of GV-stage oocytes does not trigger the increase in PtdIns(4,5)P_2. (A) Confocal images of PH-GFP labeling in a GV-stage oocyte. DNA is stained with Hoechst 33342. The corresponding bright field image is displayed on the left. (B) Changes in $[Ca^{2+}]_i$ and PH-GFP distribution at fertilization of a GV stage oocyte. Similar results were obtained in 4 oocytes. For abbreviations, see Fig. 2 legend.

dependent, increase in PtdIns(4,5)P_2. This increase was polarized to the vegetal pole, dependent upon exocytosis of cortical granules, and prevented by inhibiting PtdIns 4-kinase. These studies have implications for the mechanism of Ca^{2+} signaling and the role of PtdIns(4,5)P_2 at fertilization in mammals.

PH-GFP monitors plasma membrane PtdIns(4,5)P_2 in mouse eggs

The PH-GFP chimera selectively labeled the cortex of the eggs, suggesting that it bound to the plasma membrane-associated pool of PtdIns(4,5)P_2, in agreement with previous studies (Stauffer et al., 1998; Varnai and Balla, 1998; Holz et al., 2000; Micheva et al., 2001). In fertilized eggs expressing PH-GFP, Ca^{2+} oscillations and CGE were similar to that seen in control eggs, which suggests that expression of the exogenous protein did not interfere with the physiological process of egg activation or CGE. This is consistent with the finding that PH-GFP binding to plasma membrane PtdIns(4,5)P_2 is a dynamic process with on-off rates in the order of seconds, allowing PtdIns(4,5)P_2 to remain available for binding other proteins, such as PLC (Van der Wal et al., 2001). Together, these data demonstrate that PH-GFP can be used to monitor PtdIns(4,5)P_2 dynamics in the plasma membrane of mouse eggs.

PH-GFP provided a reliable marker for the polarity of the

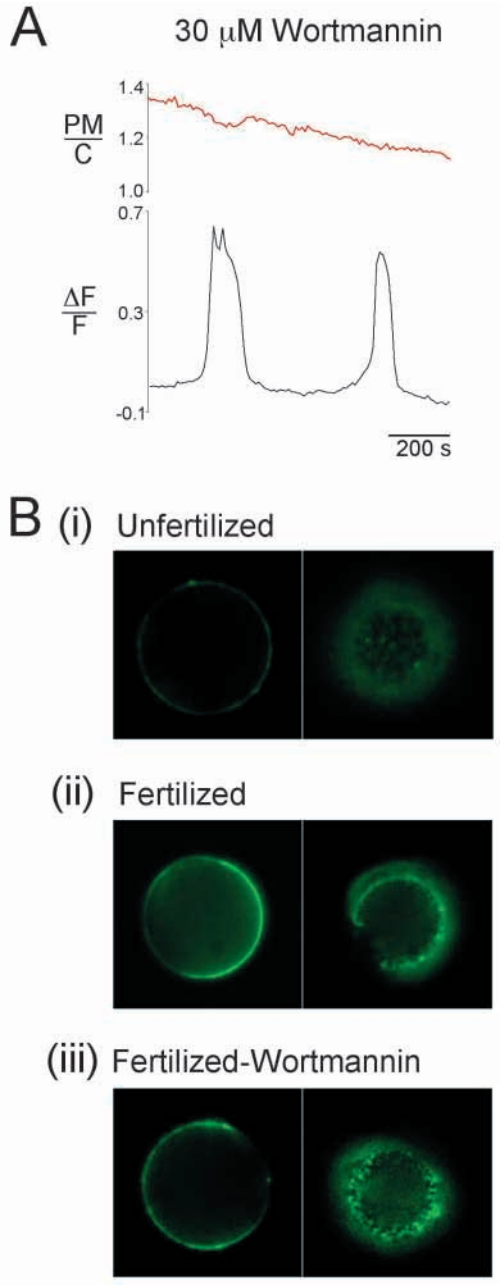

Fig. 7. Micromolar wortmannin inhibits the PtdIns(4,5)P_2 increase but not exocytosis. (A) Fertilization-induced changes in $[Ca^{2+}]_i$ and PH-GFP distribution recorded in an egg pre-treated with 30 μM wortmannin for 15 minutes. Similar results were obtained in eight eggs. For abbreviations, see Fig. 2 legend. (B) FITC-LCA labeling on (i) unfertilized MII oocyte; (ii) control fertilized egg; and (iii) wortmannin (30 μM, 15 minutes)-treated fertilized egg. See Fig. 5D legend for details on FITC-LCA labeling. Similar patterns of staining were observed in at least 12 cells, for each condition.

egg, with a reduced staining in the animal pole. This appears to reflect the absence of microvilli in this region, which effectively reduces the amount of plasma membrane available for PH-GFP labeling. This conclusion is supported by observations that a plasma membrane marker FM 1-43 also shows a decreased staining in the animal pole (G.H. and J.C., unpublished). In addition, immature oocytes that are not

polarized and have microvilli over the entire plasma membrane stain evenly with PH-GFP. Our observations of PH-GFP distribution provide no evidence for PtdIns(4,5)P_2 on intracellular membranes, although it is possible that intracellular pools of PtdIns(4,5)P_2 are not accessible to PH-GFP (Balla et al., 2000). Interestingly, eggs from *Xenopus* and sea urchin have PtdIns(4,5)P_2 in abundance in intracellular membranes (Snow et al., 1996; Rice et al., 2000). It remains to be determined whether mammalian oocytes also have stores of PtdIns(4,5)P_2 in intracellular membranes.

The dynamics of PtdIns(4,5)P_2 at fertilization: the implications for signal transduction at fertilization

Imaging PH-GFP in single living oocytes during the latent period and the first few Ca^{2+} waves did not reveal any loss of plasma membrane PtdIns(4,5)P_2. In fact the only detectable change in plasma membrane PtdIns(4,5)P_2 was an increase that follows the first Ca^{2+} transient. This may seem surprising given the requirement for PtdIns(4,5)P_2 hydrolysis for Ins(1,4,5)P_3 production but it is consistent with biochemical assays on populations of sea urchin and *Xenopus* eggs (Turner et al., 1984; Ciapa et al., 1992; Snow et al., 1996). In sea urchins, kinetic studies on PPI turnover have shown that the net increase in PtdIns(4,5)P_2 represents a small difference in a very large increase in PPI turnover at fertilization (Ciapa et al., 1992). This suggests that hydrolysis and synthesis are tightly coupled such that hydrolysis is compensated for, or independently stimulated, at fertilization of sea urchin eggs. Our experiments cannot inform on the rate of PPI turnover but do provide the advantages that PtdIns(4,5)P_2 dynamics can be monitored simultaneously with Ca^{2+} in single eggs at a relatively high temporal and spatial resolution. Thus we can be confident that any fast changes just prior to or during the Ca^{2+} transient will not go undetected due to the averaging effect of slight differences in timing in a population of eggs.

The lack of any detectable loss of plasma membrane PtdIns(4,5)P_2 suggests two main models for Ins(1,4,5)P_3 production at fertilization in mammals. The first involves plasma membrane PtdIns(4,5)P_2 hydrolysis that is rapidly compensated for by synthesis, as described above. Our data also suggests that the hydrolysis of plasma membrane PtdIns(4,5)P_2 may be small, compared with the large PPI turnover seen in sea urchins (Ciapa et al., 1992). Inhibition of type III PtdIns 4-kinase with wortmannin revealed only a limited and transient loss of PH-GFP from the plasma membrane in about half the eggs examined. Thus, shifting the balance from synthesis to hydrolysis, failed to reveal any significant loss of PtdIns(4,5)P_2 from the plasma membrane, which suggests limited PtdIns(4,5)P_2 hydrolysis. However, it is not known to what extent the wortmannin-sensitive PtdIns 4-kinase contribute and it is likely that PtdIns(4,5)P_2 is also produced via wortmannin-insensitive pathways. A limited hydrolysis of PtdIns(4,5)P_2 is also supported by the finding that low tonic levels of Ins(1,4,5)P_3, rather than larger repetitive spikes of Ins(1,4,5)P_3, provide the best mimic for fertilization-induced Ca^{2+} transients in mouse eggs [(Jones and Nixon, 2000) and this study]. In addition, we have shown that photoreleasing 20-50 μM Ins(1,4,5)P_3 can lead to the loss of PH-GFP from the plasma membrane. This is consistent with the 10-100 μM Ins(1,4,5)P_3 required to displace PH-GFP in somatic cells (van der Wal et al., 2001) and provides an upper limit on the amount of Ins(1,4,5)P_3 generated at fertilization in mammals. Species differences in the mechanism of sperm-induced Ins(1,4,5)P_3 production may explain the proposed difference in the extent of PPI turnover in mammals and sea urchins. In mammals, a sperm-derived PLC is thought to be responsible for hydrolysing PtdIns(4,5)P_2 (Jones et al., 2000) (for a review, see Carroll, 2001) while in sea urchins it is thought that the sperm activates egg PLCs (Carroll et al., 1997; Shearer et al., 1999; Jaffe et al., 2001). This additional amplification step in the signaling pathway may lead to a high rate of PtdIns(4,5)P_2 hydrolysis in sea urchin eggs compared with that provided by PLCs derived from a single sperm in mammals.

The second model suggests that plasma membrane PtdIns(4,5)P_2 is not the major source of PtdIns(4,5)P_2 used for Ins(1,4,5)P_3 production at fertilization. In our studies we are addressing only plasma membrane PtdIns(4,5)P_2 (as discussed above), whereas in sea urchins the experiments measure total cellular PtdIns(4,5)P_2. PtdIns(4,5)P_2 on intracellular membranes has been reported in *Xenopus* and sea urchin eggs (Snow et al., 1996; Rice et al., 2000). It has not been demonstrated that such intracellular pools of PtdIns(4,5)P_2 participate in Ca^{2+} signaling but intracellular pools of PtdIns(4,5)P_2 may be a specialization required for large eggs where a Ca^{2+} wave propagates through a large cytoplasmic volume (Fontanilla and Nuccitelli, 1998; Carroll et al., 1994; Deguchi et al., 2000). Hydrolysis of PtdIns(4,5)P_2 on intracellular membranes by Ca^{2+}-dependent PLC (Swann and Whitaker, 1986; Meyer and Stryer, 1988), which has been suggested to be provided by the fertilizing sperm (Rice et al., 2000), would provide Ins(1,4,5)P_3 at the wave-front to sustain Ca^{2+} release tens or hundreds of microns from the plasma membrane. Such a role for intracellular PtdIns(4,5)P_2 remains to be demonstrated but, at fertilization, where PLC is not thought to be activated at the plasma membrane by a conventional receptor-coupled mechanism, it remains a good possibility.

The increase in PtdIns(4,5)P_2 is Ca^{2+}-dependent and related to cortical granule exocytosis

Our data show that increases in plasma membrane PtdIns(4,5)P_2 always outstrip any hydrolysis at fertilization. This increase is strictly Ca^{2+} dependent such that a Ca^{2+} increase is both necessary and sufficient for the increase in PtdIns(4,5)P_2. The more rapid kinetics of the increase at fertilization compared with that of Ins(1,4,5)P_3 suggests that the fertilization Ca^{2+} transient is more effective or that the sperm activates additional signaling pathways; protein kinase C is one obvious possibility. Whatever the sperm does that stimulates the increase it is short-lived, as the majority of the synthesis takes place during the first Ca^{2+} transient.

One short-lived Ca^{2+}-dependent event at fertilization is the exocytosis of cortical granules (Kline and Kline, 1992; Kline and Stewart-Savage, 1994). A strong connection between PtdIns(4,5)P_2 synthesis at fertilization and Ca^{2+}-triggered CGE is suggested using agents that inhibit exocytosis. Three different agents that inhibit CGE in different ways, inhibit the increase in PtdIns(4,5)P_2 at fertilization while having no effects on Ca^{2+} signalling. Jasplakinolide and cytochalasin B prevent depolymerization and polymerization of actin, respectively, both of which inhibit exocytosis in a number of systems, including

eggs (Terada et al., 2000; Abbott and Ducibella, 2001). The third agent, BoNT/A, cleaves SNAP-25, a plasma membrane protein that is important for Ca^{2+}-activated exocytosis. BoNT/A strongly decreased the rate of rise of PtdIns(4,5)P_2, which is consistent with its ability to slow down and partially inhibit the exocytotic burst (Xu et al., 1998) (for a review, see Schiavo et al., 2000). The connection between exocytosis and the increase in PtdIns(4,5)P_2 is also supported by a number of indirect observations. First, both events have a similar Ca^{2+} dependence; Ins(1,4,5)P_3 stimulates both events (Lee et al., 1988; Xu et al., 1994; Abbott and Ducibella, 2001; present study) while 1 μM BAPTA-AM is inhibitory [(Kline and Kline, 1992) (present study)]. Second, the main burst of cortical granule exocytosis is stimulated by the first Ca^{2+} transient (Kline and Stewart-Savage, 1994), which is also responsible for the increase in PtdIns(4,5)P_2 (present study). Third, in situations where there is no Ca^{2+}-stimulated exocytosis (GV stage oocytes and the animal pole of MII eggs) there is no increase in PtdIns(4,5)P_2. Together, the Ca^{2+}-dependency, timing, pattern, duration and cortical location of PtdIns(4,5)P_2 increases are consistent with an involvement of CGE in the PtdIns(4,5)P_2 increase.

The mechanism by which CGE leads to an apparent increase in plasma membrane PtdIns(4,5)P_2 is not known. Inhibition of the increase by wortmannin suggests that de novo PtdIns(4,5)P_2 synthesis is responsible. This is strengthened by the fact that CGE was not inhibited by wortmannin treatment, suggesting that the PtdIns(4,5)P_2 increase was not simply due to an increase in plasma membrane. An alternative explanation is that exocytosis leads to the addition of PtdIns(4,5)P_2 to the plasma membrane that was previously unavailable to PH-GFP (Balla et al., 2000). However, the unmasking of such PtdIns(4,5)P_2 binding sites would need to be inhibited by wortmannin. Taken together our data suggest that CGE stimulates an increase in plasma membrane PtdIns(4,5)P_2, perhaps by supplying substrate or enzymes that promote PtdIns(4,5)P_2 synthesis.

A role for the transient increase in plasma membrane PtdIns(4,5)P_2 at CGE?

Preventing the increase in PtdIns(4,5)P_2 with inhibitors of exocytosis did not inhibit the generation of Ca^{2+} transients at fertilization. This suggests that the increase is not necessary for sustaining PtdIns(4,5)P_2 levels for Ins(1,4,5)P_3 production. The increase in plasma membrane PtdIns(4,5)P_2 is also not apparently necessary for exocytosis, since wortmannin inhibited the increase in PtdIns(4,5)P_2 without any obvious effect on exocytosis. A role for PtdIns(4,5)P_2 in the early, Ca^{2+}-independent, priming and docking steps of exocytosis has been suggested by studies showing that exocytosis can be recovered in permeabilised cells by providing PtdIns(4)*P* 5-kinase during the priming step (Martin, 1998). By monitoring Ca^{2+} and PtdIns(4,5)P_2 simultaneously, it is clear from our studies that the increase in PtdIns(4,5)P_2 is after the Ca^{2+} rise. This suggests that the PtdIns(4,5)P_2 increase is not necessary for priming, although basal PtdIns(4,5)P_2 may be. We suggest that the increase in PtdIns(4,5)P_2 is a consequence, rather than a cause of, exocytosis and that the role of the increase is downstream of membrane fusion. Remodelling the actin cytoskeleton (Sechi and Wehland, 2000) and endocytosis for membrane retrieval after exocytosis are known to occur after fertilization (Kline and Stewart-Savage, 1994; Bement et al., 2000; Smith et al., 2000; Lee et al., 2001) and it is here that PtdIns(4,5)P_2 synthesis may play a role.

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