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Roles of Src family kinase signaling during fertilization and the first cell cycle in the marine protostome worm *Cerebratulus*

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ABSTRACT For eggs to generate a calcium response during fertilization, the sperm of many deuterostome animals must first activate a group of egg kinases, called Src family kinases (SFKs). However, whether SFK activation is also required for fertilization-induced calcium signals in eggs of protostomes remains unknown. Thus, in this study, unfertilized oocytes of the marine protostome worm *Cerebratulus* were treated with either PP2 to inhibit SFKs or with U73122 to block phospholipase C activity downstream of SFK. Compared with control fertilizations, the inhibitors significantly reduced post-insemination levels of polar body formation and cleavage, but apparently did so via different mechanisms, based on the variable effects of these drugs on sperm incorporations and pronuclear differentiation. Moreover, confocal calcium imaging revealed that repetitive calcium waves (=oscillations) were blocked by U73122, but not by PP2, even though immunoblots indicated SFK activity was inhibited by PP2. Such findings fail to support the view that SFKs are required for initiating fertilization-induced calcium oscillations in *Cerebratulus*, and alternative mechanisms for the observed inhibition of polar body formation and cleavage in drug-treated specimens are discussed.

KEY WORDS: *egg activation, nemertean, PLC, PP2, U73122*

Introduction

During fertilization, the egg cytoplasm undergoes an elevation in calcium ions (Ca^{2+}) that is primarily regulated by the secondary messenger inositol 1,4,5 trisphosphate (IP_3) in both deuterostomes (e.g. chordates and echinoderms) and protostomes (e.g. arthropods, molluscs, and various worms) (Stricker 1999; Whitaker 2006). To initiate IP_3-mediated Ca^{2+} release, sperm can introduce into the egg a soluble factor that in mammals and a few other vertebrates represents a sperm-specific phospholipase C (PLC), termed PLCζ (Saunders *et al.* 2002; Coward *et al.* 2005; Ito *et al.* 2008). PLCζ, along with resident PLCs in the egg, generates IP_3 by phospholipid hydrolysis, and after binding to its receptors on the egg's endoplasmic reticulum (ER), IP_3 releases stored Ca^{2+} to generate a single Ca^{2+} elevation or multiple Ca^{2+} oscillations (Swann and Yu 2008).

Alternatively, or in addition, sperm may stimulate egg tyrosine kinases, called Src family kinases (SFKs), that activate PLCγ to yield IP_3 (Runft *et al.* 2002). Signaling through SFKs is required for Ca^{2+} release during fertilization in such deuterostomes as echinoderms, ascidians, and fish (Kinsey and Shen 2000; Runft and Jaffe 2000; Kinsey *et al.* 2003). Similarly, SFKs mediate the fertilization-induced Ca^{2+} response of the frog *Xenopus* (Sato *et al.* 2000, 2006), albeit by different mechanisms than in other deuterostomes (Runft *et al.* 2002). Conversely, although mammalian eggs express SFKs, such kinases are apparently not necessary for initiating Ca^{2+} oscillations (Kurokawa *et al.* 2004; Mehlmann and Jaffe 2005; Meng *et al.* 2006; Tomashov-Matar *et al.* 2007).

In protostomes, eggs of the annelid worm *Chaetopterus* contain a PLCγ (Yin and Eckberg 2009), and not only does the PLC blocker U73122 prevent sperm factor-mediated Ca^{2+} release in *Chaetopterus* (Howell *et al.* 2003), but U73122 and tyrosine kinase blockers also inhibit egg activation and cleavage (Howell *et al.* 2003; Yin and Eckberg 2009). Similarly, Ca^{2+} is elevated during fertilization and egg activation in the protostomes *Caenorhabditis* and *Drosophila* (Samuel *et al.* 2001; Horner *et al.* 2008), and oocytes of marine protostome worms in the phylum

Abbreviations used in this paper: ER, endoplasmic reticulum; MPF, maturation-promoting factor; pb, polar body; SFK, Src family kinase; SW, seawater.

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Nemertea generate Ca^{2+} oscillations upon fertilization via mechanisms apparently involving sperm factor- and IP_3-mediated signaling (Stricker 1996, 1997). However, whether SFKs are required for fertilization-induced Ca^{2+} release in protostomes remains unknown. Thus, in this study, oocytes of the nemertean *Cerebratulus* sp. were fertilized after treatments with PP2 or U73122 to inactivate SFKs or PLC, respectively, and such fertilizations were analyzed by Hoechst labeling, confocal calcium imaging, and immunoblots to assess the roles of SFK signaling.

Results

Antagonists of SFKs or PLC inhibited post-fertilization polar body formation and cleavage

PP2 and U73122 significantly reduced (P<0.05) the number of polar body forming oocytes at 30 min post-insemination, compared to controls in seawater (SW) (Fig. 1A). By contrast, fertilizations in either inactive PP2 (=PP3) or in U73122 pre-treated with 10mM dithiothreitol (DTT) to reduce U73122's activity (Lee and Shen, 1998) allowed polar body formation (Fig. 1A). Similarly, SW controls generated normal 2-cell embryos, whereas U73122 eliminated cleavage, and PP2 yielded mostly uncleaved eggs along with some overtly polyspermic cleavages (Fig. 1 B,C). PP3 or U73122+DTT also decreased normal cleavages (Fig. 1B), but significantly more (P<0.05) 2-cell embryos occurred in PP3 or U73122+DTT than in PP2 or U73122 alone.

U73122 inhibited sperm incorporation, whereas PP2 increased polyspermy

Based on Hoechst staining, sperm incorporations were significantly decreased (P<0.05) by 10μM U71322 (Fig. 1D). Although such reductions could have resulted from U71322 inhibiting sperm motility (Harayama *et al.* 2005), polar body formation and cleavage were still negligible (1.3+/-2.3%, 0+/-0%, N=3) in 5μM U73122 treatments that had increased sperm incorporations (Fig. 1D). In addition, when oocytes were pre-incubated in 10μM U73122 and washed with drug-free SW directly before insemination, sperm incorporations were elevated over those in 5μM U73122 (Fig. 1D), but polar body formation and cleavage were still blocked (0+/-0%, 0+/-0%, N=3). Similarly, oocytes were fertilized in SW before subsequent additions of U73122, and unlike the 96+/-10 % (N=3) rate of polar body formation in controls without U73122 addition, essentially no polar bodies were formed when U73122 was added 5-15 min after sperm addition (Fig. 1E) i.e., at times that occur after normal Ca^{2+} response onset (Stricker and Smythe 2003). Furthermore, fertilizations in 5μM U73122 or after 10μM U73122 washouts yielded incomplete sperm incorporations (Fig. 1 F,G), and oocytes incubated in U73122+DTT were less wrinkled than in U73122 alone (Fig. 1 H,I), collectively indicating that U73122 affected oocyte

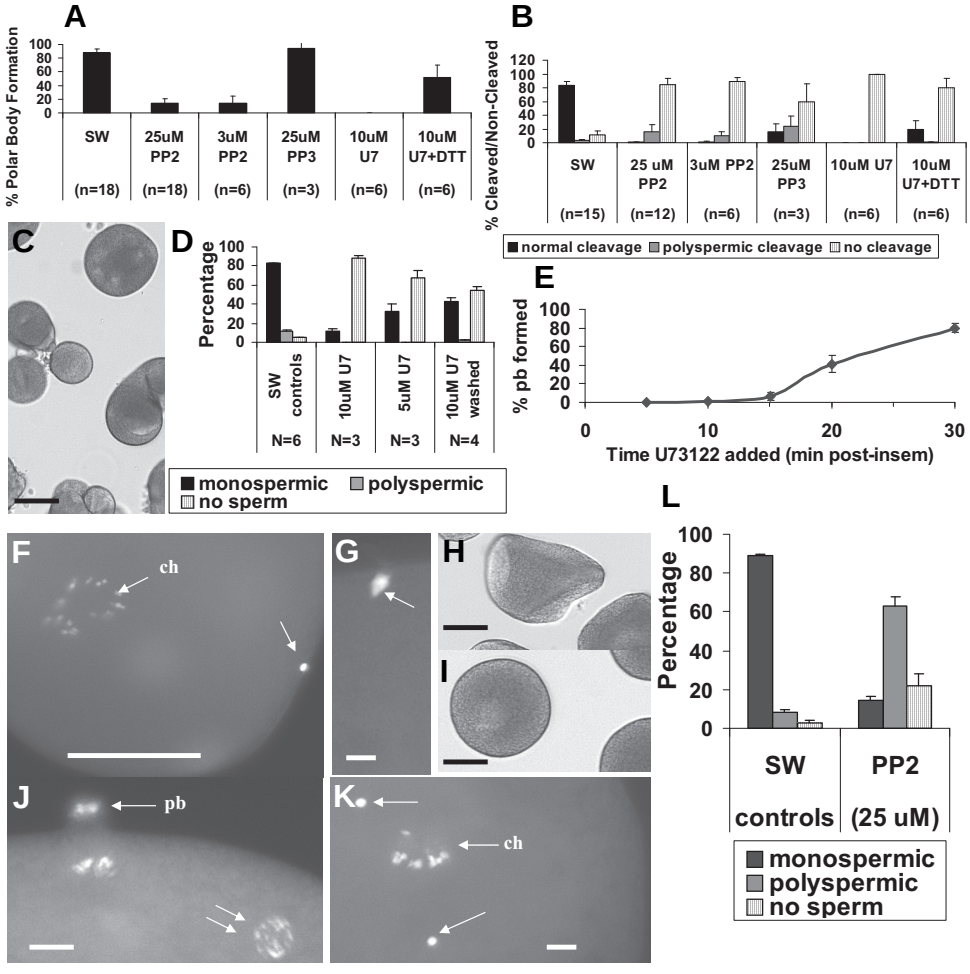

Fig. 1. Effects of inhibitors of SFKs (PP2) and PLC (U73122; "U7") on polar body (pb) formation, cleavage, sperm incorporation and pronuclear development. *Polar body formation at 30 min* **(A)** *and cleavage at 2.5 h* **(B)** *are blocked by PP2 and U73122, whereas less active drug forms (PP3) and (U7+DTT) yield levels more like SW controls; note: polar body counts were subjected to a scaling factor (see Methods and Materials).* **(C)** *Abnormal cleavage in PP2; n=number of oocyte batches counted.* **(D)** *Compared to controls, both 5 μM U73122 and 10 μM U73122 either without or with a washout in SW before sperm addition (10 μM U7 washed) significantly (P<0.05) reduce sperm incorporations.* **(E)** *Adding 10 μM U73122 at 5,10,15, or 20 min post-insemination in SW significantly (P<0.05; N=3) reduces polar body formation at 45 min, compared to controls (96+/-10 %; N=3), suggesting U73122 affects eggs, rather than simply the ability of sperm to fertilize.* **(F,G)** *Hoechst-labeled oocytes in 10 μM U73122, showing incorporated sperm (arrows) that failed to migrate toward egg chromosomes (ch).* **(H,I)** *U73122-treated oocytes before (E) and after (F) 10 mM DTT addition to reduce U73122 activity, showing less wrinkling after U73122 deactivation.* **(J-L)** *Compared to controls, PP2 treatments increase polyspermy (P<0.05; N=3)* **(L).** *(J,K) Hoechst-labeled control* **(J)** *vs. PP2-treated* **(K)** *oocyte at 60 min post-insemination, showing normal decondensed male pronucleus (double arrows) after polar body (pb) formation vs. still condensed male pronuclei (arrows) in polyspermic PP2-treated oocyte; ch, chromosomes; scale bars, 50 μm (C,F,H,I) and 10 μm (G,J,K).*

function.

Conversely, PP2 triggered significantly higher rates of polyspermy (P<0.05) (Fig. 1 J-L), with each polyspermic PP2-treated oocyte averaging 3.6+/-1.5 incorporated sperm (N=50). Furthermore, male pronuclei in PP2 treatments typically reached the oocyte interior but failed to decondense after controls had done so (Fig. 1 J,K).

Fertilization-induced Ca^{2+}oscillations were inhibited by U73122 but not by PP2

Control oocytes fertilized in SW underwent a peripheral "cortical flash" (Stricker 1996) of apparent Ca^{2+} influx during sperm-egg binding (Fig. 2A). Such flashes began ~2-4 min post-insemination and were sometimes generated after an initial artifactual rise caused by changes in oocyte shape or position during sperm addition. Subsequently, each flash was followed by ~6-12 Ca^{2+} waves that spread across the ooplasm (Fig. 2 A,B,J) and typically ceased by ~20-25 min post-insemination (Table I).

For U73122 fertilizations (Fig. 2 C-E), oocytes were incubated 30-60 min in 5μM U73122 before multiple sperm additions that ensured >50-100 sperm surrounded each oocyte. In spite of sperm availability, significantly fewer U73122-treated oocytes displayed Ca^{2+} oscillations (P<0.05) (Fig. 1B). Among non-oscillating specimens, 9/12 exhibited cortical flash-like elevations and/or non-propagating cortical "hot spots" (Glahn *et al.* 1999) (Fig. 2 C,I). Similarly, when oocytes were fertilized in SW and allowed to elevate Ca^{2+} before U73122 addition, 5/12 completed normal oscillations, whereas in the other 7, Ca^{2+} waves were prematurely terminated (N=5) or markedly dampened in amplitude and frequency (N=2) (Fig. 2 D,E).

In contrast, 15/17 PP2-treated oocytes continued to generate Ca^{2+} oscillations that traversed the ooplasm (Table 1; Fig. 2 F-H,K). Of these oscillations, 4 terminated within ~20-25 min (Table I), whereas 11 continued beyond 30 min. In addition, a prominent cortical flash was typically lacking in PP2 treatments, as most Ca^{2+} responses began with repetitive waves (Fig. 2F).

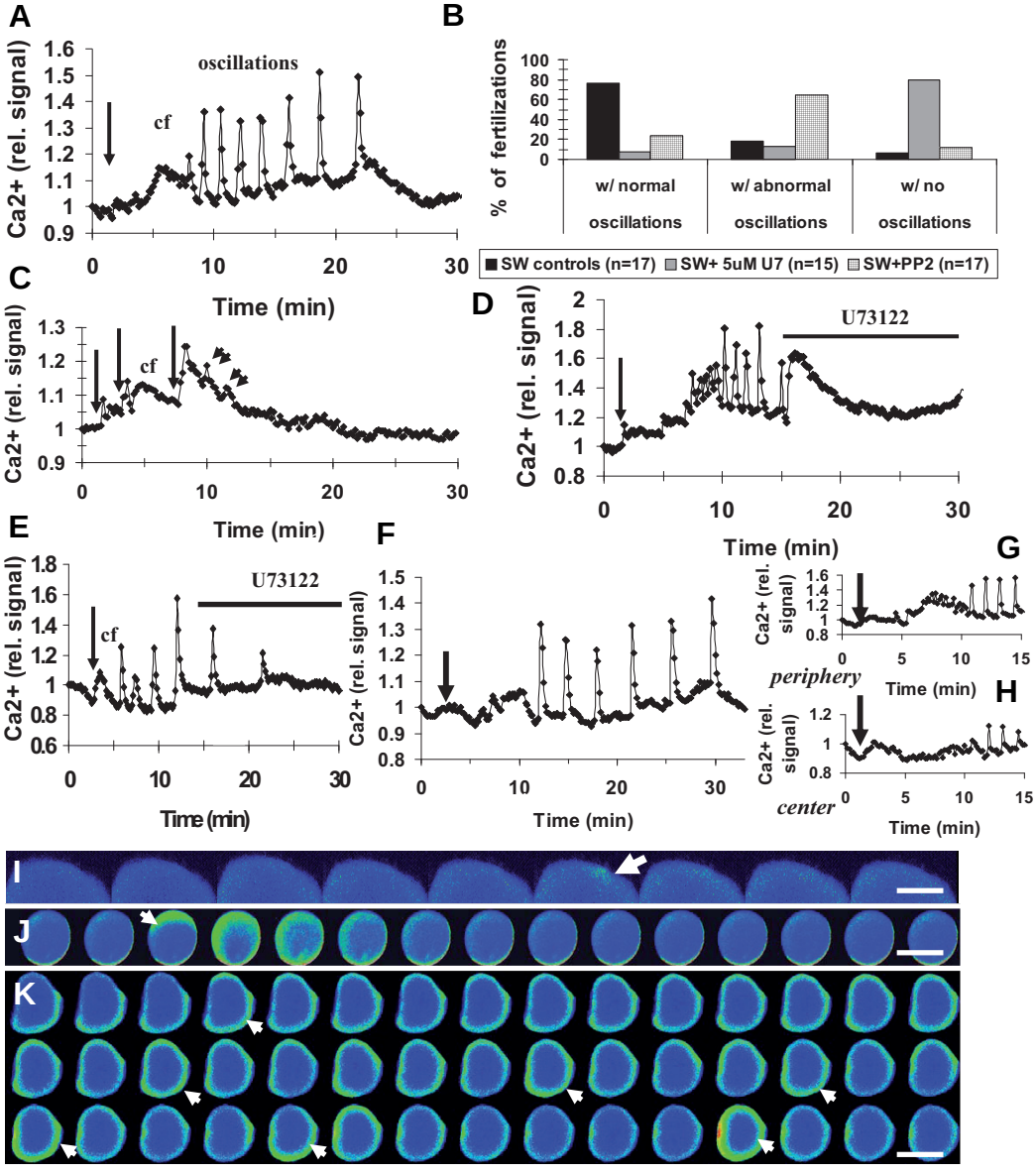

Fig. 2. Ca^{2+} dynamics. (A) *Ratioed confocal data from SW control, showing sperm addition (arrow), cortical flash (cf), and repetitive Ca^{2+} waves (=oscillations).* **(B)** *Percentages of: i) normal Ca^{2+} oscillations, ii) abnormal Ca^{2+} oscillations that did not terminate by 30 min post-insemination, or iii) no Ca^{2+} oscillations.* **(C)** *Fertilization in 5 μM U73122, showing sperm additions (arrows), cortical flash (cf), and cortical "hot spots" (double arrowheads) without Ca^{2+} oscillation production.* **(D,E)** *Fertilizations with sperm added (arrow) in SW prior to 10μM U73122 addition; note rapid termination of Ca^{2+} oscillations (D) or marked dampening in amplitude and frequency (E).* **(F)** *Fertilization-induced Ca^{2+} oscillations in PP2-treated oocyte after sperm addition (arrow)* **(G,H)** *Ca^{2+} waves from PP2-treated specimen in K, confirming global nature of Ca^{2+} waves, albeit with higher rises in periphery vs. center of oocyte.* **(I)** *Non-ratioed images of oocyte cortex during fertilization in 5 μM U73122; images were not divided by the initial image of time-lapse run to show a subtle "hot spot" of localized Ca^{2+} elevation (arrow).* **(J)** *Images divided by the initial image of the time-lapse run, showing last Ca^{2+} waves (arrows) during control fertilization.* **(K)** *Repetitive Ca^{2+} waves (arrows) produced during fertilization in 25 μM PP2 (arrows); images were left unratioed, since cell shape changes would cause distortions; scale bars, 50 μm.*

TABLE 1

FERTILIZATION-INDUCED Ca^{2+} OSCILLATIONS IN PP2-TREATED OOCYTES

	Percentage of Fertilizations Generating Ca^{2+} Oscillations	Average Number of Ca^{2+} Spikes Produced by Oscillating Oocytes during 33 min of Imaging*	Average Increase in Peak Amplitude of Ca^{2+} Spikes over Baseline Levels*	Average Time of Ca^{2+} Oscillation Onset Relative to Sperm Addition*	Average Time of Ca^{2+} Oscillation Termination Relative to Sperm Addition
SW controls	94% (16/17)	8.9 +/- 2.2 (N=16)	37.8 +/- 13.3 % (N=16)	6.2 +/- 1.2 min (N=16)	20.7 +/-1.8 min (N=16)
PP2-treated oocytes	88% (15/17)	10.2 +/- 3.8 (N=15)	64.5 +/- 25.3 % (N=15)	7.6 +/- 1.4 min (N=15)	>30 min (N=11)**

*Does not include pre-oscillation "cortical flash"

**[11/15 PP2-treated oocytes produced prolonged Ca^{2+} oscillations that lasted beyond the 33-min imaging sequence (200 images collected every 10 sec); for the 4 specimens that showed essentially normal terminations, the oscillations ended at 24 +/- 2.8 min after sperm addition].

Immunoblotting analyses verified that PP2 inhibited SFK activity and cell cycle progression

Unfertilized oocytes probed with a phospho-specific antibody against active SFKs displayed a signal in the 52-62 kD range of known SFKs (Thomas and Brugge 1997) that was reduced by PP2, but not PP3 (Fig. 3 A,B). In well-resolved blots, the 55-60 kD band comprised a doublet, and in 2/12 cases, a band of unknown significance occurred at ~52 kD (Fig. 3C, arrow), collectively suggesting oocytes possess multiple SFKs or an SFK with alternative electrophoretic mobilities. Following fertilization in SW or SW+PP3, the SFK signal remained fairly steady (Fig. 3 C-E), although 4/12 controls displayed a moderate increase (Fig. 3F). Conversely, PP2 significantly ($P<0.05$) reduced this signal (Fig. 3 A-G), as noted for somatic cells (Feistritzer *et al.* 2005).

In correlative assays of cell cycle regulators, both MAPK (mitogen-activated protein kinase) and maturation-promoting factor (MPF) activities decreased in fertilized controls (Fig. 3 C,E), as noted previously (Stricker 2009b). Alternatively, MAPK levels in PP2 averaged 33+/-34% (N=7) of control values at 30 min post-insemination, but MPF remained relatively active in 6/7 PP2 treatments (Fig. 3D), coinciding with the PP2-induced block in polar body formation (Fig. 1A).

Discussion

Although proteins in protostome eggs undergo tyrosine phosphorylation after fertilization (e.g. Shibuya *et al.* 1992; Hinton *et al.* 2003), their depicted MWs are not SFK-like. Thus, experiments described here provide the first analysis of how SFK signaling affects fertilization-induced Ca^{2+} signals in a protostome. It should be noted, however, that drugs used in this study can have ectopic effects on unintended targets (Bain *et al.* 2003). Nevertheless, until genomic data become available and baseline information regarding signaling pathways in these worms is assembled, such inhibitors provide an initial means of analysis that albeit requires cautious interpretation (Fig. 4).

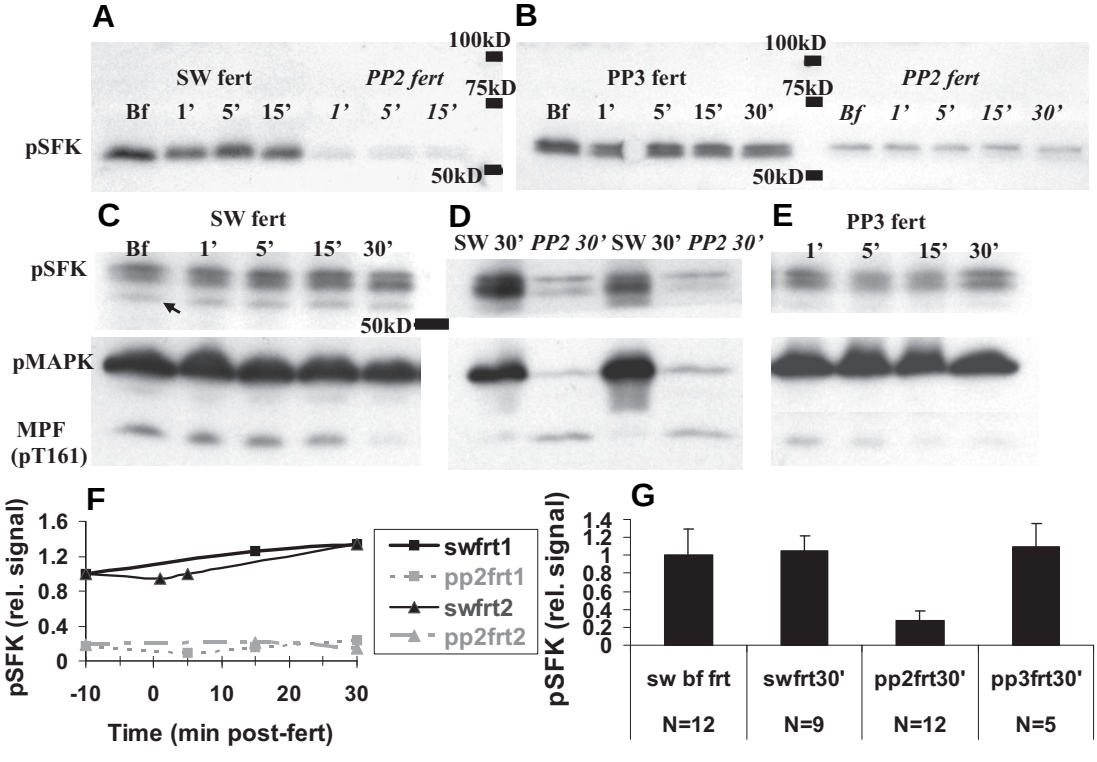

Fig. 3. SFK, MAPK, and MPF activities during the first 30 min post-fertilization. (A-E) *Control oocytes before insemination ("Bf") display putative SFK bands at 55-60 kD that are reduced by 25 μM PP2 but not by 25 μM PP3; arrow in C marks ~52-kD band of unknown significance observed in 2/12 blots. Treatments with PP2 also reduce ERK 1/2 MAPK activity ("pMAPK") at ~42 kD MW and prevent post-fertilization MPF deactivation as indicated by high phospho-Cdc2T161 signal ("pT161") at ~32 kD.* **(F)** *Two PP2 fertilizations ("pp2frt1 or 2") vs. control fertilizations in seawater alone ("swfrt1 or 2").* **(G)** *At 30 min post-fertilization, 25 μM PP2 causes statistically lower SFK activity at 55-60 kD ("pSFK") ($P<0.05$) ("pp2frt30'") than in controls before fertilization ("swbffrt") or in control zygotes ("swfrt30'") or PP3-treated oocytes ("pp3frt30'") at 30 min.*

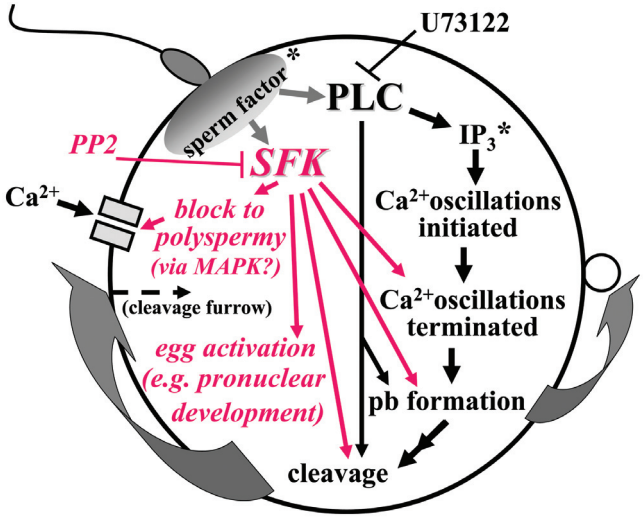

Fig. 4. Summary of results and their possible interpretations. *Asterisks indicate parts based on previously published data (Stricker 1996, 1997). Judging from experiments reported here, fertilization-induced Ca^{2+} oscillations in* Cerebratulus *sp. depend on phospholipase C (PLC), but not Src family kinase (SFK), activity. However, active SFK(s) may modulate Ca^{2+} oscillation patterns (e.g. termination timing). Moreover, SFK and/or PLC activity might affect polyspermy prevention, pronuclear development, polar body (pb) formation, and cleavage. IP_3= inositol 1,4,5 trisphosphate; MAPK=ERK 1/2 mitogen-activated.protein kinase.*

PLC and SFK inhibitors block polar body formation and cleavage with differing effects on sperm incorporation and pronuclei

U73122 treatments reduce the number of sperm-containing oocytes, as noted for mouse or sea urchin eggs (Dupont *et al.* 1996; Lee and Shen 1998). However, U73122-treated *Cerebratulus* oocytes that possess sperm are mostly monospermic, which contrasts with U73122-induced polyspermy in sea urchins and *Xenopus* (Lee and Shen 1998; Sato *et al.* 2000). Alternatively, PP2 triggers polyspermy, as described for deuterostomes (Giusti *et al.* 1999; Kinsey and Shen 2000; Sato *et al.* 2000). PP2 also causes pronuclear abnormalities and cleavage defects, which is consistent with reports of deuterostome eggs treated with similar antagonists (Talmor-Cohen *et al.* 2004: Ng *et al.* 2005) or injected with SH2 (Src Homology 2) domains to inhibit SFKs (Meng *et al.* 2006; McGinnis *et al.* 2009).

As opposed to PP2, U73122 can block Ca^{2+}oscillations without increasing polyspermy

The absence of oscillations in U73122 is not due to a lack of sperm-egg interactions, since non-oscillating specimens commonly undergo cortical flash-like increases or "hot spots" (Glahn *et al.* 1999). Similarly, >50% of *Cerebratulus* oocytes treated with U73122 after fertilization discontinue their oscillations or display oscillation dampening. Although it remains to be determined if oscillations produced by some U73122-treated specimens are simply due to inadequate drug doses (Dupont *et al.* 1996; Deng *et al.* 1998; Lee and Shen 1998), the disruption of oscillations by U73122 suggests that Ca^{2+} signaling depends on PLC activity and that blocking this activity may account for the observed inhibition of cleavage and polar body formation. However, U73122-treated oocytes can produce Ca^{2+} oscillations without forming polar bodies or cleaving, indicating a possible additional requirement for PLC during cytokinesis (Ng *et al.* 2005). Accordingly, 5-10μM U73122 doses may more effectively deactivate the putative PLC needed for cytokinesis than the PLC activity required for Ca^{2+} oscillations.

For *Cerebratulus* oocytes that incorporate sperm in U73122, few display polyspermy, as opposed to the 40-45% polyspermy rates of sea urchins or *Xenopus* (Lee and Shen 1998; Sato *et al.* 2000). Such monospermy may be related to the continued production of cortical flashes in U73122-treated specimens, since *Cerebratulus lacteus* oocytes prevent polyspermy via a prolonged oolemmal depolarization that requires a Ca^{2+} rise (Kline *et al.* 1986). This rise in turn might result from Ca^{2+} influx during the cortical flash, given that a Ca^{2+} channel blocker inhibits cortical flashes and causes polyspermy (Stricker 1996). Accordingly, PP2 disrupts cortical flashes, deactivates MAPK, and causes polyspermy, suggesting that SFK, and/or downstream MAPK, activity may mediate normal cortical flash production and polyspermy prevention. In any case, as discussed below, such polyspermy is not due to a complete lack of a fertilization-induced Ca^{2+} response, as noted for some deuterostomes with disrupted SFK signaling (Kinsey and Shen 2000; Sato *et al.* 2000).

PP2-treated oocytes produce prolonged Ca^{2+}oscillations that fail to promote cell cycle progression

Nearly all *Cerebratulus* oocytes fertilized in PP2 still produce Ca^{2+} oscillations, even though immunoblots indicate PP2 deactivates SFK. Thus, in addition to Ca^{2+}-independent mechanisms that have been proposed for SFKs' effects (Meng *et al.* 2006), PP2-treated oocytes undergoing Ca^{2+} oscillations may still fail to produce polar bodies or cleave, owing to the precise pattern of oscillations (Ducibella *et al.* 2006; Tomashov-Matar *et al.* 2007). For example, the persistent oscillations generated in PP2 may not allow the normal deactivation of MPF required for completing meiosis (Levasseur and McDougall 2000).

In any case, results reported here could signify either that fertilization-induced Ca^{2+} oscillations are generated independently of SFKs, or that SFK activity is actually required for such oscillations, but PP2 somehow allows an artifactual continuation of these Ca^{2+} signals. As potential confounding factors that cannot be precluded, Ca^{2+} oscillations might be initiated by residual SFK activity still present after PP2 incubation or by SFKs not reactive to the phospho-SFK antibody. Alternatively, PP2 may eliminate relevant SFK activity but cause additional defects that allow oscillation production. Moreover, a PP2-induced deactivation of SFK may by itself stimulate Ca^{2+} release, as suggested by one of the sea urchin SFKs that might actually inhibit, rather than trigger, Ca^{2+} release (Townley *et al.* 2009). To test these hypotheses, the type(s) of SFK(s) in *Cerebratulus* must be identified so that alternative means of inhibition such as SH2 domain injections can be utilized. In any case, findings presented here fail to support the view that SFK activity is required for generating fertilization-induced Ca^{2+} oscillations in this protostome.

Materials and Methods

Oocytes of *Cerebratulus* sp. (Stricker 2009a) were treated with calcium-free seawater to minimize spontaneous germinal vesicle break-

down (Stricker and Schatten 1989) before being matured in SW. Mature oocytes were incubated ~1hr in SW with or without inhibitor before being fertilized with diluted sperm (Stricker 2009b) in the presence or absence of the inhibitor. Polar body assays at 30 min post-fertilization were adjusted for ephemeral or hidden polar bodies, by subjecting uncorrected counts of 50-100 oocytes per treatment to a scaling factor (~2X) equaling the average of normally cleaving controls divided by the average of observed polar body-forming oocytes. Cleavage was assessed at ~2.5 h post-fertilization, and sperm incorporations were tracked via Hoechst 33342 (Stricker 1996).

For immunoblots, oocytes were probed with phospho-specific antibodies (#9101 phospho-ERK 1/2 MAPK, #9114 phospho-T161 Cdc2 MPF, #2101 phospho-Src family kinases from Cell Signaling, Beverly, MA, USA) (Stricker and Smythe 2003). Densitometry of background-subtracted bands was carried out using MetaMorph software on at least three replicates from two or more females, and statistical assessments utilized a Mann-Whitney-U test.

To assay calcium dynamics, oocytes were microinjected with Calcium Green Dextran, 10,000 MW (Invitrogen; Carlsbad, CA, USA) and imaged with a confocal microscope (Stricker 1996). Fluorescence intensities were graphed for ~15μmX15μm regions-of-interest in the peripheral third of each oocyte, and such data were normalized relative to the starting fluorescence at the onset of imaging.

Stock solutions of inhibitors were mixed in DMSO, and working dilutions (3-25μM for PP2; 5-10μM for U73122) were toward the lower end of previously used doses that have ranged up to 100μM for PP2 (Kinsey *et al.* 2003; Tomashov-Matar *et al.* 2007) and 20-30μM for U73122 (Deng *et al.* 1998; Lee and Shen 1998).

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