Release of phospholipase C ζ and $[Ca^{2+}]_i$ oscillation-inducing activity during mammalian fertilization

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Abstract

During fertilization of mammalian eggs a factor from the sperm, the sperm factor (SF), is released into the ooplasm and induces persistent $[Ca^{2+}]_i$ oscillations that are required for egg activation and embryo development. A sperm-specific phospholipase C (PLC), PLC ζ , is thought to be the SF. Here, we investigated whether the SF activity and PLC ζ are simultaneously and completely released into the ooplasm soon after sperm entry. To accomplish this, we enucleated sperm heads within 90 min of intracytoplasmic sperm injection (ICSI) and monitored the persistence of the $[Ca^{2+}]_i$ oscillations in eggs in which the sperm had been withdrawn. We also stained the enucleated sperm heads to ascertain the presence/absence of PLC ζ . Our results show that by 90 min all the SF activity had been released from the sperm, as fertilized enucleated eggs oscillated as fertilized controls, even in cases in which oscillations were prolonged by arresting eggs at metaphase. In addition, we found that the released SF activity became associated with the pronucleus (PN), as induction of PN envelope breakdown evoked comparable $[Ca^{2+}]_i$ responses in enucleated and non-manipulated zygotes. Lastly, we found that PLC ζ localized to the equatorial area of bull sperm and to the post-acrosomal region of mouse sperm and that by 90 min after ICSI all the sperm's PLC ζ immunoreactivity was lost in both species. Altogether, our findings show that during fertilization the SF activity and PLC ζ immunoreactivity are simultaneously released from the sperm, suggesting that PLC ζ may be the only $[Ca^{2+}]_i$ oscillation-inducing factor of mammalian sperm.

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Introduction

Mammalian eggs are ovulated arrested at the metaphase stage of the second meiotic division (MII). Upon fertilization, the sperm triggers repetitive and persistent changes in the intracellular concentrations of free calcium ($[Ca^{2+}]_i$), also referred to as $[Ca^{2+}]_i$ oscillations, that are required for egg activation (Jones *et al.* 1995, Schultz & Kopf 1995, Day *et al.* 2000). Egg activation entails the progressive initiation of several events, such as cortical granule exocytosis, resumption of meiosis and exit from MII arrest, extrusion of the second polar body and pronucleus (PN) formation (Schultz & Kopf 1995). The orderly completion of these events ensures the initiation of normal embryo development (Ducibella *et al.* 2002).

$[Ca^{2+}]_i$ oscillations are thought to unfold following the release into the ooplasm of a sperm-specific component, the sperm factor (SF), immediately after fusion of the gamete's membranes (Swann 1990, 1996, Stricker 1999). Initial evidence for this hypothesis stemmed from demonstrations that direct injection of sperm into the ooplasm, which circumvented sperm–egg interactions, resulted in

normal embryo development and $[Ca^{2+}]_i$ oscillations (Palermo *et al.* 1992, Nakano *et al.* 1997, Sato *et al.* 1999). Parallel and subsequent studies making use of sperm extracts from several species also found that injection of these extracts caused $[Ca^{2+}]_i$ oscillations in oocytes of widely diverse species (Swann 1990, Stricker 1997, Wu *et al.* 1997, Kyozuka *et al.* 1998, Dong *et al.* 2000, Tang *et al.* 2000, Yamamoto *et al.* 2001), and even in somatic cells (Berrie *et al.* 1996). Studies to unveil the ontogeny of this activity demonstrated that appearance of SF activity, i.e. the ability to initiate $[Ca^{2+}]_i$ oscillations, was not seen until late stages of spermiogenesis (Parrington *et al.* 2000, Yazawa *et al.* 2000). Moreover, biochemical studies conducted to ascertain the distribution of SF in the sperm suggested that it localized to the sperm perinuclear theca (PT; Kimura *et al.* 1998, Perry *et al.* 1999, 2000), which is a matrix-like multiprotein complex material surrounding the sperm nuclear membrane, and that reportedly represents the first sperm domain to mix with the ooplasm during fertilization (Sutovsky *et al.* 2003). Nonetheless, the precise distribution of SF in mammalian sperm and its molecular composition remain to be fully elucidated.

Progress toward identification of the molecular nature of the mammalian SF has been facilitated by evidence showing that sperm-induced $[Ca^{2+}]_i$ oscillations are underpinned by activation of the phosphoinositide (PI) pathway. A major signaling product of this pathway, inositol 1,4,5-trisphosphate (IP_3), results from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase(s) C (PLC). IP_3 binds and gates the IP_3 receptors (IP_3Rs) on the endoplasmic reticulum, the intracellular Ca^{2+} -store, thereby stimulating Ca^{2+} release. Evidence that the PI pathway is active in mammalian fertilization emanates from studies showing that sperm or SF-induced $[Ca^{2+}]_i$ oscillations and egg activation are abolished by functional blocking IP_3R -1 antibodies (Miyazaki *et al.* 1992, Xu *et al.* 1994), or by treatment with the PLC inhibitor U73122 (Dupont *et al.* 1996, Jones *et al.* 1998a, Lee & Shen 1998, Wu *et al.* 2001). While *a priori* one of several PLC isoforms (Rhee & Bae 1997) present in sperm could be the active PLC, and therefore the SF, the discovery of a sperm-specific PLC, PLC ζ (Saunders *et al.* 2002), points to it as the putative SF (for review, see Kurokawa *et al.* 2004, Swann *et al.* 2004, 2006). Accordingly, injection of mouse PLC ζ cRNA into mouse eggs induced fertilization-like $[Ca^{2+}]_i$ oscillations and promoted embryo development to the blastocyst stage (Saunders *et al.* 2002, Kouchi *et al.* 2004). In keeping with this finding, sperm extracts depleted of PLC ζ lacked the ability to initiate $[Ca^{2+}]_i$ oscillations (Saunders *et al.* 2002), and reducing the sperm's PLC ζ content by a transgenic RNA interference (RNAi) approach perturbed fertilization-associated $[Ca^{2+}]_i$ oscillations and reduced litter size (Knott *et al.* 2005). Importantly, expression of PLC ζ cRNAs tagged with a fluorescent marker revealed that PLC ζ accumulates in the PN (Larman *et al.* 2004, Yoda *et al.* 2004, Sone *et al.* 2005), which is consistent with the finding that, during interphase, the SF activity in fertilized mouse zygotes associates exclusively with either PN (Kono *et al.* 1996, Knott *et al.* 2003). Collectively, the data suggest that PLC ζ is the SF that underlies $[Ca^{2+}]_i$ oscillations in the mouse and in mammals. Nevertheless, whether PLC ζ represents the only $[Ca^{2+}]_i$ oscillation-inducing factor and whether its release renders sperm incompetent to induce $[Ca^{2+}]_i$ oscillations and egg activation remains to be demonstrated.

Our previous studies showed that within 15 to 60 min of fertilization, a significant portion of the sperm's $[Ca^{2+}]_i$ oscillatory activity became dissociated from the sperm and, seemingly, by 120 min the sperm lost all its ability to induce oscillations (Knott *et al.* 2003). In this study, we expand on those findings by investigating whether the released SF supports long-term $[Ca^{2+}]_i$ oscillations equivalent to those seen in fertilization. We also examined whether the released SF achieves association with the PN. Finally, we determined the localization of PLC ζ in mouse and bull sperm, and investigated whether its release from the equatorial

region of the sperm head coincides with loss of the sperm's ability to induce $[Ca^{2+}]_i$ oscillations.

Results

Complete release of SF from the sperm by 90 min post sperm entry

In a previous study, we have shown that the fertilization-associated $[Ca^{2+}]_i$ oscillatory activity of mouse and bull sperm was mostly released into the ooplasm within 60–120 min post-ICSI or post-IVF, as re-injection of the same sperm into a new egg was unable to initiate oscillations (Knott *et al.* 2003). However, a question left unanswered was whether the oscillations initiated prior to enucleation were able to persist as long as of those in zygotes in which the sperm was left undisturbed, which would confirm the notion that all the SF activity is released within the first 2 h of sperm entry. To answer this question, ICSI, sperm enucleation and $[Ca^{2+}]_i$ monitoring were performed in the time line shown in Fig. 1. To ensure that manipulation of the zygotes was not affecting $[Ca^{2+}]_i$ responses, comparable amounts of ooplasm were removed from control zygotes. As shown in Fig. 1A and B, the $[Ca^{2+}]_i$ oscillations initiated by ICSI were undisturbed by removal of a sperm-like portion of ooplasm (Fig. 1B), although all these examined zygotes showed the expected decline in frequency as interphase neared (Kono *et al.* 1996, Day *et al.* 2000, Larman *et al.* 2004, Lee *et al.* 2006). Importantly, removal of the sperm head 90 min post-ICSI did not affect the pattern or persistence of $[Ca^{2+}]_i$ oscillations, as nearly all enucleated zygotes (43/50; $87.2\% \pm 3.2$ (S.E.M)) oscillated for a few hours until oscillations began tailing off as progression into interphase occurred (Fig. 1C, $P > 0.05$). While these results are suggestive of significant release of the SF activity into the ooplasm, they do not necessarily answer whether all the sperm's $[Ca^{2+}]_i$ inducing activity is released during this period. This is so because it is well known that if arrested into a metaphase-like stage, fertilized eggs can continue to oscillate for nearly 20 h (Day *et al.* 2000), whereas under natural conditions, the oscillations cease by the approximate time of PN formation (Jones *et al.* 1995). Hence, to extend these findings, we performed that same experimental design but in the presence of 100 ng/ml colcemid, which is known to prevent MII exit (Jones *et al.* 1995, Moses & Kline 1995, Gordo *et al.* 2002). Under these conditions, oscillations in enucleated eggs persisted in excess of 8 h (Fig. 2B and E), which was comparable to the duration of oscillations in control zygotes (Fig. 2A, C and D). Altogether, the data show that within 90 min following fertilization, the totality of the SF activity becomes uncoupled from the sperm to seek its substrate.

Figure 1 Sperm enucleation after ICSI does not impact the initiation or persistence of $[Ca^{2+}]_i$ oscillations prior to PN formation. $[Ca^{2+}]_i$ profiles of eggs that underwent control ICSI (A), or from eggs in which ooplasm (B) or the sperm head (C) was removed. Enucleation here and throughout the manuscript was performed at 90 min post-ICSI. Following ICSI, eggs were cultured in KSOM for 60 min prior to enucleation. After enucleation, $[Ca^{2+}]_i$ monitoring was performed for ~180 min. The scheme representing the time line for the each of the procedures performed to complete the experiment is shown above the $[Ca^{2+}]_i$ panels. Vertical arrows denote time of ICSI or sperm enucleation.

Released SF associates with the PN during interphase in enucleated zygotes

In mouse zygotes, $[Ca^{2+}]_i$ oscillations are entrained with the cell cycle, becoming undetectable by the time zygotes proceed into interphase and form PNs (Ogonuki *et al.* 2001, Larman *et al.* 2004, Lee *et al.* 2006). At the conclusion of this stage, a rise in $[Ca^{2+}]_i$ seems to accompany the PN envelope breakdown (PNBD), an event that portends the impending first mitosis (Jones *et al.* 1995, Day *et al.* 2000, Gordo *et al.* 2002, FitzHarris

et al. 2003). While this PNBD-associated $[Ca^{2+}]_i$ rise is not observed in all fertilized zygotes (Day *et al.* 2000, Gordo *et al.* 2002) and its functional significance remains unknown (FitzHarris *et al.* 2003), it is only detectable in fertilized zygotes, which is consistent with the evidence that after fertilization, the SF activity associates almost exclusively with the PNs (Marangos *et al.* 2003). Hence, the PNBD-associated $[Ca^{2+}]_i$ rise could be used as an indicator of the cellular distribution of the SF. Hence, we wished to ascertain whether

Figure 2 Sperm enucleation does not affect the long-term duration of $[Ca^{2+}]_i$ oscillations. $[Ca^{2+}]_i$ profiles induced by ICSI in eggs from which a comparable amount ooplasm was removed (A and D), or from eggs in which the sperm head was removed (B and E), or in non-manipulated fertilized eggs (C). Following ICSI, eggs were cultured for 60 min in KSOM in the presence of colcemid (100 ng/ml) prior to enucleation. Following enucleation and during $[Ca^{2+}]_i$ monitoring, zygotes were maintained in the presence of colcemid. A break in the X-axis reflects time in culture without monitoring to decrease detrimental effects of extended u.v.-light exposure. The scheme representing the time line for the each of the procedures performed to complete the experiment is shown above the $[Ca^{2+}]_i$ panels.

enucleated zygotes were capable of mounting a $[Ca^{2+}]_i$ rise associated with PNBD. To shorten the time to PNBD, we made use of OA, a phosphatase inhibitor that has been shown to induce premature PNBD (Dyban *et al.* 1993, Moos *et al.* 1995), and that we have demonstrated causes a PNBD-associated $[Ca^{2+}]_i$ rise in fertilized zygotes (Gordo *et al.* 2002). As shown in Fig. 3A and B, OA induced a $[Ca^{2+}]_i$ rise associated with PNBD in 18/18 fertilized zygotes and in 8/10 zygotes in which a part of the ooplasm was removed. OA treatment also evoked a $[Ca^{2+}]_i$ rise associated with PNBD in the majority of enucleated zygotes (6/10; Fig. 3C, $P > 0.05$), whereas it failed to do so in control, $SrCl_2$ -activated zygotes (0/11; Fig. 3D). It is worth noting that by the time that the PNBD-associated $[Ca^{2+}]_i$ rise was detected, the nuclear envelope in all these zygotes was no longer visible, although nucleoli remnants could still be observed in a few cases. Collectively, the results suggest that in enucleated zygotes the SF achieves the correct cellular distribution.

Loss of PLC ζ coincides with loss of sperm's ability to induce $[Ca^{2+}]_i$ oscillations

The sperm-specific PLC ζ represents the strongest candidate to date to be the sperm's SF (Saunders *et al.* 2002, Larman *et al.* 2004, Kurokawa *et al.* 2005, Kuroda *et al.* 2006). However, the localization of PLC ζ in sperm, its

temporal release and, ultimately, whether its absence precludes the initiation of oscillations at fertilization has not been demonstrated. To examine PLC ζ localization in mouse and bovine sperm, we made use of two of different anti (α)-PLC ζ antibodies raised by our laboratory (Kurokawa *et al.* 2005). The specificity of these antibodies was characterized in a previous manuscript (Kurokawa *et al.* 2005) and they were shown, using Western blotting, to recognize a band of ~ 74 kDa relative molecular weight (rMW) in mouse sperm (α PLC ζ -CT), which is the expected rMW of mPLC ζ (Saunders *et al.* 2002, Kurokawa *et al.* 2005), and a band of ~ 72 kDa in bull sperm, which is consistent with the expected rMW of bPLC ζ (Malcuit *et al.* 2005). We therefore next investigated by immunofluorescence the localization of PLC ζ in mouse and bull sperm. As expected, sperm stained only with the secondary antibody, which served as negative controls, showed absence of reactivity (Fig. 4A and E). Importantly, addition of either primary antibody revealed in mouse sperm a distinct post-acrosomal band, approximately at the post-equatorial region of the sperm head (Fig. 4C and D), which is in agreement with the only previous study that examined PLC ζ localization in mouse sperm (Fujimoto *et al.* 2004). Our antibodies also labeled the sperm tail and the acrosome region (Fig. 4B–D; inset in 4B shows PNA staining), although the antigenic peptide for the NT antibody only obliterated the equatorial staining (Fig. 4B), which suggest that the post-acrosomal labeling

Figure 3 Sperm enucleation after ICSI does not affect the association of the SF with the PN. Premature PNBD was induced by the addition of 10 μ M OA. A $[Ca^{2+}]_i$ rise was observed in control-ICSI fertilized eggs (A), in eggs in which ooplasm was removed (B), and in enucleated eggs (C). A $[Ca^{2+}]_i$ rise was not induced in $SrCl_2$ -activated eggs (D). The scheme representing the time line for each of the procedures performed to complete the experiment is shown above the $[Ca^{2+}]_i$ panels.

Figure 4 PLC ζ is localized to the post-acrosomal region of mouse sperm (A–D) and to the equatorial region of bull sperm (E–H). Fresh mouse and bull sperm reactivity in the absence of primary antibody (A and E); insets show sperm nuclei stained with Hoechst 33 342. In the presence of antigenic peptide (30 μ g peptide per μ l of anti-serum of α PLC ζ -NT), a fluorescent signal was detectable in the acrosome and tail suggesting that the signal at these locations was nonspecific and due to cross-reactivity (B and F); insets show acrosomes stained with PNA-lectin (PNA). Mouse and bull sperm incubated with α PLC ζ -NT (C and G respectively) or with α PLC ζ -CT (D and H respectively) showed a distinctive and overlapping immunoreactive signal (arrow) at the post-acrosomal region (mouse) and equatorial region (bull). Scale bar in G = 10 μ m.

specifically denotes the localization of PLC ζ . In bull sperm, the antibodies also generated overlapping immunostaining patterns, with strong reactivity detected in the post acrosomal, equatorial region of the sperm head (Fig. 4G and H). As was the case for mouse sperm, non-specific fluorescence was observed to the acrosomal and tail regions (Fig. 4G and H), as it was not alleviated by pre-incubation of the antibody with its corresponding antigenic peptide (Fig. 4F, inset (PNA) shows acrosomal region). Thus, given that in sperm of both species, the addition of the NT antigenic peptide exclusively prevented

the post-acrosomal (mouse) and equatorial (bull) signals, and that the antibodies provided overlapping fluorescent signals in both species, we can therefore conclude that the described localization represents the correct PLC ζ distribution for these species.

To ascertain whether the presence of PLC ζ corresponds with the ability of sperm to initiate oscillations, we first examined whether mouse and bull sperm exposed to a brief treatment with high pH, a procedure that we have shown uncouples the SF activity from the sperm nuclei (Kurokawa *et al.* 2005), were able to

Figure 5 High-pH treatment abolishes SF activity and abrogates PLC ζ immunoreactivity in mouse (A–C) and bull sperm (D–G). Injection of a mouse (A) or a bull sperm (E) into mouse eggs initiates $[Ca^{2+}]_i$ responses and this activity is depleted by treating the sperm with high pH (B and F respectively). Importantly, pH treatment also obliterated PLC ζ immunoreactivity from sperm of both species (α PLC ζ -NT, C and D). Insets in C and D show sperm nuclear staining with Hoechst 33 258.

Figure 6 Loss of SF activity coincides with the loss of sperm PLC ζ immunoreactivity after enucleation. $[Ca^{2+}]_i$ profile of an egg after injection of a fresh bull sperm (A), or of an egg after injection with the same bull sperm enucleated 90 min after ICSI (B). Residence in the ooplasm also depleted PLC ζ immunoreactivity from bull (C) and mouse sperm (D); insets show the presence of bull and mouse sperm nuclei in the slides (Hoechst 33 258).

initiate oscillations upon injection into mouse eggs. High-pH treatment abrogated the $[Ca^{2+}]_i$ oscillatory activity of all mouse ($n=8/8$; Fig. 5A and B) and bull sperm ($n=6/6$; Fig. 5E and F) and, more importantly, it eliminated PLC ζ immunoreactivity from sperm of both species (Fig. 5C and D respectively; insets show Hoechst-stained sperm heads). Importantly, the supernatants recovered from these sperm exhibited undisturbed $[Ca^{2+}]_i$ oscillatory activity when injected in mouse MII eggs (Fig. 5G), demonstrating that the pH treatment did not eliminate PLC ζ immunoreactivity by inactivating the enzyme. In addition, PNA staining of both mouse and bull sperm after high-pH treatment resulted in the presence of specific staining (data not shown) that has been reported to develop after sperm undergo the acrosome reaction (Cheng *et al.* 1996, Baker *et al.* 2004), suggesting that not all sperm head-associated proteins were removed by the high-pH treatment.

We next investigated whether the temporal release of PLC ζ from the sperm head concurred with the previously noted release of SF activity (Knott *et al.* 2003), and whether loss of PLC ζ immunoreactivity prevented the ability of sperm to induce $[Ca^{2+}]_i$ oscillations. To examine these questions, we first performed ICSI followed by sperm head enucleation within 90 min post-ICSI. The aspirated sperm heads were either stained for PLC ζ reactivity or re-injected into MII eggs. As expected, re-injection of enucleated bull or mouse sperm heads failed to induce oscillations in mouse eggs (Fig. 6B). Importantly, all enucleated sperm heads examined lacked PLC ζ immunoreactivity (Fig. 6C and D). It is worth noting that while mouse sperm heads had started

to undergo decondensation by this time (Fig. 6D inset), this was not the case for bull sperm heads (Fig. 6C inset), suggesting that, at least for the latter, the loss of PLC ζ could not be attributed to massive exodus of sperm proteins into the ooplasm. Together, the results show that mouse and bull sperm depleted of PLC ζ are incapable of inducing $[Ca^{2+}]_i$ oscillations in eggs.

Discussion

The present study investigated whether the SF activity of mouse and bovine sperm and their PLC ζ immunoreactivity were fully released into the ooplasm within the first 90 min post-fertilization. We also examined whether the loss of both of these properties impacted the ability of these sperm to initiate $[Ca^{2+}]_i$ oscillations. We found, consistent with our previously published results (Knott *et al.* 2003), that indeed all the SF activity, i.e. the ability of the sperm to induce persistent oscillations, was released into the ooplasm within the first 2 h of sperm entry. However, in the previous manuscript, we only examined the persistence of the oscillations until the PN stage, a stage at which in mouse eggs $[Ca^{2+}]_i$ oscillations cease spontaneously (Jones *et al.* 1995, Day *et al.* 2000, Deguchi *et al.* 2000, Marangos *et al.* 2003, Lee *et al.* 2006). Therefore, here, we extended the oscillatory permissive state of eggs by arresting zygotes at a MII-like stage and appropriately prolonging the $[Ca^{2+}]_i$ monitoring period. We found that enucleated zygotes were able to sustain oscillations as long as those of fertilized and non-manipulated zygotes under any of the conditions tested. Therefore, our findings together with those in the literature allow us to propose a model of SF release during mouse fertilization whereby the SF starts to trickle from the sperm into the ooplasm soon after the fusion of the gametes (Lawrence *et al.* 1997, Jones *et al.* 1998b) and this continues uninterrupted for the next 60 to 70 min, at which time the totality of the SF activity has been deposited in the ooplasm (this study and Knott *et al.* 2003). This protracted release of the SF may be due, at least in part, to the time required to achieve full disassembly of the PT, the sperm domain thought to host the SF activity (Kimura *et al.* 1998, Perry *et al.* 1999, Knott *et al.* 2003). From a functional standpoint, the slow release of the $[Ca^{2+}]_i$ oscillation-inducing factor may ensure continued availability of the SF for the first few hours following fertilization, such that the pattern of $[Ca^{2+}]_i$ oscillations exhibits a steady pace until the progression into interphase is eminent, as signaled by the impending PN formation.

Once released into the ooplasm, the SF seemingly distributes throughout the ooplasm making possible the generation of oscillations and then, as zygotes progress into interphase, it becomes associated with the PN, as evidenced by the results showing that injection of these PNs into fresh MII eggs initiates $[Ca^{2+}]_i$ oscillations (Kono *et al.* 1996, Ogonuki *et al.* 2001, Knott *et al.*

2003). This SF association with the PN was further corroborated by the findings that inhibition of either PN formation using wheat germ agglutinin or abrogation of molecular transport into the PN abrogated the interphase-linked termination of the $[Ca^{2+}]_i$ oscillations (Marangos *et al.* 2003). Our results show that the released SF attains the expected cellular distribution even in the absence of the fertilizing sperm head, as fertilized/enucleated zygotes and non-manipulated fertilized zygotes exhibited the $[Ca^{2+}]_i$ rise associated with PNBD (Jellerette *et al.* 2004, Larman *et al.* 2004), which here was prematurely induced by exposure to OA. The association/incorporation of the SF activity with the PN is consistent with the recent demonstration that the linker region of putative SF, PLC ζ , contains a positively charged consensus nuclear localization signal (Saunders *et al.* 2002, Larman *et al.* 2004). It is presently unclear why the SF activity/PLC ζ becomes incorporated into the PN, although it might perform targeted signaling in the PN by inducing persistent stimulation of the PI pathway (Sone *et al.* 2005). Furthermore, it cannot be discounted that given the developmental risks of excessive/abnormal stimulation of the PI pathway/ $[Ca^{2+}]_i$ oscillations (Gordo *et al.* 2000, Rogers *et al.* 2004, Ozil *et al.* 2005), the PN sequestration of PLC ζ may act as an insurance mechanism to prevent the detrimental effects of persistent $[Ca^{2+}]_i$ oscillations (Marangos *et al.* 2003, Larman *et al.* 2004).

Since its discovery by Saunders *et al.* (2002), accumulating research has solidified the view that PLC ζ is likely to represent the SF in all mammals (see also Introduction, Cox *et al.* 2002, Saunders *et al.* 2002, Kurokawa *et al.* 2005, Yoneda *et al.* 2006). Nevertheless, many features of this molecule and its function remain to be elucidated. Among the least investigated characteristics of PLC ζ is its localization in sperm. The SF activity has been shown to be associated with the PT of the sperm head (Perry *et al.* 2000, Knott *et al.* 2003) and, consistent with those findings, the only report that examined the localization of PLC ζ found that it distributes to the post-acrosomal region of mouse sperm (Fujimoto *et al.* 2004). This is an appropriate distribution for the putative $[Ca^{2+}]_i$ oscillation-inducing factor, since reportedly the contents of this region quickly mingle with the ooplasm (Manandhar & Toshimori 2003, Sutovsky *et al.* 2003). In the present study, we extend those results by examining the localization of PLC ζ in mouse and bull sperm with antibodies raised against peptide sequences from opposite ends of the molecule (Kurokawa *et al.* 2005). We selected sperm from these species as they show greatly different sperm shapes, with the round shape of bull sperm more reminiscent of the sperm shape of humans and other large mammals. Our immunofluorescence results confirm the post-acrosomal localization of PLC ζ in mouse sperm and, for the first time, show that in bull sperm PLC ζ is distributed to the equatorial region, a region that is also expected to gain rapid access to the ooplasm (Sutovsky *et al.* 2003). It is important to note that

our localization studies were performed in non-capacitated and non-acrosome-reacted sperm, changes that may alter the localization/conformation of PLC ζ to favor its release/activation. Thus, PLC ζ localization studies should also be performed in future studies in capacitated and acrosome-reacted sperm. Lastly, it is worth noting that the presence of a 53 kDa immunoreactive polypeptide was reported in extracts from sperm tails using an anti-PLC ζ antibody (Fujimoto *et al.* 2004), and detection of this polypeptide by our antibodies may account for some of the non-specific staining observed in our preparations. Nonetheless, it is unlikely that this protein represents a fragment of PLC ζ as this reactivity was not abolished by the corresponding antigenic peptide.

Whether or not the loss of PLC ζ affects the ability of sperm to initiate oscillations remains untested since a knockout mouse is not yet available. Moreover, the temporal release of PLC ζ during fertilization is also an outstanding question. To start answering these questions, we depleted the content of PLC ζ from sperm using a high-pH treatment, which we have shown releases all the SF activity from the sperm into the supernatant (Kurokawa *et al.* 2005). PLC ζ was also depleted in a more physiological manner by enucleating the fertilizing sperm from the ooplasm after a time that is known to deplete the sperm's oscillatory activity (Knott *et al.* 2003). Under both conditions and in sperm of both species, the loss of the sperm's $[Ca^{2+}]_i$ oscillatory activity coincided with the loss of PLC ζ immunoreactivity, which occurred by approximately 90 min post-fertilization. Together, these results strengthen the view that PLC ζ is the putative factor responsible for the initiation of the $[Ca^{2+}]_i$ oscillations at fertilization in mammals. Future studies should examine the distribution of PLC ζ within the ooplasm soon after fertilization. We were unable to perform these studies given the high cross-reactivity of our antibodies with egg proteins.

In summary, our results demonstrate that both the SF activity and the PLC ζ content are emptied into the ooplasm within 90 min of sperm entry and that absence of PLC ζ reactivity coincides with loss of $[Ca^{2+}]_i$ oscillatory activity of mammalian sperm. These results support the notion that PLC ζ is necessary and sufficient to induce egg activation in mammals.

Materials and Methods

Gamete collection

Metaphase II arrested eggs were recovered from B6D2F1 (C57BL/6J $\times$ DBA/2J) female mice (6–8 weeks old) that were induced to ovulate by injection of 5 IU of human chorionic gonadotropin (hCG; Sigma), which was administered 46–48 h after an injection of 5 IU pregnant mare serum gonadotropin (Sigma). Eggs were collected from the oviducts 14–15 h post-hCG in HEPES-buffered tyrode-lactate solution (TL-HEPES) supplemented with 5% heat-treated fetal calf serum (FCS;

Gibco, BRL). Cumulus cells were removed by a brief treatment with 0.1% bovine testes hyaluronidase (Sigma). Mouse sperm were collected from the cauda epididymides of B6D2F1 or CD-1 male mice (11- to 24-weeks old) and washed two times with injection buffer (IB, 75 mM KCl and 20 mM HEPES, pH 7.0). Bull sperm were obtained from frozen semen samples (kindly donated by Dr Marvin Pace, American Breeder Services, DeForest, WI, USA) following selection using Percoll gradients (90–45%) according to the procedure described by others (Parrish *et al.* 1985). The selected live and motile sperm were washed twice and then resuspended in a Dulbecco's PBS (DPBS). Bull sperm were decapitated by a 30 s sonication on ice (XL2020; Heat Systems, Farmingdale, NY, USA). High-pH treatment was used to deplete the $[Ca^{2+}]_i$ oscillation-inducing activity from mouse and bull sperm using a procedure previously described by our laboratory (Kurokawa *et al.* 2005). In brief, after sonication and centrifugation, sperm pellets were washed with sperm buffer (75 mM KCl, 20 mM HEPES, 1 mM EGTA, 10 mM glycerophosphate, 1 mM dithiothreitol, 200 μ M phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin, 10 mg/ml leupeptin, pH 7.0), followed by a 30-min incubation in a high salt solution (1 M KCl, 10 mM Tris, pH 7.4) at 4 °C after which sperm were exposed to an alkaline carbonate solution (100 mM Na_2CO_3 , pH 11.5) for 10 min at 4 °C. The sperm suspension was then neutralized with 0.5 M Tris (pH 3.0) and washed with DPBS. All procedures were performed according to standard animal protocols approved by the University of Massachusetts Animal Care Committee.

Intracytoplasmic sperm injection (ICSI) and sperm enucleation

ICSI was performed as previously described (Kimura & Yanagimachi 1995, Kurokawa & Fissore 2003) using Narishige manipulators (Medical Systems Corp., Great Neck, NY, USA) mounted on a Nikon Diaphot microscope (Nikon Inc., Garden City, NY, USA). All manipulations were carried out in 50 μ l drops of HEPES-buffered CZB media (Chatot *et al.* 1989) under light mineral oil at room temperature (RT). Sperm were washed in IB and mixed with one part IB containing 12% polyvinyl pyrrolidone (MW 360 kDa; Sigma). Single sperm was aspirated into a blunt-ended pipette driven by a Piezo electric unit (Burleigh, Rochester, NY, USA). Several Piezo pulses were applied to separate the head from the tail and different intensity pulses were used to penetrate the zona pellucida and plasma membrane. After ICSI, zygotes were cultured in potassium simplex optimized medium (Specialty Media, Lavallete, NJ, USA) for 90 min. Enucleation, a term here exclusively reserved to indicate removal of the sperm head from the ooplasm, was carried out using the same set up and as previously described by our laboratory (Knott *et al.* 2003). Prior to enucleation, eggs were incubated in 3 μ g/ml Hoechst 33 342 for 5 min at RT. Enucleation was accomplished by bringing the pipette near the Hoechst-stained sperm head, the precise location of which was established by brief pulses of u.v. light, followed by aspiration using an IM-55-2 Narishige syringe. Control eggs underwent the same manipulation procedure, but a comparable volume of ooplasm was aspirated. Enucleated sperm heads used for immunostaining were freed of the surrounding cytoplasm by administration of Piezo pulses.

$[Ca^{2+}]_i$ monitoring

$[Ca^{2+}]_i$ monitoring was carried out as previously described (Jellerette *et al.* 2004). Eggs were first loaded with 1 μ M Fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) supplemented with 0.02% pluronic acid (Molecular Probes) for 20 min at RT and then transferred into 50 μ l drops of TL-HEPES (without FCS) placed on a glass coverslip sealed over an opening in the bottom of a culture dish and covered with mineral oil. Eggs were monitored simultaneously using a 20 $\times$ objective on a Nikon Diaphot inverted microscope (Nikon Corp., Tokyo, Japan) fitted for fluorescence measurements. A 75 W Xenon lamp provided the excitation light. The excitation wavelength was alternated between 340 and 380 nm by a filter wheel (Ludl Electronic Products, Hawthorne, NY, USA) and fluorescence ratios were obtained every 20 or 30 s. The emitted light was passed through a 510 nm barrier filter and collected with either a cooled Photometrics SenSys CCD or a cool SNAP ES digital camera (Roper Scientific, Tucson, AZ, USA). SimplePCI software (Compix Imaging Inc., Cranberry, PA, USA) was used to monitor $[Ca^{2+}]_i$ and synchronize the rotation of the filter wheel. $[Ca^{2+}]_i$ values are reported as the ratio of 340/380 nm fluorescence in the whole egg.

Antibodies and other chemicals

Two different anti-PLC ζ rabbit sera were raised (Kurokawa *et al.* 2005): one against a 19-mer sequence (GYRRVPLFSKSGAN-LEPSS) on the C-terminus of mouse (m) PLC ζ (accession no. NP_473403; Saunders *et al.* 2002) and the other against a 19-mer sequence (MENKWFSLMVRDDFKGGKI) on the N-terminus of pig (p) PLC ζ (accession no. BAC78817; Kurokawa *et al.* 2005). The antibodies specifically recognized PLC ζ in mouse and porcine sperm in Western blots respectively (Kurokawa *et al.* 2005). Okadaic acid (OA; Sigma), a phosphatase inhibitor, was dissolved in TL-HEPES medium and used at 10 μ M as a final concentration.

Sperm immunofluorescence

Sperm were fixed in 3.7% paraformaldehyde for 30 min at 4 °C followed by permeabilization with 0.1% (v/v) Triton X-100-DPBS for 10 min at RT. The sperm suspension was then spotted as 50 μ l drops onto 0.1% poly L-lysine pre-coated glass slides (Erie Sci., Portsmouth, NH, USA) and allowed to attach to the slide for 20 min at 37 °C. Sperm were incubated in 5% normal goat serum (NGS) in DPBS for 3 h at 4 °C and then incubated overnight at 4 °C with anti-pPLC ζ (NT; 1:100) or anti-mPLC ζ (CT; 1:100) in 5% NGS. After several washes with 0.1% (v/v) Tween 20-DPBS (DPBS-T), a secondary, Alexa Fluor 555-labeled goat anti-rabbit antibody (1:200) was added for 1 h at RT. Detection of the acrosome was performed by incubating the same sperm samples with 20 μ g/ml Alexa Fluor 488-conjugated peanut agglutinin (PNA)-lectin (from *Arachis hypogaea* peanut; Molecular Probes) in 5% NGS for 30 min at RT. After several washings in DPBS-T, samples were counterstained with 5 μ g/ml Hoechst 33 258 and mounted using the Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were obtained using a Zeiss Axiovert 200 M

microscope with a 63× oil immersion objective and a Hamamatsu Orca AG-cooled CCD Camera under the control of Openlab software (Improvision, Lexington, MA, USA).

Statistical analysis

Comparisons of $[Ca^{2+}]_i$ parameters were performed using Student's *t*-test. All experiments were repeated at least three times. Statistical comparisons were carried out using the Sigmaplot software (SPW 8.0, SPSS Inc., Chicago, IL, USA). Significance was set at $P < 0.05$.

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