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Proteolytic processing of phospholipase Cζ and $[Ca^{2+}]_i$ oscillations during mammalian fertilization

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Abstract

Phospholipase Cζ (PLCζ) is a sperm-specific PLC capable of causing repetitive intracellular Ca^{2+} ($[Ca^{2+}]_i$) release ($[Ca^{2+}]_i$ oscillations) in mammalian eggs. Accumulating evidence suggests that PLCζ is the sperm factor responsible for inducing egg activation. Nevertheless, some sperm fractions devoid of 72-kDa PLCζ showed $[Ca^{2+}]_i$ oscillation-inducing and PLCζ-like PLC activity (Kurokawa et al., (2005) *Dev. Biol.* 285, 376–392). Here, we report that PLCζ remains functional after proteolytic cleavage at the X–Y linker region. We found that N-terminal (33 and 37 kDa) and C-terminal fragments (27 kDa), presumably the result of PLCζ cleavage at the X–Y linker region, were present in fresh sperm as well as in sperm extracts and remained associated as functional complexes. Protease V8 cleaved 72-kDa PLCζ into 33/37 and 27 kDa fragments, while PLC activity and $[Ca^{2+}]_i$ oscillation-inducing activity persisted until degradation of the fragments. Immunodepletion or affinity depletion of these fragments abolished PLC activity and $[Ca^{2+}]_i$ oscillation-inducing activity from sperm extracts. Lastly, co-expression of cRNAs encoding residues 1–361 and 362–647 of mouse PLCζ, mimicking cleavage at the X–Y linker region, induced $[Ca^{2+}]_i$ oscillations and embryo development in mouse eggs. Our results support the hypothesis that PLCζ is the sole mammalian sperm factor and that its linker region may have important regulatory functions during mammalian fertilization.

Keywords: Calcium; Egg activation; Fertilization; Embryo development; Inositol 1,4,5-trisphosphate; Phospholipase Cζ; Proteolytic cleavage; Sperm factor; Src kinase

Introduction

In mammals, it is postulated that the fertilizing sperm delivers into the ooplasm a soluble protein, termed sperm factor, which evokes repetitive intracellular Ca^{2+} ($[Ca^{2+}]_i$) release, also known as $[Ca^{2+}]_i$ oscillations. These $[Ca^{2+}]_i$ oscillations induce egg activation, which comprises a series of events including the release of the cortical granules and the resumption of the cell cycle and that ultimately leads to embryo development (Schultz and Kopf, 1995; Ducibella et al., 2002). Earlier studies have shown that the sperm factor induces $[Ca^{2+}]_i$ oscillations by stimulating production of inositol 1,4,5-trisphosphate (IP_3), an event that requires the participation of a phospholipase C (PLC) (Dupont et al., 1996; Jones et al., 1998; Rice et al., 2000). IP_3 binds and gates IP_3 receptors (IP_3Rs), which are located in the endoplasmic reticulum, the internal Ca^{2+} store of eggs, thereby inducing Ca^{2+} release. Consistent with this proposed sequence of events, treatment of eggs with a PLC inhibitor (Dupont et al., 1996; Wu et al., 2001) or abrogation of IP_3R-1 function (Miyazaki et al., 1992; Wu et al., 1997) prevents the initiation of $[Ca^{2+}]_i$ oscillations. However, given that both sperm and eggs express several PLC isoforms, it has remained controversial whether the sperm factor is a sperm PLC or is an activator of an egg PLC (for a review, see Kurokawa et al., 2004a). Toward resolving this issue, the recent discovery of the sperm-specific

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PLCζ in the mouse (Saunders et al., 2002), along with the findings that injection of PLCζ recombinant protein or of its cRNA into eggs initiates sperm-like $[Ca^{2+}]_i$ oscillations (Saunders et al., 2002; Kouchi et al., 2004) and that its immunodepletion from cytosolic sperm extracts abolishes the ability of these extracts to cause oscillations (Saunders et al., 2002), has solidified the view that the sperm factor in mammals is a sperm PLC. However, whether PLCζ represents the only active component in all mammalian sperm/sperm extracts requires additional investigation.

The sperm-specific PLCζ is one of the smallest of the known PLCs consisting of only four EF-hands, the catalytic X- and Y-domains, and a C2 domain (Fig. 1A), all of which are common to the other PLC isoforms (β, γ, δ, ε and η). Besides the absence of the pleckstrin homology (PH) domain, the most salient feature of PLCζ is the exceptionally high Ca^{2+} sensitivity of its enzymatic activity; the enzyme is ~100-fold more sensitive to Ca^{2+} than PLCδ whose activity is also regulated by Ca^{2+} (Kouchi et al., 2004). Accordingly, PLCζ should be active at the basal $[Ca^{2+}]_i$ concentrations prevailing in cells, ~100 nM, which is consistent with the view that it serves as the initiator of oscillations at fertilization. Nevertheless, it is still unclear what molecular determinants of PLCζ confer such a high Ca^{2+} sensitivity, although mutational studies have revealed that both EF-hand and C2 domains, where important Ca^{2+}-binding sites are located, are required for full activation of PLCζ (Kouchi et al., 2005; Nomikos et al., 2005). Likewise, it is unknown how the activity of the enzyme is regulated *in vivo*. Provided that PLCζ is active at resting $[Ca^{2+}]_i$ levels, it seems important that its enzymatic activity be carefully controlled prior to interaction of the gametes so that premature acrosome reaction is averted. In this regard, Miyazaki and co-workers recently showed that *in vitro* PLCζ activity is diminished by interaction of the C2 domain with PI(3)P and PI(5)P, although the physiological relevance of this mechanism remains to be tested (Kouchi et al., 2005).

We have previously demonstrated that two sperm fractions (SF), the cytosolic fraction (SF^C) and the high-pH soluble fraction (SF^{pH}), can be obtained by conventional sonication/freeze-thawing and biochemical processing of porcine (p) sperm (Kurokawa et al., 2005); both SFs were equally capable of triggering $[Ca^{2+}]_i$ oscillations and activation when microinjected into mouse eggs. To our surprise, although both SF^C and SF^{pH} showed PLCζ-like *in vitro* PLC activity, immunoblotting studies revealed that only trace amounts of full-length PLCζ (72-kDa PLCζ) were present in SF^{pH} compared to SF^C (Kurokawa et al., 2005). Moreover, additional chromatographic processing of these fractions showed that other active fractions also lacked 72-kDa PLCζ (Kurokawa et al., 2005). Hence, we envisioned two possibilities: (1) there is an unidentified sperm-specific PLC isoform that also contributes to the sperm-induced $[Ca^{2+}]_i$ oscillations; or (2) PLCζ undergoes some kind of post-translational modification, such as proteolytic cleavage, which disables its immunological detection but not its phosphoinositide-hydrolyzing activity. In the present study, we show evidence that PLCζ remains functional even after proteolytic cleavage at the linker region that connects the X- and Y-domains. Both SF^C and SF^{pH} contain N-terminal and C-terminal PLCζ fragments that form functional complexes as their depletion abrogates $[Ca^{2+}]_i$ oscillation-triggering activity as well as PLC activity from SF^{pH} fractions. Moreover, SF^C treated with *Staphylococcus aureus* protease V8 retains the ability to induce $[Ca^{2+}]_i$ oscillations even after 72-kDa PLCζ, but not the fragments, is no longer detectable by immunoblotting. We also found that co-expression of cRNAs encoding residues 1–361 and 362–647 of mouse PLCζ (mPLCζ), which approximates cleavage of the linker region, induces $[Ca^{2+}]_i$ oscillations and embryo development in mouse eggs. Collectively, our results confirm that PLCζ is the only molecule in our sperm extracts capable of inducing $[Ca^{2+}]_i$ oscillations and further show that its proteolytic processing may play a role during mammalian fertilization.

Materials and methods

Gamete collection

Eggs at the second stage of meiosis (MII) were obtained from CD1 female mice (8–12 weeks old) superovulated by injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, MO) followed 48 h later by 5 IU of human chorionic gonadotropin (hCG; Sigma). Eggs were harvested 14 h post-hCG into a Hepes-buffered tyrode–lactate solution (TL-Hepes) supplemented with 5% heat-treated calf serum (Parrish et al., 1988). Cumulus cells were removed by brief treatment with 0.1% bovine testes hyaluronidase (Sigma). Porcine sperm were collected from freshly ejaculated semen and were immediately washed two times either with injection buffer (IB: 75 mM KCl and 20 mM HEPES, pH 7.0) for sperm extract preparation or with TL-Hepes for immunoblotting.

All procedures involving live animal handling and euthanasia were performed according to standard animal protocols approved by the University of Massachusetts Animal Care Committee.

Preparation of porcine sperm extracts

SF^C was prepared as previously described (Wu et al., 2001). Briefly, after washing, the sperm suspension was sonicated in sperm buffer (75 mM KCl, 20 mM Hepes, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM DTT, 200 μM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, pH 7.0) at 4 °C and the lysate ultracentrifuged. The clear supernatant was concentrated with ultrafiltration membranes (Centriprep-30; Amicon, Beverly, MA). These extracts were then mixed for 30 min at 4 °C with ammonium sulfate at 50% final saturation; the precipitates were collected by centrifugation and kept at −80 °C. Upon use, the pellets were resuspended with IB, washed and re-concentrated with ultrafiltration membranes.

SF^{pH} was obtained as we reported previously (Kurokawa et al., 2005). In brief, after sonication, the porcine sperm pellets were washed with sperm buffer twice and then once with sperm buffer containing 1 M KCl. The resulting pellets were treated with 100 mM Na_2CO_3 (pH 11.5). After the treatment, the sperm suspension was neutralized, dialyzed against IB and concentrated. Samples were aliquoted and kept at −80 °C.

cDNA constructs

Full length mPLCζ (mPLCζ WT; Accession No. AF435950), a variant lacking three N-terminal EF-hand domains (mPLCζ ΔEF; Accession No. AK006672), and the regions corresponding to residues 248–647, 362–647 and 454–647 of mPLCζ were amplified by PCR. The regions corresponding to residues 1–557 (mPLCζ ΔC2), 1–247, 1–361 and 1–453 of mPLCζ were made by introducing a stop codon to the respective sites of mPLCζ WT using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Likewise, D210R and S405A mutations were generated using the QuikChange kit according to the