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Functional, biochemical, and chromatographic characterization of the complete $[Ca^{2+}]_i$ oscillation-inducing activity of porcine sperm

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Abstract

A cytosolic sperm protein(s), referred to as sperm factor (SF), is delivered into eggs by the sperm during mammalian fertilization to induce repetitive increases in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) that are referred to as $[Ca^{2+}]_i$ oscillations. $[Ca^{2+}]_i$ oscillations are essential for egg activation and early embryonic development. Recent evidence shows that the novel sperm-specific phospholipase C (PLC), PLCζ, may be the long sought after $[Ca^{2+}]_i$ oscillation-inducing SF. Here, we demonstrate the complete extraction of SF from porcine sperm and show that regardless of the method of extraction a single molecule/complex appears to be responsible for the $[Ca^{2+}]_i$ oscillation-inducing activity of these extracts. Consistent with this notion, all sperm fractions that induced $[Ca^{2+}]_i$ oscillations, including FPLC-purified fractions, exhibited high in vitro PLC activity at basal Ca^{2+} levels (0.1–5 μM), a hallmark of PLCζ. Notably, we detected immunoreactive 72-kDa PLCζ in an inactive fraction, and several fractions capable of inducing oscillations were devoid of 72-kDa PLCζ. Nonetheless, in the latter fractions, proteolytic fragments, presumably corresponding to cleaved forms of PLCζ, were detected by immunoblotting. Therefore, our findings corroborate the hypothesis that a sperm-specific PLC is the main component of the $[Ca^{2+}]_i$ oscillation-inducing activity of sperm but provide evidence that the presence of 72-kDa PLCζ does not precisely correspond with the Ca^{2+} releasing activity of porcine sperm fractions.

Keywords: Egg activation; Fertilization; Oocyte

Introduction

In response to fertilization, mammalian eggs exhibit repetitive increases in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) that last for several hours after sperm entry; this $[Ca^{2+}]_i$ signal is commonly referred to as $[Ca^{2+}]_i$ oscillations (Miyazaki et al., 1993; Stricker, 1999; Runft et al., 2002). The sperm-initiated $[Ca^{2+}]_i$ oscillations are required to induce all events of egg activation, a process which includes cortical granule exocytosis, resumption and completion of meiosis, DNA replication, and that culminates in the first mitotic cleavage (for review, see Schultz and Kopf, 1995). Evidence suggests that the fertilizing sperm, upon gamete fusion, delivers into the ooplasm a soluble substance that is responsible for initiating the $[Ca^{2+}]_i$ oscillations. In support of this concept, direct sperm injection into eggs, even though it circumvents the required interaction and fusion of gametes, evokes $[Ca^{2+}]_i$ responses similar to those induced by fertilization (Kimura and Yanagimachi, 1995; Nakano et al., 1997; Kurokawa and Fissore, 2003). Moreover, injection of sperm extracts (SEs), but not of extracts from other tissues, also triggers $[Ca^{2+}]_i$ oscillations and egg activation (Swann, 1990; Homa and

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Swann, 1994; Wu et al., 1997, 1998; Jones et al., 2000). Nonetheless, the molecular identity of the sperm's $[Ca^{2+}]_i$ oscillations-inducing factor is still under investigation and is therefore presently called sperm factor (SF).

The Ca^{2+} release and oscillations induced by the sperm are widely thought to be mediated by the inositol 1,4,5-trisphosphate receptor 1 (IP_3R-1), a ligand-gated Ca^{2+} channel located in the endoplasmic reticulum membrane, the main intracellular Ca^{2+} store (Miyazaki et al., 1992; Mehlmann et al., 1996; Wu et al., 1997; Parrington et al., 1998; Fissore et al., 1999). The natural ligand of IP_3Rs is inositol 1,4,5-trisphosphate (IP_3), one of the products of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC) enzymes (for review, see Berridge et al., 2000). There is a growing list of PLC isoforms (Rhee, 2001; Hwang et al., 2005) and, consistent with a pivotal role in early development, both male and female gametes express several PLC isoforms (Dupont et al., 1996; Mehlmann et al., 1998; Wang et al., 1998; Fukami et al., 2001; Wu et al., 2001; Parrington et al., 2002; Saunders et al., 2002). Evaluation of the functional role of these enzymes in fertilization, which was performed by means of PLC isoform-specific inhibition, revealed that one of the PLC isoforms present in eggs, PLCγ, was responsible for IP_3 production and Ca^{2+} release during fertilization in sea urchin, starfish, ascidian, and *Xenopus* (Carroll et al., 1997, 1999; Shearer et al., 1999; Runft and Jaffe, 2000; Sato et al., 2003). However, similar studies failed to implicate any of the traditional PLCs in mammalian fertilization (Williams et al., 1998; Mehlmann et al., 1998). Therefore, despite the realization of the essential role of IP_3R-1-mediated Ca^{2+} release in mammalian fertilization, identification of the PLC isoform responsible for the underlying IP_3 production has remained elusive (for review, see Runft et al., 2002; Kurokawa et al., 2004a).

In spite of the initial failure to identify the participating PLC, two findings convincingly pointed to a critical role of these enzymes in mammalian fertilization. First, sperm- and SE-initiated $[Ca^{2+}]_i$ oscillations were blocked by addition of the general PLC inhibitor U-73122, but not by exposure to its inactive analog (Dupont et al., 1996; Wu et al., 2001). Second, the capacity of SEs to initiate $[Ca^{2+}]_i$ oscillations closely correlated with enhanced in vitro PLC activity in active fractions (Jones et al., 2000). Interestingly, this PLC activity seemed to effectively operate in the presence of low environmental Ca^{2+} levels (Rice et al., 2000), which is in contrast to the high Ca^{2+} level required for the activity of other PLC isoforms (Allen et al., 1997). Therefore, it was of significance when Lai and co-workers identified and characterized a novel sperm-specific PLC, PLCζ (Saunders et al., 2002), which showed a 100-fold higher sensitivity to Ca^{2+} than other PLC isoforms (Kouchi et al., 2004). PLCζ with a molecular mass of 74 kDa is the smallest of the PLC subtypes expressed in vertebrates, although it exhibits the modular domain organization characteristic of PLCs including four EF hand motifs, X and Y catalytic domains, and a C2 domain (Saunders et al., 2002); similar to PLCδ subtypes, PLCζ is devoid of the regulatory domains present in the β and γ isoforms (Rhee, 2001). Consistent with its proposed role in fertilization, injection of PLCζ cRNA or its recombinant protein triggered fertilization like $[Ca^{2+}]_i$ oscillations in mouse eggs, whereas injection of the same concentration of cRNA PLCδ1, a closely related isoform expressed in testis (Lee et al., 1999), failed to do so (Saunders et al., 2002; Kouchi et al., 2004). In addition, immunodepletion of PLCζ from hamster SEs (hSEs) curtailed the ability of these extracts to trigger $[Ca^{2+}]_i$ oscillations (Saunders et al., 2002). Furthermore, overexpression of tagged fusion proteins revealed that PLCζ accumulated into pronuclei (Larman et al., 2004; Yoda et al., 2004), which is in agreement with earlier observations that pronuclei of fertilized zygotes, but not of chemically activated counterparts, have the ability to trigger $[Ca^{2+}]_i$ oscillations when introduced into new metaphase II eggs (Kono et al., 1995; Knott et al., 2003). Taken together, these findings implicate PLCζ as the long sought after SF responsible for the initiation of oscillations during mammalian fertilization.

In spite of the aforementioned evidence, it still remains to be demonstrated whether or not PLCζ represents the physiological, and possibly exclusive trigger of $[Ca^{2+}]_i$ oscillations during fertilization and after injections of SEs. In regard to the latter, studies using mouse sperm have argued that fractions capable of inducing $[Ca^{2+}]_i$ responses can be recovered from at least two very different subcellular localizations. One of these fractions is called "cytosolic" or "soluble", as is collected after breaking off the sperm membranes (Swann, 1990; Wu et al., 1997), whereas the other fraction remains associated with the sperm nucleus and its recovery requires more aggressive extraction procedures (Kuretake et al., 1996; Perry et al., 1999, 2000; Kurokawa and Fissore, 2003). It is worth noting that thus far the presence of PLCζ has mainly been demonstrated in soluble SEs (Saunders et al., 2002).

In this study, we investigated whether or not 72-kDa porcine PLCζ (pPLCζ) represents the lone $[Ca^{2+}]_i$ oscillation-inducing component in porcine SEs (pSEs) that include the complete oscillatory ability of porcine sperm. Our findings corroborate the hypothesis that a sperm-specific PLC with high sensitivity to Ca^{2+} is responsible for the initiation of $[Ca^{2+}]_i$ oscillations in mammalian fertilization but also show that the presence of immunoreactive 72-kDa pPLCζ does not always correspond with the $[Ca^{2+}]_i$ oscillatory activity of pSEs.

Materials and methods

Gamete collection

Metaphase II eggs were obtained from B6D2F1 (C57BL/6J × DBA/2J) and CD-1 female mice (8–12 weeks old)