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PLCζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development

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SUMMARY

Upon fertilisation by sperm, mammalian eggs are activated by a series of intracellular Ca^{2+} oscillations that are essential for embryo development. The mechanism by which sperm induces this complex signalling phenomenon is unknown. One proposal is that the sperm introduces an exclusive cytosolic factor into the egg that elicits serial Ca^{2+} release. The 'sperm factor' hypothesis has not been ratified because a sperm-specific protein that generates repetitive Ca^{2+} transients and egg activation has not been found. We identify a novel, sperm-specific phospholipase C, PLCζ, that triggers Ca^{2+} oscillations in mouse eggs indistinguishable from those at fertilisation. PLCζ removal from sperm extracts abolishes Ca^{2+} release in eggs. Moreover, the PLCζ content of a single sperm was sufficient to produce Ca^{2+} oscillations as well as normal embryo development to blastocyst. Our results are consistent with sperm PLCζ as the molecular trigger for development of a fertilised egg into an embryo.

Key words: Fertilisation, Sperm factor, Phospholipase C, Ca^{2+} oscillations, Egg activation, Mouse

INTRODUCTION

Activation of egg development in all animals and plants is produced by the fertilising spermatozoa triggering an acute rise in cytosolic free Ca^{2+} concentration (Stricker, 1999). In mammals, the unification of sperm and egg leads to a distinctive series of cytosolic Ca^{2+} oscillations that are a prerequisite for normal embryo development (Miyazaki et al., 1993; Stricker, 1999). This striking Ca^{2+} signalling phenomenon arises from increases in inositol 1,4,5-trisphosphate (IP_3) levels, which activate IP_3 receptor-mediated Ca^{2+} release from intracellular stores in the egg (Miyazaki et al., 1993; Brind et al., 2000; Jellerette et al., 2000). However, the basic mechanism that results in stimulation of phosphoinositide (PI) metabolism following sperm-egg interaction has not been determined in any species.

The sperm factor hypothesis of signalling at fertilisation proposes that spermatozoa contain a soluble Ca^{2+} releasing factor that enters the egg after the gamete membranes fuse together and generates Ca^{2+} oscillations (Swann, 1990; Stricker, 1999). This is consistent with the finding that cytoplasmic fusion of sperm and egg is a prelude to Ca^{2+} release (Lawrence et al., 1997; Jones et al., 1998a). Direct support for this hypothesis comes from experiments where microinjection into eggs of either single spermatozoa, or soluble sperm extracts, triggers Ca^{2+} oscillations similar to those at fertilisation in mammalian and some non-mammalian eggs (Swann, 1990; Wu et al., 1997; Wu et al., 1998; Stricker, 1997; Nakano et al., 1997; Kyozuka et al., 1998; Tang et al., 2000). The mammalian sperm factor that generates Ca^{2+} oscillations is protein based (Swann, 1990), acts across species (Wu et al., 1997), and can cause Ca^{2+} release in somatic cells (Berrie et al., 1996) and in cell-free systems such as sea urchin egg homogenates (Jones et al., 1998b). Sperm specifically express a Ca^{2+} oscillation-inducing protein, because microinjecting mRNA isolated from spermatogenic cells, but not mRNA from other tissues, elicits fertilisation-like Ca^{2+} oscillations in mouse eggs (Parrington et al., 2000). Despite intensive biochemical investigation, the molecular identity of the putative sperm factor has remained elusive (Stricker, 1999). Different proteins, including a 33 kDa protein (Parrington et al., 1996) and a truncated form of the *Kit* receptor (Sette et al., 1997), have previously been sperm factor candidates. However, neither these two, nor any other sperm proteins, have been shown to generate Ca^{2+} oscillations in eggs (Wu et al., 1998; Wolosker et al., 1998), the single-most distinctive feature of mammalian fertilisation (Stricker, 1999).

In intact eggs and egg homogenates, mammalian sperm extracts trigger Ca^{2+} release by stimulating IP_3 production (Jones et al., 1998b; Rice et al., 2000; Jones et al., 2000; Wu et al., 2001), indicating involvement of a PI-specific phospholipase C (PLC) in the signal transduction mechanism. The high level of PLC enzyme activity measured biochemically in sperm extracts suggests that the sperm factor

may itself be a PLC (Jones et al., 1998b; Rice et al., 2000). However, the PLCβ, γ and δ isoforms that exist in sperm are absent from chromatographic fractions of sperm extract that specifically cause Ca^{2+} oscillations (Wu et al., 2001; Parrington et al., 2002). In addition, when the purified, recombinant PLCβ1, γ1, γ2 or δ1 proteins are added to egg homogenates or microinjected into eggs, they fail to cause Ca^{2+} release (Jones et al., 2000). A PLCδ4 splice variant found in sperm functions in the acrosome reaction, rather than in Ca^{2+} release in eggs at fertilisation (Fukami et al., 2001). These observations led us to investigate the possible existence of a distinct, uncharacterised sperm PLC isoform. Our studies reveal that a new PLC isoform (PLCζ), specifically expressed in mammalian sperm, uniquely possesses all the essential properties of the sperm factor. These results are consistent with sperm PLCζ as the physiological trigger of egg activation, and thus an essential protein for mammalian fertilisation and embryo development.

MATERIALS AND METHODS

Characterisation of a novel sperm PLC

Rabbit antisera was raised to a 19-mer sequence, GYRRVPLFSKSGANLEPSS, within the mouse testis ESTs identified as homologous to PLC (see below). Mammalian tissues and sperm cytosolic proteins (Parrington et al., 1996), were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with anti-peptide antisera. Heparin affinity and gel filtration column chromatography of soluble sperm proteins (10 mg) used an AKTA FPLC system (Amersham Pharmacia) (Parrington et al., 1996) in 50 mM sodium phosphate, 0.15 M NaCl, pH 7. Fluorometric Ca^{2+} release assays using fluo-3 in sea urchin homogenates (Jones et al., 1998b) was monitored using an LS50B (Perkin-Elmer).

Molecular cloning and sequence analysis of mouse sperm PLCζ

Blast searches of the mouse EST database using mammalian PLC sequences (www.ncbi.nlm.nih.gov/BLAST) identified 12, novel PLC-related sequences (Accession Numbers, AV282878, AV278700, AV278207, AV272100, AV271735, AV270614, AV270212, AV263382, AV263095, AV258739, AV258594 and AV045146). These mouse ESTs were 232-294 basepairs with identical 3′ sequences and all derived from testis. The full-length sequence encoding this novel PLC, named PLCζ, was obtained by two-step RACE PCR amplification with *pfu* polymerase from a mouse spermatid cDNA library (35 ng) in lambdaZAPII. The single amplified DNA of 2.2 kb was cloned into pCR-XL-TOPO (Invitrogen), ten independent colonies were sequenced on both strands, and analysed for open reading frame by MacVector 6.5 (Oxford Molecular), for PLC homology and phylogeny by ClustalW sequence alignment (www.clustalw.genome.ad.jp) and domain structure by RPS-Blast (www.ncbi.nlm.nih.gov/structure/cdd). The GenBank Accession Number for PLCζ is AF435950.

Northern blot and polymerase chain reaction analysis

A 1.2 kb probe from the 5′ end of mouse PLCζ, prepared by PCR as above, was cloned into pCR-BluntII-TOPO (Invitrogen) and sequenced. Antisense digoxigenin-labelled RNA synthesised from this plasmid (DIG Nucleic acid labelling system, Roche Molecular Biochemicals) was used to probe a male mouse tissue polyA$^+$-RNA blot with equal loading of 2 μg polyA$^+$-RNA/lane (MessageMap Northern, Stratagene). Hybridised probe was detected using the DIG Luminescence Detection Kit (Roche Molecular Biochemicals) and displayed using QuantityOne software (BioRad). Polymerase chain reaction amplification using 30 cycles was performed with oligonucleotide primers that define a 0.9 kb region within PLCζ, using cDNA prepared from mouse spermatids or mouse testis devoid of spermatids in the lambda ZAPII vector (10 ng). Negative and positive controls comprised reactions without DNA template and with PLCζ plasmid DNA (1 ng), respectively.

Complementary RNA synthesis and in vitro translation

The 1941 bp open reading frame of mouse PLCζ was cloned into pCR-Blunt II-TOPO, sequenced and subcloned (pTarget, Promega) to generate pTarget-mPLCζ. Complementary RNA (cRNA) was synthesised from linearised pTarget-mPLCζ (Ribomax RNA synthesis, Promega) in the presence of 3 mM $m^7G(5')ppp(5')G$, isopropanol precipitated and resuspended in DEPC-treated water containing 4 U/μl RNasin (Promega). Mutagenesis of ^{210}Asp to ^{210}Arg in PLCζ to produce ^{D210R}PLCζ was achieved using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Constructs and cRNAs for rat PLCδ1 and $^{\Delta PH}$PLCδ1, which encoded the full-length (756 amino acids) and PH domain-deleted PLCδ1 (Δ1-132), respectively, and ^{D210R}PLCζ were produced in pTarget as above. cRNA (2 μg) was expressed in vitro (Reticulocyte lysate system, Promega) in the presence of [^{35}S]methionine (Amersham Pharmacia). Radiolabelled protein, analysed by SDS-PAGE and autoradiography, was displayed using QuantityOne software (BioRad).

Epitope tagging, bacterial expression and PLCζ quantitation

The 1941 bp open reading frame of mouse PLCζ was subcloned into pGBK-T7 (Clontech) with an in-frame Myc epitope tag at the 5′-end. The Myc-PLCζ was further subcloned into pcDNA3.1 and sequence-verified before cRNA synthesis from the T7 site (Ribomax) for egg microinjection, as described above. For bacterial expression, Myc-PLCζ was subcloned into pBAD (Invitrogen) with an in-frame hexahistidine tag at the 3′ end. The Myc-PLCζ-Histag protein was produced in 0.2% w/v arabinose-induced, BL21(DE3)pLysS *E. coli*, after extraction of the pelleted bacteria by five freeze-thaw and ultrasonication cycles, then purified by nickel affinity chromatography (ProBond, Invitrogen). Protein quantitation was performed using the BCA protein assay (Pierce).

Densitometric analysis of the Myc-PLCζ band expressed in eggs microinjected with different cRNA concentrations (Fig. 6C), Myc-PLCζ-Histag protein purified from *E. coli*, and calibrated sperm extract PLCζ derived from 10^4-10^6 mouse sperm, employed a Myc monoclonal antibody (1:2000, Santa Cruz Biotechnology) and rabbit anti-PLCζ antiserum (1:1000), respectively, using QuantityOne software (BioRad). A calibration standard plot, from analysis by immunoblot densitometry (Malek et al., 1997) using the Myc antibody, was constructed using defined amounts of Myc-PLCζ-Histag protein, purified from *E. coli*, to enable the calculation of the relative Myc-PLCζ content in batches of 100 microinjected eggs. For the quantitation analysis, expression of the Myc-PLCζ protein was assumed to be linear with time after cRNA microinjection, as was shown for microinjected EGFP cRNA expressed in mouse eggs (Aida et al., 2001). This assumption was necessary because the c-Myc-PLCζ protein was below the detection limit within 3 hours of cRNA microinjection (data not shown). Hence, for a single mouse egg, the calculated 440-750 fg of Myc-PLCζ protein expressed 5 hours after microinjection with 0.02 mg/ml cRNA, was equivalent to 44-75 fg expressed at 0.5 hours, the time when the first Ca^{2+} transient is normally observed (Fig. 5B). A separate calibration plot using the anti-PLCζ antibody was constructed with different Myc-PLCζ-Histag protein concentrations to enable estimation of the relative PLCζ content in defined numbers of mouse sperm (Fig. 6D).

Immunodepletion of PLCζ from sperm extracts

Soluble extracts (Parrington et al., 1999) prepared from hamster sperm were incubated for 1 hour at 4°C with control IgG or anti-PLCζ. antibody that had been covalently attached to Protein G beads (1

mg/ml, Seize X Kit, Pierce). The PLCζ content of the supernatant and precipitated beads was determined by immunoblot analysis with anti-PLCζ antibody. Antibody-treated sperm supernatants were also analysed for Ca^{2+} release activity by fluo-3 fluorometry with sea urchin egg homogenates, as described above, and for ability to generate Ca^{2+} oscillations by microinjection into mouse eggs, as described below. Maximal immunodepletion of the sperm PLCζ protein was achieved by using an optimised ratio of antibody beads to sperm extract for each experiment (n=4). The optimal ratio was empirically determined for each sperm extract preparation as the minimum concentration of sperm extract (0.3-0.8 mg/ml) that still retains Ca^{2+} release activity after treatment with the control IgG beads.

Preparation and handling of gametes

Mouse egg procedures were carried out either in Hepes-buffered KSOM or amino acid supplemented KSOM (Summers et al., 2000). Female MF1 mice were superovulated by injection with 5 IU of PMSG followed 48 hours later by HCG (Intervet). Eggs were collected 13.5-14.5 hours after HCG, maintained in 100 μl droplets of H-KSOM under mineral oil at 37°C and cRNA microinjection performed within 1 hour. Expression of Myc-PLCζ in eggs was examined 5 hours after cRNA microinjection, by adding SDS sample buffer to pelleted eggs and incubating at 95°C for 5 minutes prior to SDS-PAGE, immunoblot then densitometric analysis with the Myc monoclonal antibody, as described above. Calibrated mouse sperm pellets were resuspended in 10 mM Tris-HCl pH 7.5, 15 mM dithiothreitol (Perry et al., 1999), then subjected to five freeze-thaw cycles in liquid N_2 and centrifuged at 20,000 ***g*** at 4°C for 10 minutes, before densitometric analysis of the soluble extract with PLCζ antibody, as described above. For in vitro fertilisation studies, sperm were capacitated for 2-3 hours before being added to eggs. Egg activation and development studies were in H-KSOM containing 2 μM cytochalasin D for 4 hours. Further development to two-cell stage, morula and blastocyst stage was carried out in 50 μl droplets of KSOM under mineral oil at 37°C in a 5% CO_2 incubator.

Measurement of intracellular Ca^{2+} in MII-arrested mouse eggs

Eggs loaded with 4 μM Fura red-AM (Molecular Probes) for 10 minutes were washed in H-KSOM and placed on a Nikon Diaphot stage. Loading media included sulfinpyrazone to prevent dye compartmentalisation and extrusion (Lawrence et al., 1997). cRNA solutions in 120 mM KCl, 20 mM Hepes, pH 7.4, were microinjected to 3-5% of egg volume as previously described (Swann, 1990). Protein synthesis was inhibited in control experiments (Lawrence et al., 1998; Jones et al., 1995) where eggs were preincubated in solution containing 10 μM cycloheximide for 30 minutes before microinjection with PLCζ cRNA (0.02 mg/ml; n=9). Injection volume was estimated from the displacement caused by bolus injection. Ca^{2+} measurements were performed on a CCD-based imaging system as previously described (Lawrence et al., 1997), or a Zeiss Axiovert 100 with illumination from a monochromator (Photonics) controlled by MetaFluor v4.0 (Universal Imaging Corp).

RESULTS

Identification of a novel sperm PLC

Analysis of the mouse EST database for PLC-related sequences reveals twelve testis-derived expressed sequence tags (ESTs) with identical 3′ ends, apparently from a single mouse testis gene, encoding the C terminus of a putative novel PLC (see Materials and Methods). The putative testis PLC sequence is not found in ESTs from any other tissue. An antiserum raised to an unique peptide antigen deduced from

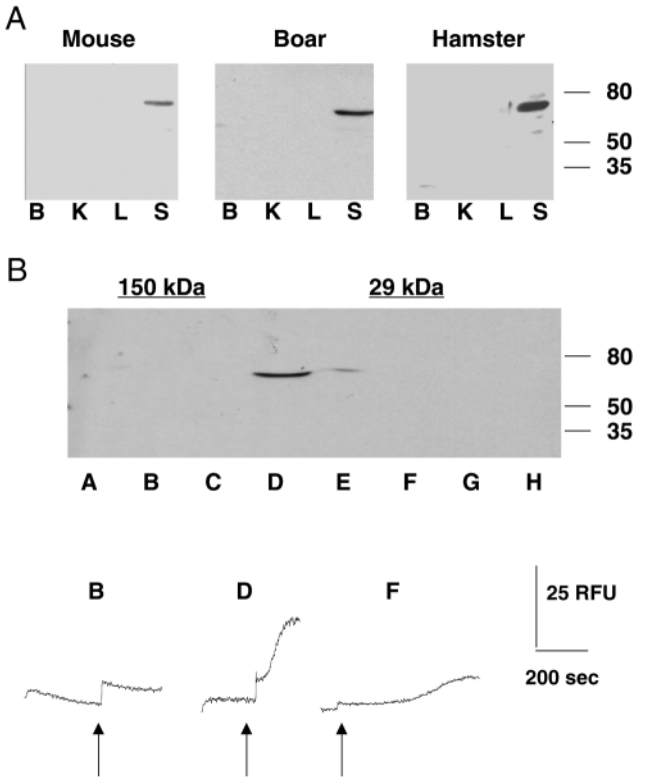

Fig. 1. Identification of a novel sperm PLC. (A) Immunoblot analysis of heparin-eluted soluble extracts of brain, kidney, liver and sperm (lanes B, K, L, S; 50 μg/lane) from mouse, boar and hamster, using antibody raised to the novel sperm PLC. Molecular weight markers in kDa, on the right. (B) Immunoblot of heparin-eluted soluble sperm proteins fractionated by gel filtration column chromatography on Sephacryl S-200 column. The underlined 150 kDa and 29 kDa indicate elution positions of gel filtration standards alcohol dehydrogenase and carbonic anhydrase, respectively. Shown below is the corresponding Ca^{2+} release activity of column fractions B, D and F assayed fluorometrically in sea urchin egg homogenates. Scale bars indicate time (seconds) and relative fluorescence units (RFU) (Jones et al., 1998b). Arrows indicate time of addition.

the mouse testis ESTs recognises a single protein band of ~70 kDa) in immunoblots of mouse, boar and hamster sperm (Fig. 1A). Soluble protein extracts from several other tissues are devoid of this immunoreactivity, suggesting that the ~70 kDa protein is specifically enriched in sperm (Fig. 1A). Gel filtration chromatography of sperm extracts shows that the immunoreactive sperm protein elutes between the 150 kDa and 29 kDa markers, consistent with a ~70 kDa monomer in solution (Fig. 1B). Importantly, the ~70 kDa protein specifically co-migrates with Ca^{2+} release activity in fluorometric assays using egg homogenate (Fig. 1B). This is in contrast to previous chromatographic studies where antibodies to the PLCβ, γ and δ isoforms showed that they did not co-migrate with Ca^{2+} releasing activity (Wu et al., 2001; Parrington et al., 2002). The elution profile further indicates that the ~70 kDa sperm protein is unrelated to the recently discovered PLCε, which has a molecular mass of ~250 kDa (Lopez et al., 2001; Song et al., 2001), and therefore it could be a new PLC.

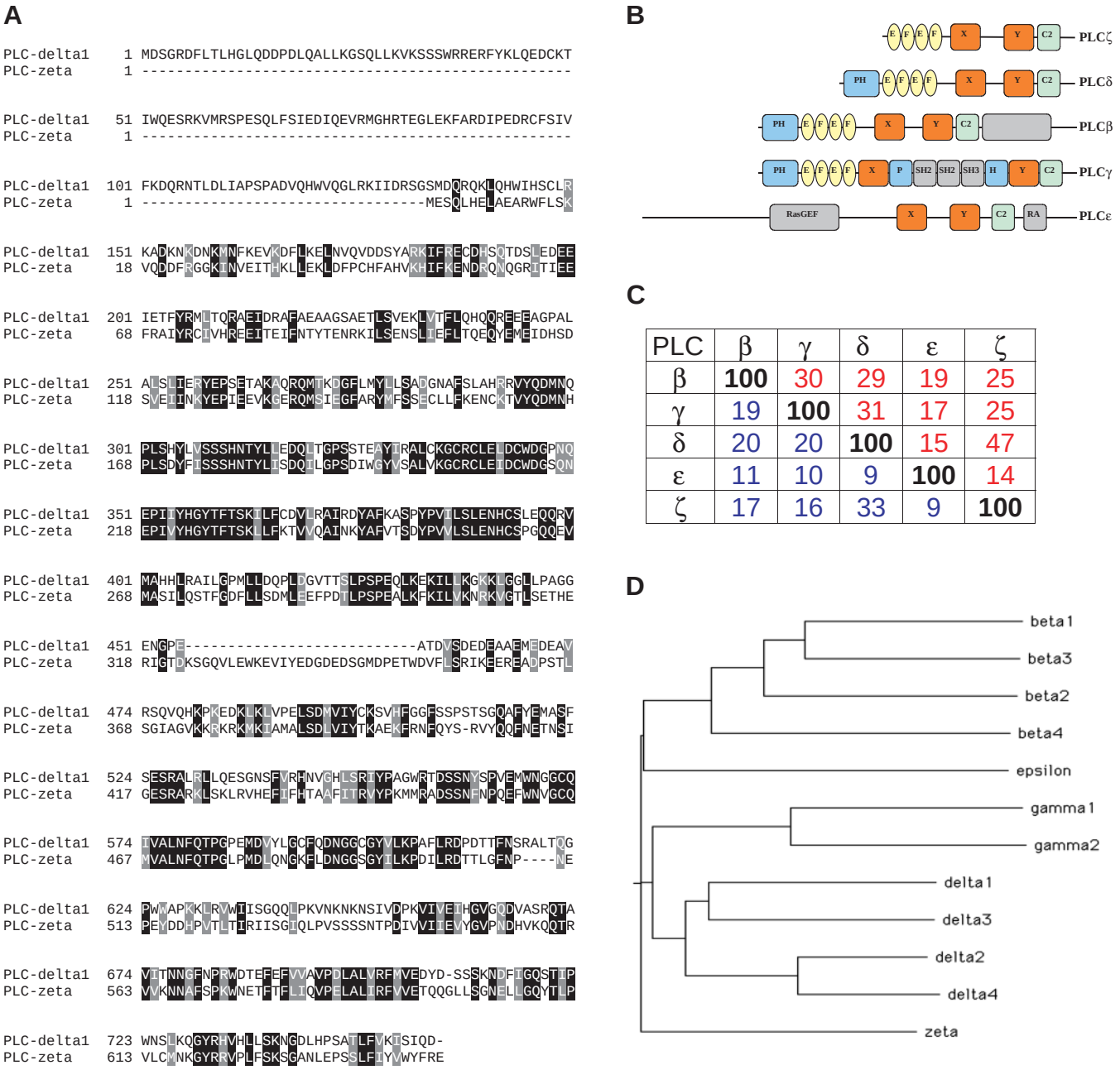

Fig. 2. Molecular cloning of mouse sperm PLCζ. (A) Clustal alignment of mouse sperm PLCζ with rat PLCδ1 (Accession number, P10688). Identical amino acids are shown in shaded black boxes, conservative substitutions in grey. (B) Schematic illustrating the predicted domain features of mouse PLCζ and mammalian PLC isoforms β, γ, δ, and ε. (C) Sequence identity (blue) and similarity (red) between mammalian PLC isoforms (β3, P51432; γ2, AAH07565; δ1, P10688; ε, AAG17145; and ζ). (D) Dendrogram illustrating phylogeny of Clustal aligned mammalian PLC sequences. Tree branch lengths, indicating amino acid substitutions per residue, were 0.298 for ζ; 0.309-0.322 for δ1-4; 0.397 for ε; 0.400-0.413 for β1-4; and 0.412-0.417 for γ1-2.

Molecular cloning of sperm PLCζ

The complete cDNA sequence encoding a novel sperm PLC homologue was cloned from a mouse spermatid cDNA library by PCR using oligonucleotide primers designed with the mouse ESTs identified above. Within the ~2.2 kb sequence, untranslated regions at the 5′ and 3′ ends, of 194 basepairs (bp) and 52 bp (excluding polyA$^+$-tract), respectively, are found flanking a single open reading frame (ORF) of 1941 bp. The ORF encodes a novel protein sequence of 647 amino acids, with a predicted molecular mass of 74 kDa and pI of 5.3 (Fig. 2A). The novel 74 kDa protein includes the C-terminal peptide sequence used to produce the antiserum and is consistent with the native sperm protein of ~70 kDa detected in immunoblots (Fig. 1A). Blastp sequence analysis suggests that the sperm protein is a novel PLC isoform, smaller than all those previously identified (PLCβ, γ, δ and ε) (Katan and Williams, 1997; Rebecchi and Scarlata, 1998; Rhee, 2001), which we accordingly assign PLCζ.

Clustal alignment of PLCζ with PLCδ1 reveals that the most notable difference of this new isoform is that it lacks an N-terminal PH domain (Fig. 2A,B). A single PH domain is found at the N terminus of all the PLCβ, γ and δ isoforms, and the PH domain of PLCδ1 has been shown to be involved in membrane phospholipid interactions (Katan and Williams, 1997; Rebecchi and Scarlata, 1998; Rhee, 2001). The sequence analysis also indicates that PLCζ possesses the typical X and Y catalytic domains found in all known PLCs (residues 168-307 and 386-502 of PLCζ, respectively). The X and Y domains are between a tandem pair of N-terminal EF hand-like domains and a C-terminal C2 domain (residues 20-150 and 521-625, respectively), both of which are present in most PLCs (Fig. 2B). The X and Y domains of PLCζ contain the PLCδ1 active site residues, corresponding to ^{178}His, ^{210}Asp and ^{223}His, that have been shown to be involved in catalysis by site-directed mutation studies of PLCδ1 (Ellis et al., 1993; Ellis et al., 1998) and are conserved across the entire PLC family (Katan, 1998; Rebecchi and Pentyala, 2000). Another distinction between PLCζ and PLCδ1 is the extended X-Y linker sequence in PLCζ (residues 308-385), which has a high proportion of charged residues (Fig. 2A). The X-Y linker region is the only part of PLCδ1 that was not determined in the 3D crystal structure (Williams, 1999). Multiple alignment of PLCζ with the other mammalian PLC isoforms shows that it has the highest degree of similarity with the PLCδ group (33% identity with PLCδ1) and the lowest with PLCε (9% identity; Fig. 2C). The classification of PLCζ as a distinct isoform is supported by phylogeny analysis of the twelve identified mammalian PLCs, which suggests that ζ is the least divergent PLC isoform from a hypothetical precursor, with the rank order ζ<δ<β<ε<γ (Fig. 2D). In accordance with this observation, the domain structure of ζ is similar to plant PLCs that also lack an N-terminal PH domain but retain normal enzymatic properties (Rebecchi and Pentyala, 2000). No plant PLCs with domain structures of the mammalian β, γ, δ or ε isoforms have been identified (Rebecchi and Pentyala, 2000).

Northern blot analysis with mouse tissue mRNAs shows that PLCζ is present as a relatively abundant 2.3 kb transcript only in the testis (Fig. 3A). The transcript abundance is consistent with the significant number of mouse testis ESTs found in the database. The transcript size of 2.3 kb for PLCζ matches the spermatid cDNA clone with a 1941 bp ORF plus ~300 bp of untranslated sequence (Fig. 2A). The PLCζ transcript distribution also is congruent with immunoblot analysis of a panel of mouse tissues which suggests testis-specificity, as PLCζ protein expression is not detected in any sample other than sperm (Fig. 3B). Sperm cell-specificity of PLCζ expression within testis was examined by performing PCR on cDNA from mouse spermatids and mouse testis devoid of spermatids. PLCζ amplification is observed with spermatid cDNA but not with testis cDNA devoid of spermatids (Fig. 3C), suggesting that PLCζ expression within testis is sperm cell-specific. No PLC isoform has previously been found to be sperm specific, although a splice variant of PLCδ4 enriched in testis (Nagano et al., 1999) was shown to be involved in the zona pellucida-induced acrosome reaction (Fukami et al., 2001).

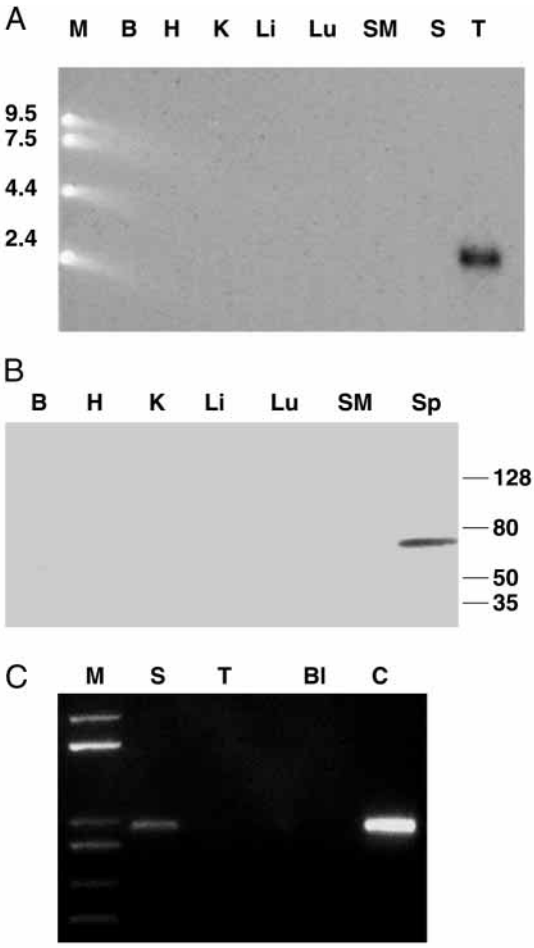

Fig. 3. Sperm-specific expression of mouse PLCζ. (A) Northern blot analysis of PLCζ transcript distribution in mouse. Lanes from left to right: RNA standard markers, brain, heart, kidney, liver, lung, skeletal muscle, spleen, testis (2 μg polyA$^+$-RNA/lane). Molecular weight markers in kb are on the left. (B) Immunoblot analysis of PLCζ protein distribution in mouse. Left to right: brain, heart, kidney, liver, lung, skeletal muscle, sperm (50 μg protein/lane). Molecular weight markers in kDa are on the right. (C) Polymerase chain reaction detection of PLCζ in cDNA from mouse spermatid and mouse testis devoid of spermatids. Left to right: DNA markers (2.0, 1.6, 1.0, 0.8, 0.6 and 0.5 kb, top to bottom), spermatid cDNA (10 ng), testis cDNA (10 ng), blank (no DNA) and positive control (1 ng PLCζ plasmid).

PLCζ triggers Ca^{2+} oscillations in eggs

The defining character of the mammalian sperm factor is the ability to elicit Ca^{2+} oscillations that mimic the fertilisation-associated transients displayed by mammalian eggs (Swann, 1990; Fissore et al., 1998). To examine whether sperm PLCζ could trigger such Ca^{2+} oscillations, we introduced PLCζ complementary RNA (cRNA) by microinjection into MII-arrested mouse eggs, as described previously for spermatogenic cell mRNA (Parrington et al., 2000). Eggs microinjected with a pipette concentration of 2 mg/ml PLCζ cRNA, corresponding to <0.1 mg/ml in the egg after a 3-5% injection volume, underwent a prolonged series of Ca^{2+} oscillations that commence within 15-20 minutes (Fig. 4A, top trace). The high oscillation frequency is similar to that observed upon microinjection of concentrated sperm extracts into mouse eggs (Tang et al., 2000). Ca^{2+} oscillations of similar amplitude, but lower frequency, are obtained with a 1000-fold

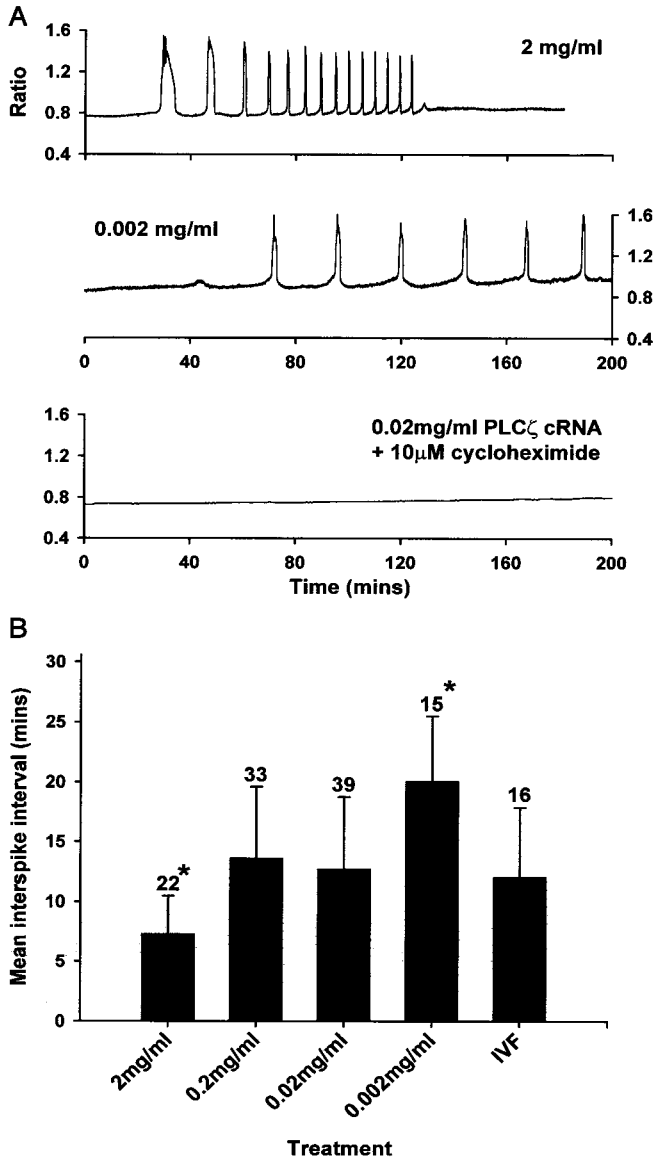

Fig. 4. PLCζ triggers Ca^{2+} oscillations in MII-arrested mouse eggs. (A) Dose-dependent Ca^{2+} oscillations in fura-red loaded mouse eggs triggered by microinjection of cRNA encoding mouse sperm PLCζ (2 and 0.002 mg/ml, top and middle trace, respectively) and after preincubation with 10 μM cycloheximide (0.02 mg/ml, bottom trace). **(**B) Mean interspike interval of Ca^{2+} oscillations in eggs following microinjection of various PLCζ cRNA concentrations (2-0.002 mg/ml in pipette, i.e. <0.1-0.0001 mg/ml in egg) compared with the interval observed upon in vitro fertilisation (IVF). Number of microinjected eggs is shown above each condition. *, significantly different from IVF at the 5% level (Student's unpaired *t*-test).

dilution to 0.002 mg/ml PLCζ cRNA (Fig. 4A, middle trace; 0.0001 mg/ml in egg). None of the eggs treated with cycloheximide to block protein synthesis showed any Ca^{2+} transients after PLCζ cRNA-microinjection (0.02 mg/ml, n=9; Fig. 4A, bottom trace). Robust Ca^{2+} oscillations were observed in 100% of the eggs microinjected with the four different PLCζ cRNA concentrations tested, ranging from 0.002-2 mg/ml (Fig. 4B). Importantly, the frequency, but not the amplitude, of Ca^{2+} oscillations varies with PLCζ cRNA concentration, directly matching the same phenomenon observed with different concentrations of sperm extract (Swann, 1990). The highest pipette concentration used, 2 mg/ml, produces Ca^{2+} oscillations with a mean interspike interval of 7.3±3.2 minutes (Fig. 4B). The lowest pipette concentration of PLCζ cRNA that gives oscillations within 2 hours of injection (0.002 mg/ml), displayed a mean interspike interval of 20.1±5.4 minutes (Fig. 4B). Both of these values are significantly different to the mean interspike interval produced with in vitro fertilisation (IVF) of mouse eggs (12.1±5.8 minutes). However, the interspike intervals for 0.2 and 0.02 mg/ml PLCζ cRNA (13.6±3.2 and 12.7±6.0 minutes, respectively) are not significantly different from IVF (Fig. 4B).

Fertilisation-like Ca^{2+} signals via PLCζ

The Ca^{2+} oscillations at fertilisation (Cuthbertson and Cobbold, 1985) display some unique features. The first Ca^{2+} transient invariably lasts longer than subsequent oscillations (Fig. 5A), and exhibits a set of intriguing, smaller sinusoidal increases on top of the main peak (Fig. 5A-I). Microinjection of a pipette concentration of PLCζ cRNA that produces an interspike interval matching IVF (i.e. 0.02 mg/ml; Fig. 4B) results not only in the same, longer initial Ca^{2+} transient, but also displays a similar pattern of smaller sinusoidal increases (Fig. 5B and Fig. 5B-I). The first Ca^{2+} increase after 0.02 mg/ml PLCζ cRNA microinjection matches the first IVF transient in both average duration (PLCζ 2.8±0.6 minutes, n=39 versus IVF 3.0±0.7 minutes, n=16), and also in reproducibly producing the cluster of smaller Ca^{2+} increases superimposed on the first transient (Fig. 5B-I). A concentration of 0.02 mg/ml PLCζ cRNA was used for subsequent microinjection experiments, unless stated otherwise, to provide the precise Ca^{2+} signalling conditions that are stereotypical of fertilisation.

The ability of sperm PLCζ to initiate Ca^{2+} oscillations in eggs is specific to this novel PLC isoform because microinjecting a PLCδ cRNA (2 mg/ml), structurally the most similar mammalian isoform to PLCζ (Fig. 2B), does not trigger a Ca^{2+} increase in any of the 14 eggs tested (Fig. 5C). The lack of effect of PLCδ1 cRNA is consistent with the inability of microinjected PLCδ1 protein to cause any Ca^{2+} changes in mouse eggs (Jones et al., 2000). As the lack of an N-terminal PH domain is the most distinctive difference between PLCζ and PLCδ1 (Fig. 2B), the function of PLCζ could possibly be mimicked by a truncated PLCδ1 without the PH domain. Therefore, a deletion construct of PLCδ1 minus the N-terminal 132 residue PH domain ($^{\Delta PH}$PLCδ1) was prepared, resembling the domain structure of sperm PLCζ. Microinjection of $^{\Delta PH}$PLCδ1 cRNA into eggs does not result in any detectable Ca^{2+} changes (Fig. 5D, 2 mg/ml, n=12 eggs), suggesting that additional factors unique to sperm PLCζ are crucial for Ca^{2+} mobilisation in mammalian eggs. To determine whether catalytically active sperm PLCζ is required for Ca^{2+} mobilisation in the egg, ζ cRNA with a mutation at ^{210}Asp, a putative active site residue critical for PLCζ enzyme function (Katan, 1998; Williams, 1999; Rebecchi and Pentyala, 2000), was microinjected. Mutation of the corresponding residue in PLCδ1, ^{343}Asp to ^{343}Arg, was shown to be the most severe of numerous site-directed alterations, causing a 180,000-fold reduction in PIP_2-mediated hydrolysis of PLCδ1 (Ellis et al., 1998). The microinjection of ^{D210R}PLCζ cRNA into eggs, even

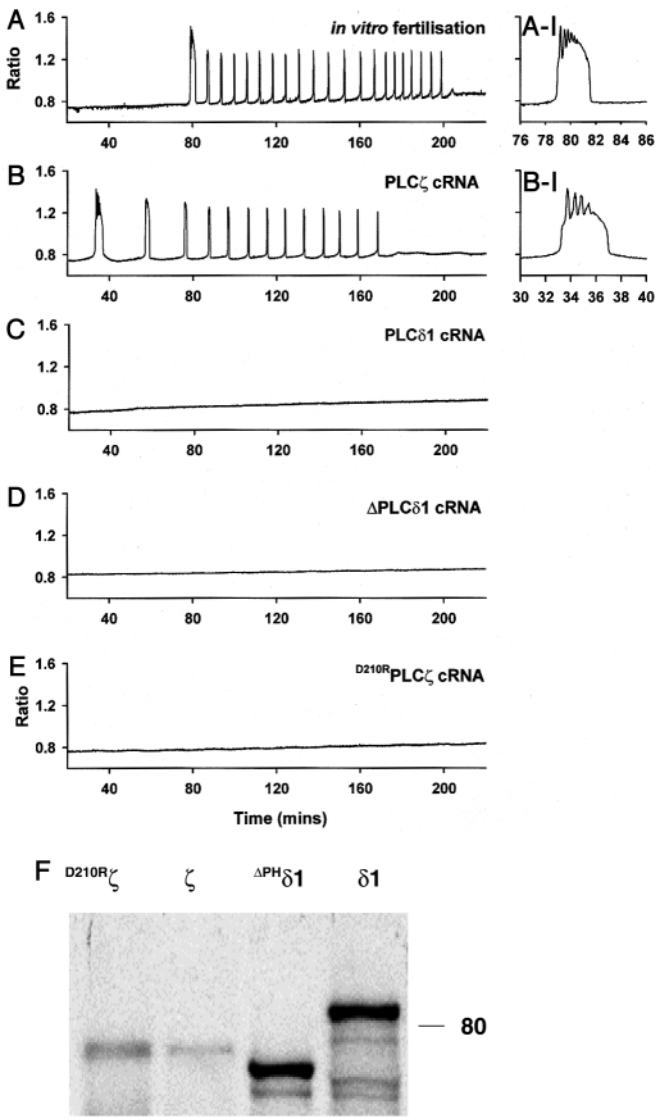

Fig. 5. In vitro fertilisation consistent with PLCζ-induced Ca^{2+} oscillations. Ca^{2+} changes in fura-red loaded mouse eggs that were either (A) in vitro fertilised with mouse sperm, or microinjected with cRNA encoding (B) PLCζ at 0.02 mg/ml; (C) PLCδ1 at 2 mg/ml; (D) $^{\Delta PH}$PLCδ1 at 2 mg/ml (PH domain-deleted PLCδ1); (E) ^{D210R}PLCζ at 2 mg/ml. (A-I,B-I) Expanded traces of the longer-duration, first Ca^{2+} transient taken from A,B, respectively. (F) Autoradiograph following SDS-PAGE of [^{35}S]-labelled protein expressed in vitro from cRNA, lanes from left to right, of ^{D210R}PLCζ, PLCζ, $^{\Delta PH}$PLC δ1 and PLCδ1 corresponding to predicted protein sizes of 74, 74, 70 and 85 kDa, respectively.

at high concentration (2 mg/ml), does not produce any Ca^{2+} increase (Fig. 5E, n=22). This suggests that ^{210}Asp is crucial for PLCζ enzyme activity, and that IP_3 production is necessary for Ca^{2+} release to occur in eggs (Miyazaki et al., 1983; Stricker, 1999; Brind et al., 2000; Jellerette et al., 2000). The four different cRNAs used in this study, PLCζ, ^{D210R}PLCζ, $^{\Delta PH}$PLCδ1 and PLCδ1, were also expressed in vitro in rabbit reticulocyte lysates, illustrating that they are correctly synthesised and yield the predicted protein sizes of 74, 74, 70 and 85 kDa, respectively (Fig. 5F).

Physiological level of PLCζ in a single sperm

The sperm factor hypothesis predicts that a single sperm contains sufficient activating factor to initiate Ca^{2+} release upon sperm-egg fusion (Swann, 1990; Stricker, 1999). The observation of sperm PLCζ cRNA triggering fertilisation-like Ca^{2+} oscillations in eggs (Figs 4 and 5) is of physiological significance only if the PLCζ protein expressed in a single egg is similar to the native PLCζ present in a single sperm. In order to quantitate the PLCζ expressed in microinjected eggs, a Myc epitope tag was introduced at the N terminus of PLCζ (Lopez et al., 2001). Microinjected Myc-PLCζ cRNA at different concentrations is as effective at generating Ca^{2+} oscillations in eggs (Fig. 6A, 0.02 mg/ml) as the untagged PLCζ (Fig. 4B), indicating that the N-terminal attachment of the Myc tag is not deleterious to PLCζ activity, as was shown for Myc-PLCε (Lopez et al., 2001). Furthermore, the Myc-PLCζ protein expressed in eggs is readily detected in immunoblots using an anti-Myc monoclonal antibody, as a single band with the predicted mass of 78 kDa, whereas uninjected eggs exhibit no immunoreactivity (Fig. 6B). Comparison of the relative mobility of native mouse sperm PLCζ (Fig. 6c, 74 kDa) and recombinant Myc-PLCζ protein [Fig. 6C, 78 kDa (74 kDa PLCζ + 4 kDa Myc tag)] indicates that the deduced ORF of the PLCζ cDNA clone (Fig. 2A, 74 kDa) represents the complete sperm PLCζ sequence. Densitometric analysis of the immunoreactive 78 kDa Myc-PLCζ protein expressed in eggs (Fig. 6C, 100 eggs microinjected with each Myc-PLCζ cRNA concentration), compared with calibrated amounts of purified recombinant Myc-PLCζ protein produced in bacteria, enabled the determination of 44-75 fg/egg (n=4) as the amount of PLCζ protein that triggers Ca^{2+} oscillations using 0.02 mg/ml cRNA (see Materials and Methods). This cRNA concentration is the one that most closely mimics the IVF response, though tenfold lower levels (i.e. 4-8 fg PLCζ protein/egg using 0.002 mg/ml cRNA) are also able to cause Ca^{2+} oscillations (Fig. 4).

The PLCζ content of sperm was also determined by densitometry with a PLCζ polyclonal antibody using a defined number of mouse sperm and compared with calibrated amounts of recombinant PLCζ protein (Fig. 6D). Using densitometric values within the recombinant PLCζ protein calibration plot, obtained from samples comprising 10^4-10^6 mouse sperm, a single mouse sperm was calculated to contain 20-50 fg PLCζ protein (n=4). The level of PLCζ able to produce Ca^{2+} oscillations in a single egg similar to fertilisation (4-75 fg, i.e. with 0.002-0.02 mg/ml cRNA) is therefore in the same range as the single sperm content of PLCζ (20-50 fg). The observed quantitative correlation indicates that the PLCζ from a single sperm is sufficient to produce the Ca^{2+} oscillations observed upon sperm-egg fusion.

Sperm PLCζ depletion abrogates Ca^{2+} oscillations

The features of sperm PLCζ at the functional (Figs 4 and 5) and quantitative (Fig. 6) level are fully consistent with characteristics observed for the sperm factor present in mammalian sperm extracts (Swann, 1990; Berrie et al., 1996; Wu et al., 1997; Stricker, 1997; Wu et al., 1998; Jones et al., 1998b; Kyozuka et al., 1998; Tang et al., 2000). However, it remains possible that sperm components other than PLCζ are also involved in causing Ca^{2+} release in eggs. To address whether the PLCζ in sperm is uniquely responsible for Ca^{2+} mobilisation in eggs, the PLCζ content of sperm extracts was

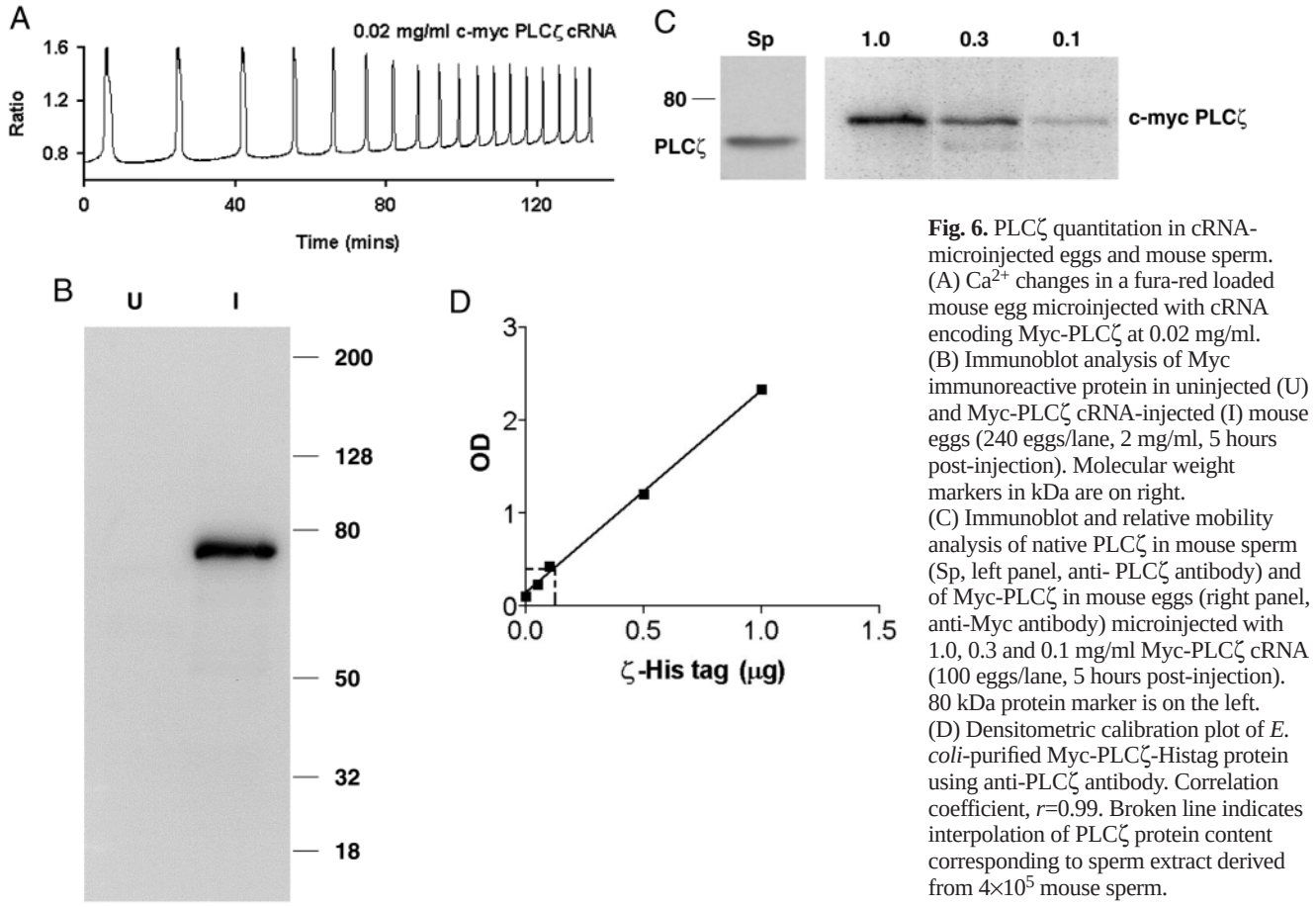

Fig. 6. PLCζ quantitation in cRNA-microinjected eggs and mouse sperm. (A) Ca^{2+} changes in a fura-red loaded mouse egg microinjected with cRNA encoding Myc-PLCζ at 0.02 mg/ml. (B) Immunoblot analysis of Myc immunoreactive protein in uninjected (U) and Myc-PLCζ cRNA-injected (I) mouse eggs (240 eggs/lane, 2 mg/ml, 5 hours post-injection). Molecular weight markers in kDa are on right. (C) Immunoblot and relative mobility analysis of native PLCζ in mouse sperm (Sp, left panel, anti- PLCζ antibody) and of Myc-PLCζ in mouse eggs (right panel, anti-Myc antibody) microinjected with 1.0, 0.3 and 0.1 mg/ml Myc-PLCζ cRNA (100 eggs/lane, 5 hours post-injection). 80 kDa protein marker is on the left. (D) Densitometric calibration plot of *E. coli*-purified Myc-PLCζ-Histag protein using anti-PLCζ antibody. Correlation coefficient, r=0.99. Broken line indicates interpolation of PLCζ protein content corresponding to sperm extract derived from 4×10^5 mouse sperm.

specifically depleted using an anti-PLCζ antibody. Immunoblot analysis indicates that sperm extract supernatant retains the PLCζ protein after control antibody treatment, in contrast to PLCζ antibody-treated supernatant where the PLCζ is absent (Fig. 7A, S– and S+, respectively). Analysis of the corresponding precipitated antibody samples reveals that the sperm PLCζ is effectively removed by PLCζ antibody, but not by the control antibody (Fig. 7A, P+ and P–, respectively). Assessment of Ca^{2+} release activity in antibody-treated sperm extracts using sea urchin egg homogenate assays shows that PLCζ-depleted samples lack any Ca^{2+} mobilising activity, whereas a robust Ca^{2+} release is observed with the control antibody-treated sperm extract containing PLCζ protein (Fig. 7B, S+ and S–, respectively). Moreover, microinjection of antibody-treated sperm extracts into mouse eggs illustrates that the ability of untreated samples to generate IVF-like Ca^{2+} oscillations (Fig. 7C, top trace) is fully preserved in control antibody-treated samples (Fig. 7C, second trace, n=13), while PLCζ-depletion effectively abrogates Ca^{2+} release activity (Fig. 7C, bottom two traces, n=13). These PLCζ antibody depletion experiments (n=4) suggest that PLCζ is the sole component of sperm extracts possessing the ability to cause Ca^{2+} release in mouse eggs. Taken together with evidence that the PLCζ level in a single mouse sperm is sufficient to trigger IVF-like Ca^{2+} oscillations in a single mouse egg (Figs 4-6), the immunodepletion data provides compelling evidence that PLCζ is synonymous with the previously described mammalian sperm factor (Swann, 1990; Berrie et al., 1996; Wu et al., 1997; Stricker, 1997; Wu et al., 1998; Jones et al., 1998b; Kyozuka et al., 1998; Tang et al., 2000).

PLCζ activates normal embryo development

The activation of mammalian eggs is caused by sperm-induced Ca^{2+} oscillations at fertilisation (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992). Microinjection of sperm extract into eggs also produces activation and the consequent cellular processes leading to embryo development (Stice and Robl, 1990; Fissore et al., 1998; Sakurai et al., 1999). The isolated sperm factor molecule therefore is predicted to support embryo development after egg activation, providing a crucial test for any putative sperm factor candidate (Fissore et al., 1998). Because eggs that are microinjected with PLCζ cRNA (0.02mg/ml) display all the properties of Ca^{2+} oscillations indistinguishable from those of IVF (Fig. 4B, Fig. 5B) and is equivalent to the PLCζ content of a single sperm (Fig. 6), their ongoing development was monitored for several days after PLCζ-microinjection. PLCζ-microinjected eggs underwent activation (Fig. 8A) because normal development proceeds to the two-cell stage within 24 hours (78%, n=147), and many reach the morula or blastocyst stages by 4-5 days (62%, n=76). None of the eggs microinjected with buffer control reach the two-cell stage, indicating activation as an artefact of microinjection procedure has not occurred (data not shown). The proportion of PLCζ-induced embryos that develop to either the two-cell, or morula and blastocyst stages, is the same as for eggs that are either parthenogenetically activated (Bos-

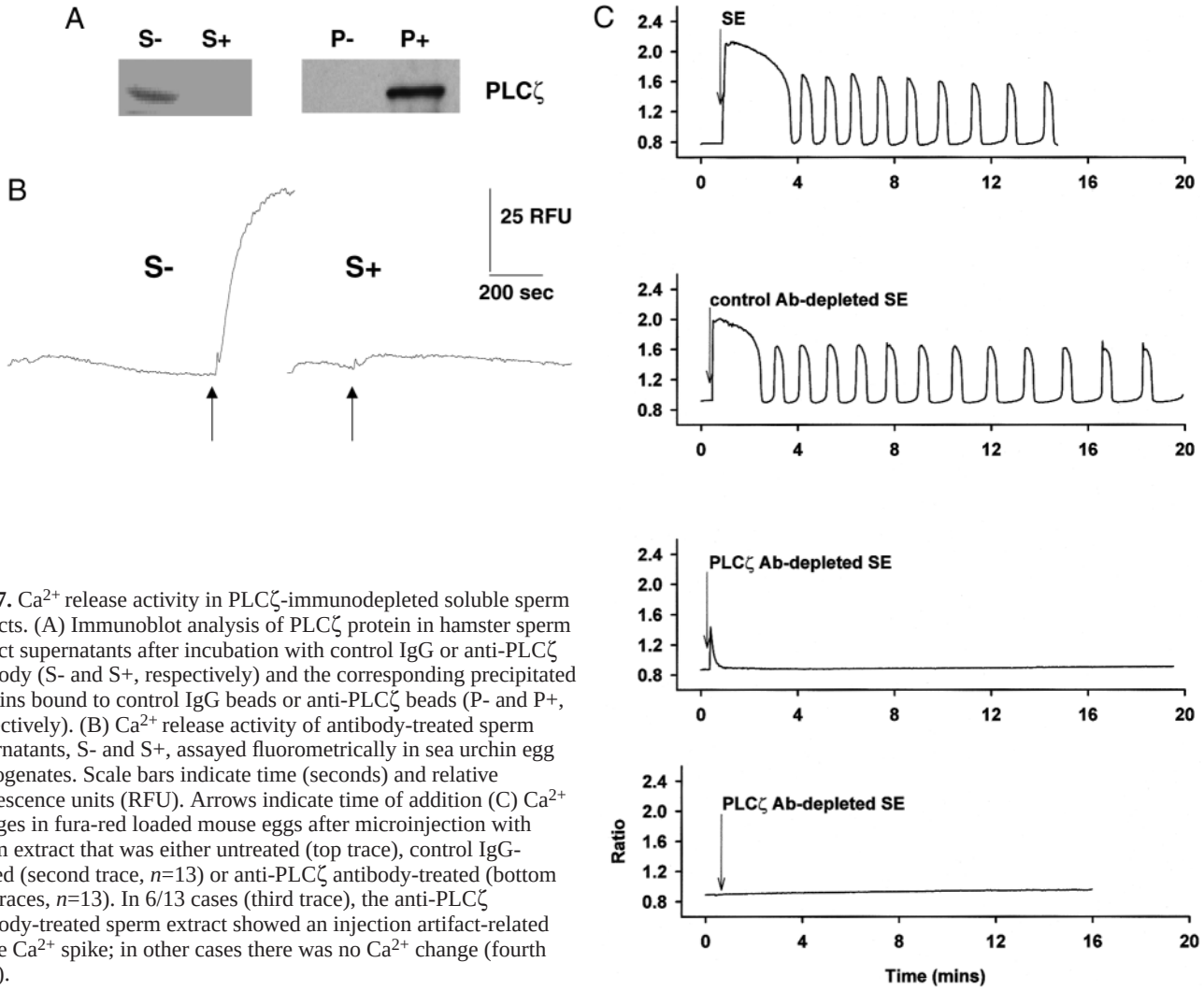

Fig. 7. Ca^{2+} release activity in PLCζ-immunodepleted soluble sperm extracts. (A) Immunoblot analysis of PLCζ protein in hamster sperm extract supernatants after incubation with control IgG or anti-PLCζ antibody (S- and S+, respectively) and the corresponding precipitated proteins bound to control IgG beads or anti-PLCζ beads (P- and P+, respectively). (B) Ca^{2+} release activity of antibody-treated sperm supernatants, S- and S+, assayed fluorometrically in sea urchin egg homogenates. Scale bars indicate time (seconds) and relative fluorescence units (RFU). Arrows indicate time of addition (C) Ca^{2+} changes in fura-red loaded mouse eggs after microinjection with sperm extract that was either untreated (top trace), control IgG-treated (second trace, *n*=13) or anti-PLCζ antibody-treated (bottom two traces, *n*=13). In 6/13 cases (third trace), the anti-PLCζ antibody-treated sperm extract showed an injection artifact-related single Ca^{2+} spike; in other cases there was no Ca^{2+} change (fourth trace).

Mikich et al., 1997) by strontium ions (*n*=75), or when embryos are collected at the one-cell stage from female mice after in vivo fertilisation (*n*=101) upon mating with males (Fig. 8A). Photomicrographs taken at 24 hours and 5 days after PLCζ-microinjection into mouse eggs show the appearance of normal embryo development to the two-cell stage and blastocyst stage (left and right panel, respectively, Fig. 8B). There are no morphological differences to embryos obtained after fertilisation with sperm (data not shown). Thus, after inducing Ca^{2+} oscillations in the egg, sperm PLCζ-microinjection also triggers the entire cascade of events required for activation and embryo development, in the same manner as sperm at fertilisation.

The possibility remains that a novel action of PLCζ other than PIP_2 hydrolysis is responsible for egg activation, such as a protein-protein interaction with a distinct egg molecule. To test whether an enzymatically active PLCζ is required for egg activation and embryo development, the ^{D210R}PLCζ cRNA (0.02mg/ml), which has been shown to be defective in triggering Ca^{2+} oscillations (Fig. 5E) (Ellis et al., 1998; Katan, 1998; Williams, 1999; Rebecchi and Pentyala, 2000), was microinjected and egg activation assessed after 24 hours. None of the ^{D210R}PLCζ cRNA-microinjected eggs were found to proceed to the pronuclear or two-cell stage (Fig. 8C, *n*=20), suggesting that the enzymatic function of sperm PLCζ is crucial for egg activation.

DISCUSSION

Cytoplasmic oscillations in intracellular free Ca^{2+} is a remarkable signalling phenomenon observed in many cell types that can regulate a wide variety of physiological processes (Berridge et al., 2000). However, as the original observation of Ca^{2+} oscillations at mammalian fertilisation (Cuthbertson and Cobbold, 1985), the molecular mechanism has remained an enigma. A popular model of Ca^{2+} signalling at fertilisation involves a sperm surface ligand interacting with a receptor on the egg plasma membrane. The ligand-bound membrane receptor couples with an egg PLC to stimulate IP_3 production and Ca^{2+} release, analogous to the signalling pathway found ubiquitously in somatic cells (Berridge et al., 2000). However, the egg and sperm molecules required for the operation of this ‘receptor’ model have not been identified despite extensive studies (Stricker, 1999).

The second major hypothesis involves a sperm cytosolic

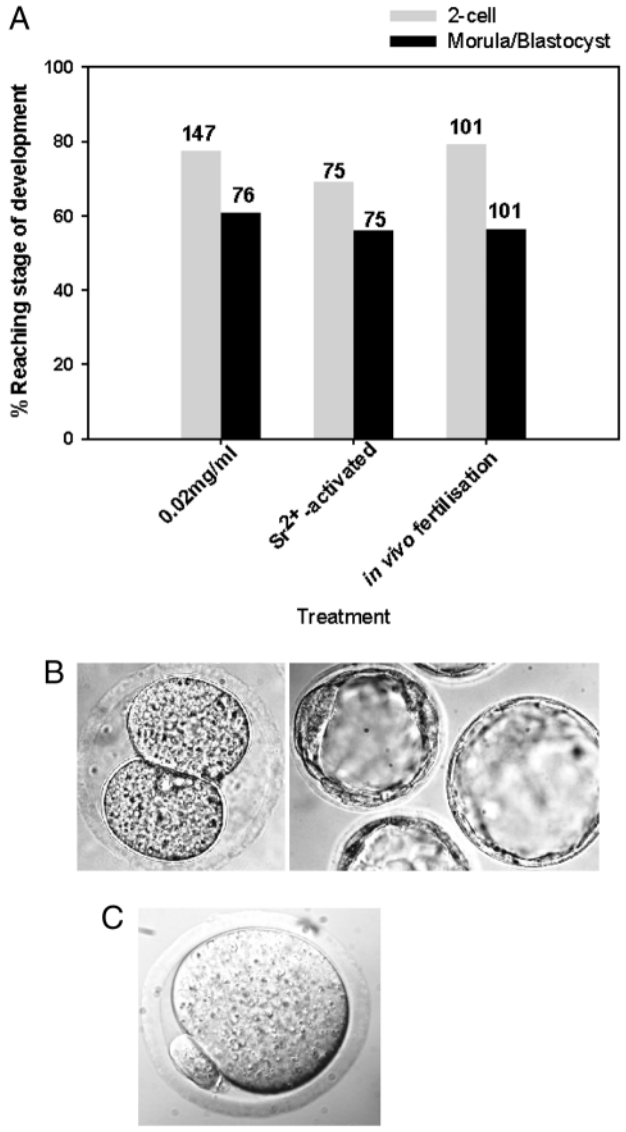

Fig. 8. Activation and embryo development to blastocyst in PLCζ-injected mouse eggs. (A) Mouse eggs were either microinjected with PLCζ cRNA (0.02 mg/ml), or parthenogenetically activated with strontium (5 mM, 4 hours) or fertilised with sperm in vivo, then placed in a 5% CO_2 incubator at 37°C. Percentage of eggs reaching the two-cell stage after 24 hours, and morula/blastocyst stage after 96 hours, was recorded for each treatment. Number of microinjected eggs is shown above each condition. (B) Micrographs illustrating mouse embryos at the two-cell stage (left) and blastocyst stage (right), at 24 hours and 96 hours, respectively, after egg microinjection with PLCζ cRNA (0.02 mg/ml). (C) Micrograph illustrating mouse egg 24 hours after microinjection with ^{D210R}PLCζ cRNA (0.02 mg/ml).

protein that enters the egg and causes Ca^{2+} release (Swann, 1996; Yamamoto et al., 2001). This 'sperm factor' model, though hindered by initial quandaries (Parrington et al., 1996; Sette et al., 1997), has gained increasing credence due to the numerous studies demonstrating the potency of sperm extracts in effecting Ca^{2+} release in eggs (Stice and Robl, 1990; Swann, 1990; Nakano et al., 1997; Stricker, 1997; Wu et al., 1997; Jones et al., 1998b; Kyozuka et al., 1998; Wu et al., 1998; Parrington et al., 1999; Dong et al., 2000; Jones et al., 2000; Rice et al., 2000; Tang et al., 2000; Wu et al., 2001; Yamamoto et al., 2001; Parrington et al., 2002). Moreover, the sperm factor model is congruous with the amount of activity contained in a single sperm (Nixon et al., 2000) and is further supported by the technique of intracytoplasmic sperm injection (ICSI), a clinically effective IVF procedure that has produced thousands of live births (Bonduelle et al., 1999). In the ICSI method, which bypasses the possibility of sperm-egg membrane interaction, a single spermatozoa is injected directly into a human egg to cause Ca^{2+} oscillations, activation and development to term (Bonduelle et al., 1999; Yanagida et al., 2001). Interestingly, the ICSI practice of breaking off the sperm tail to enhance the rate of egg activation (Dozortsev et al., 1995; Yanagida et al., 2001) could be explained by the facilitated release of sperm cytosolic contents, including the sperm factor.

These two major models for Ca^{2+} signalling at fertilisation have developed an overlap following recent observations indicating that a PLC activity is indeed involved in triggering Ca^{2+} oscillations, but the PLC is in the sperm, not the egg (Jones et al., 1998b; Rice et al., 2000; Wu et al., 2001; Parrington et al., 2002). However, comprehensive analysis of known PLC isoforms by different approaches have all concluded that none of them could be the sperm factor (Mehlmann et al., 1998; Jones et al., 2000; Fukami et al., 2001; Mehlmann et al., 2001; Wu et al., 2001; Parrington et al., 2002). There remains the possibility of an undiscovered sperm PLC with the requisite Ca^{2+} signalling properties, and this was directly addressed in the present study.

The revelation of abundant testis-derived ESTs with PLC homology led to our characterisation of a novel sperm PLC isoform (Fig. 1). The new isoform, PLCζ, is the smallest PLC identified to date, most closely resembling the PLCδ class, but without an N-terminal PH domain and a longer X-Y domain linker sequence (Fig. 2). The tissue transcript and protein expression profile indicates sperm-specific enrichment of the PLCζ protein, consistent with a gamete-specific role (Fig. 3). Functional analysis by expression in mammalian eggs provides exquisite evidence that PLCζ possesses the mandatory properties of the sperm factor. PLCζ exhibits the unique ability to produce Ca^{2+} oscillations with the characteristic interspike interval (Fig. 4), and the intriguing, first transient profile-specificity (Fig. 5), found in Ca^{2+} signalling at fertilisation. The inability of the PH-domain-deleted PLCδ1 to mimic fertilisation Ca^{2+} transients, suggests an exclusive functional specificity for the sperm PLCζ domains inside mammalian eggs (Fig. 5). Similarly, the functionally ineffective PLCζ with a catalytic site mutation (Fig. 5) is consistent with the previously shown vital role of a PLC and IP_3 production in mobilising Ca^{2+} in eggs (Miyazaki et al., 1993; Brind et al., 2000; Jellerette et al., 2000). Quantitative correlation of the PLCζ level that produces an IVF-like Ca^{2+} response with that found in a single sperm (Fig. 6), together with demonstration of the unique role of the PLCζ within sperm extracts in effecting Ca^{2+} release in eggs (Fig. 7), directly support the tenet that sperm PLCζ has a physiologically relevant role in egg activation. Furthermore, the normal development of PLCζ-microinjected eggs to the blastocyst stage (Fig. 8) shows that Ca^{2+} oscillations, which are triggered solely via PLCζ, are both necessary and sufficient to initiate the entire network of cellular

processes that operate from egg activation through early embryo development to blastocyst. These decisive features of PLCζ argue that it is an important component of the augured mammalian sperm factor and also that there is a physiological role for PLCζ in egg activation and embryo development during mammalian fertilisation.

Discovery of PLCζ as a novel mediator of intracellular Ca^{2+} regulation will enable an increased understanding of the propagation mechanism of large amplitude, low-frequency cytosolic Ca^{2+} oscillations (Berridge et al., 2000). Identification of PLCζ as a component of the putative physiological sperm factor should help to reveal the molecular mechanisms involved in subsequent stages of embryo development after egg activation. Analysis of human sperm PLCζ may also provide a new framework for understanding some cases of male factor infertility where the sperm are ineffective in stimulating development (Rybouchkin et al., 1996; Battaglia et al., 1997). Finally, PLCζ could be applied in approaches to improve egg activation rates, for example, after somatic cell nuclear transfer into enucleated eggs, in the production of stem cells for therapy of human diseases (Aldhous, 2001).

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REFERENCES

Aida, T., Oda, S., Awaji, T., Yoshida, K. and Miyazaki, S. (2001). Expression of a green fluorescent protein variant by injection of RNA with an added long poly(A) tail. *Mol. Hum. Reprod.* **7**, 1039-1046.

Aldhous, P. (2001). Can they rebuild us? *Nature* **410**, 622-625.

Battaglia, D. E., Koehler, J. K., Klein, N. A. and Tucker, M. J. (1997). Failure of oocyte activation after intracytoplasmic sperm injection using round-headed sperm. *Fertil. Steril.* **68**, 118-122.

Berridge, M. J., Lipp, P. and Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell. Biol.* **1**, 11-21.

Berrie, C. P., Cuthbertson, K. S. R., Parrington, J., Lai, F. A. and Swann, K. (1996). A cytosolic sperm factor triggers calcium oscillations in rat hepatocytes. *Biochem. J.* **313**, 369-372.

Bonduelle, M., Camus, M., de Vos, A., Staessen, C., Tournaye, H., van Asche, E., Verheyen, G., Liebaers, I. and van Steirteghem, A. (1999). Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. *Hum. Reprod.* **Suppl. 1**, 243-264.

Bos-Mikich, A., Whittingham, D. G. and Jones, K. T. (1997). Meiotic and mitotic Ca^{2+} oscillations affect cell compaction in resulting blastocyts. *Dev. Biol.* **182**, 172-179.

Brind, S., Swann, K. and Carroll, J. (2000). Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm and adenophostin A but not to increase in intracellular Ca^{2+} or egg activation. *Dev. Biol.* **223**, 251-265.

Cuthbertson, K. S. R. and Cobbold, P. H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. *Nature* **316**, 541-542.

Dong, J. B., Tang, T. S. and Sun, F. Z. (2000). Xenopus and chicken sperm contain a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. *Biochem. Biophys. Res. Commun.* **268**, 947-951.

Dozortsev, D., Rybouchkin, A., de Sutter, P., Qian, C. and Dhont, M. (1995). Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. *Hum. Reprod.* **10**, 403-407.

Ellis, M. V., Carne, A. and Katan. M. (1993). Structural requirements of phosphatidylinositol-specific phospholipase C delta 1 for enzyme activity. *Eur. J. Biochem.* **213**, 339-347.

Ellis, M. V., James, S. R., Perisic, O., Downes, C. P., Williams, R. L. and Katan, M. (1998). Catalytic domain of phosphoinositide-specific phospholipase C (PLC). Mutational analysis of residues within the active site and hydrophobic ridge of plcdelta1. *J. Biol. Chem.* **273**, 11650-11659.

Fissore, R. A., Gordo, A. C. and Wu, H. (1998). Activation of development in mammals: is there a role for a sperm cytosolic factor? *Theriogenology* **49**, 43-52.

Fukami, K., Nakao, K., Inoue, T., Kataoka, Y., Kurokawa, M., Fissore, R. A., Nakamura, K., Katsuki, M., Mikoshiba, K., Yoshida, N. and Takenawa, T. (2001). Requirement of phospholipase Cδ4 for the zona pellucida induced acrosome reaction. *Science* **292**, 920-923.

Jellerette, T., He, C. L., Wu, H., Parys, J. B. and Fissore, R. A. (2000). Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. *Dev. Biol.* **223**, 238-250.

Jones, K. T., Carroll, J., Merriman, J. A., Whittingham. D. G. and Kono, T. (1995). Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development* **121**, 3259-3266.

Jones, K. T., Soeller, C. and Cannell, M. B. (1998a). The passage of Ca^{2+} and fluorescent markers between the sperm and egg after fusion in the mouse. *Development* **125**, 4627-4635.

Jones, K. T., Cruttwell, C., Parrington, J. and Swann, K. (1998b). A mammalian sperm cytosolic phospholipase C activity generates inositol trisphosphate and causes Ca^{2+} release in sea urchin egg homogenates. *FEBS Lett.* **437**, 297-300.

Jones, K. T., Matsuda, M., Parrington, J., Katan, M. and Swann, K. (2000). Different Ca^{2+} releasing abilities of sperm extracts compared with tissue extracts and phospholipase C isoforms in sea urchin egg homogenate and mouse eggs. *Biochem. J.* **346**, 743-749.

Katan, M. and Williams, R. L. (1997). Phosphoinositide-specific phospholipase C: structural basis for catalysis and regulatory interactions. *Semin. Cell Dev. Biol.* **8**, 287-296.

Katan, M. (1998). Families of phosphoinositide-specific phospholipase C: structure and function. *Biochim. Biophys. Acta* **1436**, 5-17.

Kline, D. and Kline, J. T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell division in mouse eggs. *Dev. Biol.* **149**, 80-89.

Kyozuka, K., Deguchi, R., Mohri, T. and Miyazaki, S. (1998). Injection of sperm extract mimics spatiotemporal dynamics of Ca^{2+} responses and progression of meiosis at fertilization of ascidian oocytes. *Development* **125**, 4099-4105.

Lawrence, Y., Whitaker, M. and Swann, K. (1997). Sperm-oocyte fusion is the prelude to the initial Ca^{2+} increase at fertilization in the mouse. *Development* **124**, 223-241.

Lawrence, Y., Ozil, J. P. and Swann, K. (1998). The effects of a Ca^{2+} chelator and heavy-metal-ion chelators upon Ca^{2+} oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca^{2+} increases. *Biochem. J.* **335**, 335-342.

Lopez, I., Mak, E. C., Ding, J., Hamm, H. E. and Lomasney, J. W. (2001). A novel bifunctional phospholipase C that is regulated by $G_{\alpha}12$ and stimulates the Ras/mitogen-activated protein kinase pathway. *J. Biol. Chem.* **276**, 2758-2765.

Malek, A. M., Izumo, S., Alper, S. L. (1997). Quantitative densitometric analysis using a commercially available handheld CCD digital camera. *Biotechniques* **6**, 1150-1153.

Mehlmann, L. M., Carpenter, G., Rhee, S. G. and Jaffe, L. A. (1998). SH2 domain-mediated activation of phospholipase C is not required to initiate Ca^{2+} release at fertilization of mouse eggs. *Dev. Biol.* **203**, 221-232.

Mehlmann, L. M., Chattopadhyay, A., Carpenter, G. and Jaffe, L. A. (2001). Evidence that phospholipase C from the sperm is not responsible for initiating Ca^{2+} release at fertilization in mouse eggs. *Dev. Biol.* **236**, 492-501.

Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev. Biol.* **158**, 62-78.

Nagano, K., Fukami, K., Minagawa, T., Watanabe, Y., Ozaki, C. and Takenawa, T. (1999). A novel phospholipase C delta4 (PLCδ4) splice variant as a negative regulator of PLC. *J. Biol. Chem.* **274**, 2872-2879.

Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwubara, Y. and Miyazaki, S. (1997). Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol. Hum. Reprod.* **3**, 1087-1093.

Nixon, V. L., McDougall, A. and Jones, K. T. (2000). Ca^{2+} oscillations and the cell cycle at fertilization of mammalian and ascidian eggs. *Biol. Cell* **92,** 187-196.

Parrington, J., Swann, K., Shevchenko, V. I., Sesay, A. K. and Lai, F. A. (1996). A soluble sperm protein that triggers calcium oscillations in mammalian eggs. *Nature* **379**, 364-368.

Parrington, J., Jones, K. T., Lai, F. A. and Swann, K. (1999). The soluble sperm factor that causes Ca^{2+} release from sea urchin egg homogenates also triggers Ca^{2+} oscillations after injection into mouse eggs. *Biochem. J*. **341**, 1-4.

Parrington, J., Lai, F. A. and Swann, K. (2000). The soluble mammalian sperm factor protein that triggers Ca^{2+} oscillations in eggs: Evidence for expression in mRNA(s) coding for sperm factor protein(s) in spermatogenic cells. *Biol. Cell* **92**, 1-9.

Parrington, J., Jones, M. L., Tunwell, R., Devader, C., Katan, M. and Swann, K. (2002). The sperm factor that causes Ca^{2+} release in eggs is not constituted by one of the common isoforms of phospholipase C present in mammalian sperm. *Reproduction* **123**, 31-39.

Perry, A. C. F., Wakayama, T. and Yanagimachi, R. (1999). A novel trans-complementation assay suggests full mammalian oocyte activation is coordinately initiated by multiple, submembrane sperm compartments. *Biol. Reprod.* **60**, 747–755.

Rebecchi, M. J. and Scarlata, S. (1998). Pleckstrin homology domains: a common fold with diverse functions. *Annu. Rev. Biophys. Biomol. Struct*. **27**, 503-528.

Rebecchi, M. J. and Pentyala, S. N. (2000). Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol. Rev*. **80**, 1291-1335.

Rhee, S. G. (2001). Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* **70**, 281-312.

Rice, A., Parrington, J., Jones, K. T. and Swann, K. (2000). Mammalian sperm contain a Ca^{2+} sensitive phospholipase C activity that can generate $InsP_3$ from PIP_2 associated with intracellular organelles. *Dev. Biol.* **227**, 125-135.

Rybouchkin, A., Dozortsev, D., Pelinck, M. J., de Sutter, P. and Dhont, M. (1996). Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. *Hum. Reprod*. **11**, 2170-2175.

Sakurai, A., Oda, S., Kuwabara, Y. and Miyazaki, S. (1999). Fertilization, embryonic development, and offspring from mouse eggs injected with round spermatids combined with Ca^{2+} oscillation-inducing sperm factor. *Mol. Hum. Reprod.* **5**, 132-138.

Sette, C., Bevilacqua, A., Bianchini, A., Mangia, F. and Geremia, R. (1997). Parthenogenetic activation of mouse eggs by microinjection of a truncated *c-kit* tyrosine kinase present in spermatozoa. *Development* **124**, 2267-2274.

Song, C., Hu, C. D., Masago, M., Kariya, K., Yamawaki-Kataoka, Y., Shibatoghe, M., Wu, D., Satoh, T. and Kataoka, T. (2001). Regulation of a novel human phospholipase C, PLCε, through membrane targeting by Ras. *J. Biol. Chem.* **276**, 2752-2757.

Stice, S. L. and Robl, J. M. (1990). Activation of mammalian oocytes by a factor obtained from rabbit sperm. *Mol. Reprod. Dev.* **25**, 272-280.

Stricker, S. A. (1997). Intracellular injections of a soluble sperm factor trigger calcium oscillations and meiotic maturation in unfertilized oocytes of a marine worm. *Dev. Biol*. **186**, 185-201.

Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol*. **211**, 57-76.

Summers, M. C., McGinnis, L. K. M., Lawitts, J. A., Raffin, M. and Biggers, J. D. (2000). IVF of mouse ova in a simple optimized medium supplemented with amino acids. *Hum. Reprod*. **15**, 1791-1801.

Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* **110**, 1295-1302.

Swann, K. (1996). Soluble sperm factors and Ca^{2+} release in eggs at fertilization. *Rev. Reprod.* **1**, 33-39.

Tang, T. S., Dong, J. B., Huang, X. Y. and Sun, F. Z. (2000). Ca^{2+} oscillations induced by a cytosolic sperm factor are mediated by a maternal machinery that functions only once in mammalian eggs. *Development* **127**, 1141-1150.

Williams, R. L. (1999). Mammalian phosphoinositide-specific phospholipase C. *Biochim. Biophys. Acta* **1441**, 255-267.

Wolosker, H., Kline, D., Bain, Y., Blackshaw, S., Cameron, A. S., Frahlich, T. J., Schnaar, R. L. and Snyder, S. H. (1998). Molecularly cloned mammalian glucosamine 6 phosphate deaminase localizes to the transporting epithelium and lacks oscillin activity. *FASEB J*. **12**, 91-99.

Wu, H., He, C. L. and Fissore, R. A. (1997). Injection of a porcine sperm factor triggers calcium oscillations in mouse oocytes and bovine eggs. *Mol. Reprod. Dev*. **46**, 176-189.

Wu, H., He, C. L., Jehn, B., Black, S. J. and Fissore, R. A. (1998). Partial characterization of the calcium-releasing activity of porcine sperm cytosolic extracts. *Dev. Biol*. **203**, 369-381.

Wu, H., Smyth, J., Luzzi, V., Fukami, K., Takenawa, T., Black, S. L., Allbritton, N. L. and Fissore, R. A. (2001). Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod*. **64**, 1338-1349.

Yamamoto, S., Kubota, H. Y., Yoshimoto, Y. and Iwao, Y. (2001). Injection of a sperm extract triggers egg activation in the newt *Cynops pyrrhogaster. Dev. Biol*. **230**, 89-99.

Yanagida, K., Katayose, H., Hirata, S., Yazawa, H., Hayashi, S. and Sato, A. (2001). Influence of sperm immobilization on onset of Ca^{2+} oscillations after ICSI. *Hum. Reprod.* **16,** 148-152.