Sperm phospholipase C ζ from humans and cynomolgus monkeys triggers Ca²⁺ oscillations, activation and development of mouse oocytes

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Fusion with a fertilizing spermatozoon induces the mammalian oocyte to undergo a remarkable series of oscillations in cytosolic Ca²⁺, leading to oocyte activation and development of the embryo. The exact molecular mechanism for generating Ca²⁺ oscillations has not been established. A sperm-specific zeta isoform of phospholipase C (PLC ζ) has been identified in mice. Mouse PLC ζ triggers Ca²⁺ oscillations in mouse oocytes and exhibits properties synonymous with the 'sperm factor' that has been proposed to diffuse into the oocyte after gamete fusion. The present study isolated the PLC ζ homologue from human and cynomolgus monkey testes. Comparison with mouse and monkey PLC ζ protein sequences indicates a

shorter X–Y linker region in human PLC ζ and predicts a distinctly different isoelectric point. Microinjection of complementary RNA for both human and cynomolgus monkey PLC ζ elicits Ca²⁺ oscillations in mouse oocytes equivalent to those seen during fertilization in mice. Moreover, human PLC ζ elicits mouse egg activation and early embryonic development up to the blastocyst stage, and exhibits greater potency than PLC ζ from monkeys and mice. These results are consistent with the proposal that sperm PLC ζ is the molecular trigger for egg activation during fertilization and that the role and activity of PLC ζ is highly conserved across mammalian species.

Introduction

The earliest detected signalling event during mammalian oocyte activation by spermatozoa is the release of Ca²⁺ from intracellular stores (Stricker, 1999; Runft *et al.*, 2002). In fertilized mammalian oocytes, this Ca²⁺ release occurs shortly after sperm–oocyte membrane fusion and is manifested as a striking series of cytosolic Ca²⁺ oscillations (Miyazaki *et al.*, 1993; Swann, 1996; Stricker, 1999). The oscillations in cytosolic free Ca²⁺ are regular, continue for several hours, and cease around the time of formation of the pronuclei (Carroll, 2001). Ca²⁺ oscillations are essential for stimulating embryo development, as their inhibition with *N,N'*-(1,2-ethanediylbis(oxy-2,1-phenylene))bis(*N*-(carboxymethyl)-glycine (BAPTA) blocks oocyte activation (Kline and Kline, 1992) and the premature termination of Ca²⁺ oscillations prevents the formation of pronuclei (Lawrence *et al.*, 1998).

The explicit mechanism underlying fertilization-induced Ca²⁺ oscillations remains to be fully defined, but is believed to involve activation of the phosphoinositide pathway

leading to increased production of inositol 1,4,5-trisphosphate (InsP₃) (Stricker, 1999; Runft *et al.*, 2002). There are three distinct hypotheses to explain the Ca²⁺ release observed at fertilization: the 'conduit', 'contact' and 'content' models (Jaffe, 1991). The 'conduit' model proposes that sperm–oocyte fusion leads to increased influx of Ca²⁺ into the oocyte, leading indirectly to Ca²⁺ release (Jaffe, 1991). This model appears unlikely in mammalian oocytes, as increased Ca²⁺ influx alone is not effective in triggering Ca²⁺ oscillations (Swann, 1996), and sperm–oocyte fusion causes Ca²⁺ release in the absence of extracellular Ca²⁺ (Jones *et al.*, 1998a). The 'contact' model proposes that a receptor on the oocyte surface couples to either a G-protein or a tyrosine kinase and, upon sperm binding, this receptor activates an oocyte phospholipase C β (PLC β) or PLC γ , respectively, resulting in an increase in intracellular InsP₃ (Runft *et al.*, 2002). However, this hypothesis seems unlikely in mouse oocytes, as antibodies to Gq proteins fail to block fertilization-induced Ca²⁺ release (Williams *et al.*, 1998). The PLC γ pathway has been proposed on the basis that SH2 protein constructs are able to block Ca²⁺ release at fertilization in invertebrate oocytes (Carroll *et al.*, 1997; Shearer *et al.*, 1999). However, this inhibition does not occur in frog or mouse eggs and oocytes (Mehlmann *et al.*, 1998; Runft *et al.*, 1999). In addition, in mammals, there is

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no direct evidence of a sperm-specific plasma membrane receptor capable of stimulating the InsP_3 production pathway.

An alternative hypothesis is that the spermatozoon itself contains a soluble factor that, upon sperm–oocyte fusion, diffuses into the oocyte cytosol and stimulates the InsP_3 pathway (Swann, 1990; Fissore *et al.*, 1998; Stricker, 1999). Evidence for this hypothesis in mammals comes from the finding that microinjecting protein-based sperm extracts into oocytes from a number of mammalian species elicits Ca^{2+} oscillations similar to those seen at fertilization (Swann, 1990; Homa and Swann, 1994; Wu *et al.*, 1997; Tang *et al.*, 2000). The microinjection of mRNA from spermatogenic cells also triggers Ca^{2+} oscillations in mouse oocytes (Parrington *et al.*, 2000) and the introduction of whole spermatozoa into mammalian oocytes (intracytoplasmic sperm injection, ICSI), which avoids any surface contact between the oocyte and spermatozoon, also elicits a similar set of Ca^{2+} oscillations (Tesarik *et al.*, 1994; Nakano *et al.*, 1997). The Ca^{2+} -releasing sperm factor appears to be a sperm-specific protein, as extracts from other tissues do not elicit Ca^{2+} increases when injected into oocytes (Swann, 1990; Wu *et al.*, 1997). However, this mechanism is not species-specific, because sperm extracts from hamsters, humans, pigs, cows, frogs and chickens can each trigger Ca^{2+} oscillations in mouse oocytes (Homa and Swann, 1994; Wu *et al.*, 1997, 1998; Tang *et al.*, 2000).

Previous candidates for the Ca^{2+} -releasing sperm factor have been a 33 kDa glucosamine-6-phosphate deaminase (Parrington *et al.*, 1996) and a truncated form of the c-kit transmembrane receptor (tr-kit) (Sette *et al.*, 1997). However, both these candidates now seem unlikely to be the sperm factor (Wolosker *et al.*, 1998; Parrington *et al.*, 1999). More recent studies have suggested that the sperm factor itself may have a PLC activity (Jones *et al.*, 1998b; Rice *et al.*, 2000). However, all of the established somatic PLC isozymes tested have failed to elicit Ca^{2+} oscillations in oocytes at concentrations that approximate the PLC activity of sperm extracts (Jones *et al.*, 2000; Parrington *et al.*, 2002; Runft *et al.*, 2002). The known somatic PLC isozymes also fail to correlate with the Ca^{2+} -releasing activity of sperm extracts that have been chromatographically fractionated (Wu *et al.*, 2001; Parrington *et al.*, 2002).

A novel, sperm-specific isoform of PLC, named PLC ζ , has been identified, and immunodepletion was used to attribute the Ca^{2+} -releasing activity of mouse and hamster sperm extracts to the presence of PLC ζ (Saunders *et al.*, 2002). Microinjection of complementary RNA (cRNA) of the mouse PLC ζ (mPLC ζ) triggered Ca^{2+} oscillations similar to those observed at fertilization in mouse oocytes, and evoked early embryonic development to the blastocyst stage (Saunders *et al.*, 2002). These data indicate that PLC ζ correlates with the 'sperm factor' in mice. In the present study, human and simian PLC ζ (hPLC ζ and sPLC ζ , respectively) were identified, together with the genomic organization and tissue expression profile of human PLC ζ . Human and simian PLC ζ are both effective at inducing Ca^{2+}

oscillations in mouse oocytes, and human PLC ζ can also trigger development to blastocyst. Human PLC ζ is more potent in causing Ca^{2+} oscillations than the mouse and monkey isoforms.

Materials and Methods

Molecular cloning and sequence analysis of human PLC ζ

Blast searches of the human expressed sequence tag (EST) database (www.ncbi.nlm.nih.gov/BLAST) using known mammalian PLC sequences revealed several, closely related, novel sequences of 408–559 bp with distinct homology to the delta class of phosphoinositide-specific PLCs, and all sequences were derived from testis (accession numbers AA861064, AA398866, AA609626, AA620685 and AA435866). The full sequence of a putative PLC expressed by the testis was derived by two-step, nested PCR amplification using an adult human testes large insert cDNA library (500 ng) in the lambda TriplEx2 vector (HL5503u; Clontech, Oxford). The oligonucleotide primers were derived from conserved regions of the ESTs, and primers were designed against the library vector adapter sequence. PCR amplification was carried out with *Taq* DNA polymerase using conditions of 32 cycles, and temperatures of 94°C (1 min), 70°C (45 s) and 72°C (3 min). The testis library template-specific, amplified DNA products were cloned into pCR-BluntII-TOPO (Invitrogen, Paisley); the nucleotide sequence was determined (Prism Big Dye kit; Applied Biosystems International, Warrington) and then analysed for open reading frame (ORF) using MacVector 6.5 (Oxford Molecular, Oxford). Oligonucleotide primers were designed to incorporate the putative start and stop codons of a novel, testis-derived PLC sequence. The full-length PLC was amplified using *Pfu* polymerase, using 32 cycles of 94°C (1 min), 62°C (1 min) and 72°C (3 min). The single, amplified approximately 1.9 kb band was cloned into pCR-XL-TOPO and the insert DNA sequenced along both strands as described above. The 1.9 kb insert was subcloned into pTarget (Promega, Southampton) to generate pTarget-hPLC ζ . Homology sequence analysis and alignment with related PLCs was performed using ClustalW (www.clustalw.genome.ad.jp) and domain structure by RPS-Blast (www.ncbi.nlm.nih.gov/structure/cdd). The Genbank accession number for human PLC ζ is AF532185.

Identification and cloning of simian PLC ζ

A cynomolgus monkey cDNA library was prepared from size-selected, adult *Macaca fascicularis* testis cDNAs of >1.5 kb, and several novel, full-length insert DNA sequences were determined. Blast searching with the hPLC ζ sequence revealed two homologous simian sequences derived from the adult *M. fascicularis* testis cDNA library (accession numbers AB070108 and AB070109). The ORF within these two cynomolgus monkey cDNA clones were amplified by PCR with *Pfu* DNA polymerase as described

above, cloned into pcDNA3.1-V5-His-TOPO (Invitrogen) (pcDNA-s ζ) and the insert DNA sequenced along both strands, as described above. Homology sequence analysis and alignment was performed using ClustalW (www.clustalw.genome.ad.jp) and domain structure by RPS-Blast (www.ncbi.nlm.nih.gov/structure/cdd).

Northern and western blot analysis

A 950 bp fragment from the 3' terminal of the hPLC ζ ORF was amplified by PCR with *Pfu* DNA polymerase as described above, and cloned into pCR-BluntII-TOPO. Antisense cRNA, labelled with digoxigenin, was synthesized from the linearized plasmid (DIG Nucleic Acid labelling system; Roche Molecular Biochemicals, Lewes) and used as a riboprobe for hybridization analysis with a human male tissue RNA blot (Origene, Rockville, MD), containing equal loading of 20 μ g total RNA (2 μ g)⁻¹ polyA⁺ mRNA per lane, and standardized with an actin probe. The hybridized probe was detected using a DIG luminescence detection kit (Roche Molecular Biochemicals). For Western blot analysis, rabbit antisera was raised to a keyhole limpet haemocyanin (KLH)-conjugated 19mer peptide sequence, GYRRIPLSRMGESLEPAS, corresponding to an amino acid sequence located near to the carboxy-terminal end of the putative human PLC ζ sequence. Human sperm extracts (Parrington *et al.*, 1999) were added to SDS sample buffer and proteins separated by 10% polyacrylamide gel electrophoresis, transferred to PVDF membrane, and probed with rabbit anti-hPLC ζ peptide antisera (Saunders *et al.*, 2002).

Complementary RNA synthesis and in vitro translation

Complementary RNA (cRNA) encoding the complete ORF of human and monkey PLC ζ was produced from the linearized hPLC ζ and sPLC ζ plasmid constructs described above, using a Ribomax RNA synthesis kit (Promega), in the presence of 3 mmol m⁷G(5')ppp(5')G I⁻¹ (37°C, 2 h). Synthesized cRNA products were analysed by electrophoresis (1% native agarose gel, 50% formaldehyde-formamide in loading buffer), isopropanol precipitated, and resuspended in DEPC-treated water containing 4U RNasin μ l⁻¹ (Promega). Complementary RNAs were assayed by *in vitro* expression of PLC ζ protein (Reticulocyte Lysate System, Promega) in the presence of [³⁵S]methionine, and analysed by 10% SDS-PAGE and autoradiography, as described by Saunders *et al.* (2002).

Preparation and handling of gametes

Experiments were carried out on mouse oocytes in either Hepes-buffered KSOM (H-KSOM) or amino acid supplemented KSOM (Summers *et al.*, 2000). All compounds were from Sigma, unless stated otherwise. Female MF1 mice were superovulated by an injection of 5 iu eCG (PMSG; Intervet, Milton Keynes) and injection with hCG (Intervet) 48 h later. Oocytes were collected after a further 13.5–14.5 h, as

described by Lawrence *et al.* (1998) and maintained in 100 μ l droplets of H-KSOM under mineral oil at 37°C. The cRNA injections were carried out between 14.5 and 15.5 h after hCG injection. Activation of oocytes for development studies was carried out in H-KSOM containing 2 μ mol cytochalasin D I⁻¹ for 4 h, to prevent second polar body extrusion. Further development to two-cell and blastocyst stages was carried out in 50 μ l droplets of KSOM under mineral oil at 37°C in a 5% CO₂ incubator.

Measurement of intracellular Ca²⁺ and microinjection

Intracellular calcium changes were measured with Fura red-AM (Molecular Probes, Eugene, OR). Stock solutions of 1 mmol I⁻¹ dissolved in DMSO plus 5% (w/v) pluronic F127 (Molecular Probes) were used to load the oocytes, with a final concentration of 4 μ mol Fura red-AM I⁻¹ for 10 min. The loading medium was also supplemented with sulfinpyrazone, which helps to prevent compartmentalization and extrusion of the dye (Lawrence *et al.*, 1998). Ca²⁺ measurements were carried out on charge coupled device-based imaging systems using a Nikon Diaphot with epifluorescence controlled by Newcastle Photometrics Multipoint System, Newcastle-upon-Tyne, as described by Lawrence *et al.* (1998), or a Zeiss Axiovert 100 with illumination from a monochromator (Photonics, Robertsbridge) controlled by MetaFluor v4.0 (Universal Imaging Corp, Marlow). After loading with Fura red-AM, oocytes were washed in H-KSOM and placed on the stage of a Nikon Diaphot. Oocytes were microinjected as described by Swann (1990). The cRNA solutions were diluted with an injection buffer (120 mmol KCl I⁻¹; 20 mmol Hepes I⁻¹, pH 7.4). The volume injected was estimated from the diameter of cytoplasmic displacement caused by the bolus injection. All injections were 3–5% of the oocyte volume.

Results

Identification and characterization of a novel human PLC ζ sequence

Previous studies showed that various somatic PLC isoforms were ineffective at triggering Ca²⁺ oscillations in eggs, indicating that a putative sperm-specific PLC isoform remains to be identified (see Introduction). In early 1999, a Blast search of the human EST database for uncharacterized PLC-like sequences identified a group of novel, related sequences, specifically derived from testis, displaying homology to the δ isoforms of PLC. The full-length sequence of this PLC was obtained by rapid amplification of cDNA ends (RACE)-PCR from a human testis cDNA library, using oligonucleotide primers designed to the human ESTs and flanking lambda vector sequence. Nucleotide sequence analysis of the 2.2 kb full-length cDNA revealed a single ORF of 1824 bp, flanked by 5' and 3' untranslated regions of approximately 200 and 120 bp, respectively. The ORF encodes a protein of 608 amino acids (Fig. 1), with a

Fig. 1. Molecular cloning of human and simian phospholipase C ζ (PLC ζ). ClustalW alignment of human, simian and mouse PLC ζ sequences and rat PLC δ 1 (accession numbers AF532185, AB070108, AF435950 and P10688, respectively). Identical amino acids are shown in shaded black boxes, conservative substitutions are boxed in grey.

Fig. 2. Sequence comparison and domain analysis of phospholipase C ζ (PLC ζ). (a) Percentage identity of the human, simian and mouse PLC ζ and rat PLC δ 1 protein sequences is shown in red. Percentage similarity after combining sequence identity with conservative substitutions is shown in blue. (b) Schematic diagram depicting a linear representation of the domain structures of human, simian and mouse PLC ζ and rat PLC δ 1. aa: amino acids.

deduced molecular mass of 70 kDa and a predicted isoelectric point of 9.2. The molecular mass of the human protein is similar to that of mouse PLC ζ (74 kDa; Saunders *et al.*, 2002) but smaller than any previously characterized mammalian PLC β , γ , δ , ϵ isoforms (85–230 kDa).

ClustalW sequence alignment of the human protein with mouse PLC ζ showed a striking sequence identity, confirming it as the human homologue of PLC ζ (hPLC ζ ; Fig. 1). Similarly, the *M. fascicularis* testis sequences of 641 and 640 amino acids (accession numbers AB070108 and AB070109, respectively), both with deduced molecular mass of 74 kDa, also aligned optimally with human and mouse PLC ζ and were thus identified as simian PLC ζ homologues (sPLC ζ ; Fig. 1). The only difference between the two simian sequences was that EEEKF in residues 334–339 of AB070108 corresponded to EEE-RF in AB070109. Unless stated otherwise, further reference to studies with simian PLC ζ sequences in this report implies the 641 residue form, AB070108, as no other functionally significant differences have been observed with the 640 residue, AB070109.

Comparison of the PLC ζ sequences from the three

Fig. 3. Structure of the human *plc-ζ* gene. The fifteen *plc-ζ* exons identified within the 179 456 bp contig (accession number AC023940) are shown aligned to a 54.8 kb region of human chromosome 12 (12p12.3). Exons are labelled E1 to E15. The start and stop codons for human phospholipase C ζ (hPLC ζ) are located within E2 and E15, respectively. The solid lines between exons represent the introns (see Table 2).

species revealed a higher percentage identity between the primate sequences, hPLC ζ and sPLC ζ (90%), than between the primate and the rodent sequences, mPLC ζ (70 and 71% for comparison between the mouse and the human and simian sequences, respectively; Fig. 2a). Identity of all three PLC ζ s with PLC δ 1 was 33%. As originally observed for mouse PLC ζ (Saunders *et al.*, 2002), the multiple sequence alignment with PLC δ 1 (Fig. 1) demonstrates that all the PLC ζ s lack a plextrin-homology (PH) domain at the N-terminus, a domain found in most other animal PLCs (Fig. 2b). The other conserved PLC domains (the EF-hand region, the X and Y catalytic domains, and the C2 domain) were present in the human, simian and mouse PLC ζ sequences (Fig. 2b). The shorter length of the human compared with mouse and simian PLC ζ sequences (608 versus 647 and 641, respectively) is due to a smaller X–Y linker segment, the region located between the X and Y catalytic domains (Figs 1 and 2b). A summary of the molecular properties of mouse, human and simian PLC ζ s is presented (Table 1).

A Blast search of the public domain human genome database identified a sequence of 179 456 bp, assembled in two ordered pieces (accession number AC023940) and containing the entire human PLC ζ cDNA sequence. The genomic organization of the human *plc-ζ* gene (Fig. 3, Table 2) was deduced from this 179 kb contig as comprising 15 exons, encompassed within a region spanning 54.8 kb

Table 1. Summary of the molecular properties of mammalian PLCζs

Species	AA	MW	pI	Exons	mRNA (kb)	Acc. no.
Mouse PLCζ	647	74 609	5.30	nd	2.3	AF435950
Simian PLCζ	641	74 545	7.46	nd	2.3	AB070108
Simian PLCζ	640	74 445	7.88	nd	2.3	AB070109
Human PLCζ	608	70 406	9.26	15	2.2	AF532185

AA: amino acid sequence length; MW: deduced molecular weight; pI: predicted isoelectric point; Exons: number of exons identified; mRNA: transcript size; Acc. no.: Genbank accession number; nd: denotes not determined.

Table 2. Sequence coordinates and length of exons and introns comprising the human *plc-zeta* gene localized to chromosome 12p12.3

Chromosome 12 co-ordinates	Exon number	Intron number	Length (bp)
4443338 – 4443286	1		53
4443285 – 4442862		1	424
4442861 – 4442712	2		150
4442711 – 4441697		2	1015
4441696 – 4441564	3		133
4441563 – 4428887		3	12 677
4428886 – 4428667	4		220
4428666 – 4424987		4	3680
4424986 – 4424784	5		203
4424783 – 4418341		5	6443
4418340 – 4418195	6		146
4418194 – 4410669		6	7526
4410668 – 4410519	7		150
4410518 – 4407131		7	3388
4407130 – 4407045	8		86
4407044 – 4406922		8	123
4406921 – 4406853	9		69
4406852 – 4405305		9	1548
4405304 – 4405151	10		154
4405150 – 4401621		10	3530
4401620 – 4401503	11		118
4401502 – 4400434		11	1069
4400433 – 4400263	12		171
4400262 – 4393572		12	6691
4393571 – 4393443	13		129
4393442 – 4389634		13	3809
4389633 – 4389484	14		150
4389483 – 4388679		14	805
4388678 – 4388535	15		144

located on the short arm of chromosome 12 (12p12.3). The ATG initiation methionine codon is located in exon 2 and the TAA stop codon in exon 15 (Fig. 3, Table 2).

A short intron sequence of 123 bp occurs between exons 8 and 9 and is in-frame, indicating that it may form the central part of a single larger exon that incorporates both exons 8 and 9. In accord with this suggestion, the in-frame

translation of the 123 bp sequence yields a sequence of 41 residues that is homologous to the corresponding segment within the X–Y linker sequence in *M. fascicularis* PLCζ (73% identity, Fig. 4). This finding raises the possibility that alternative splicing of this region occurs to produce multiple forms of hPLCζ. An attempt was made to demonstrate specifically the presence of this 123 bp sequence in the

Human intron 8 translation

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1  KVEEWEEVADGEEEEEEEEEEEEEEEDKFKESSEVLESVLG 41
318 KVEEWEEVADLEEEEEEEEE-----KFKESSEVLESVLG 350

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Simian X-Y linker

Fig. 4. ClustalW alignment of translated human intron 8. The 41 residue translation of the in-frame 123 bp intron 8 sequence is shown aligned to the corresponding region within the X-Y linker of the simian phospholipase C ζ (sPLC ζ) sequence. Identical amino acids are shown in shaded black boxes; conservative substitutions are boxed in grey.

Fig. 5. Expression of human phospholipase C ζ (hPLC ζ) in testis and spermatozoa. (a) Northern blot analysis of PLC ζ transcript abundance and distribution in human tissues. Lanes from left to right are: B: brain; C: colon; H: heart; Lu: lung; Li: liver; K: kidney; SI: small intestine; Mu: muscle; PI: placenta; Sp: spleen; St: stomach; T: testis. RNA size markers in kb are indicated on the left. (b) Immunoblot analysis of PLC ζ expression in human spermatozoa. Human sperm protein extract (50 μ g protein per lane) was separated by SDS-PAGE, transferred to membrane and probed with anti-PLC ζ antiserum. Protein size markers in kDa are indicated on the right.

human cDNA library, which was derived from testes pooled from 25 unrelated males, aged 25–64. However, using PCR primers immediately flanking the X–Y linker region of hPLC ζ , no evidence was found for expression of this 123 bp

sequence in the testis cDNA library (data not shown). In addition, the distinctive lack of a PH domain in the three PLC ζ sequences identified so far (Fig. 2b) was consistent with the inability to identify any sequence homology to the PLC δ 1 PH domain within the 179 kb human genomic contig containing the 15 exons of the *plc* gene (data not shown).

Northern blot analysis using a digoxigenin-labelled, anti-sense cRNA probe derived from the 3'-end of hPLC ζ displayed specific hybridization only to a 2.2 kb mRNA expressed in relative abundance in human testis (Fig. 5a). This finding is consistent with the 2.2 kb cDNA obtained by full-length RACE-PCR (Fig. 1). No other band was detected in any of the other 11 human tissues, which was a similar result to that obtained in the hybridization analysis of mouse tissues with mPLC ζ that identified a testis-specific 2.3 kb transcript (Saunders *et al.*, 2002). The expression in human spermatozoa of a protein corresponding to hPLC ζ was confirmed by immunoblot analysis of human sperm extracts. Antisera raised to a peptide sequence deduced from the hPLC ζ of 608 residues recognized a single protein band of approximately 70 kDa in human spermatozoa (Fig. 5b), in congruence with the 70 kDa protein predicted from the 1824 bp ORF (Fig. 1, Table 1).

Triggering of Ca²⁺ oscillations in mouse oocytes by human PLC ζ

Microinjection of mouse PLC ζ cRNA into mouse oocytes triggers Ca²⁺ oscillations indistinguishable from those associated with fertilization (Saunders *et al.*, 2002). The ability of hPLC ζ to cause Ca²⁺ changes was examined by microinjecting cRNA encoding hPLC ζ into MII-arrested mouse oocytes at a pipette concentration of 20 μ g hPLC ζ cRNA ml⁻¹, which corresponds to 0.001 mg ml⁻¹ in the oocyte after a 3–5% injection volume. A representative Ca²⁺ recording for each of the four different concentrations of hPLC ζ cRNA that were microinjected is shown (Fig. 6a–d). At a concentration of 20 μ g ml⁻¹, hPLC ζ cRNA triggered high frequency Ca²⁺ oscillations within 10–15 min of microinjection (mean interspike interval: 4.21 ± 1.79 min). As was observed with mouse PLC ζ cRNA and hamster sperm extract microinjection, Ca²⁺ oscillations of lower frequency were obtained with lower concentrations of

Fig. 6. Ca^{2+} oscillations in mouse oocytes microinjected with human phospholipase C ζ (hPLC ζ) cRNA. (a) Dose-dependent Ca^{2+} oscillations in metaphase II-arrested mouse oocytes after microinjection of hPLC ζ cRNA. Traces show the cytosolic Ca^{2+} oscillations observed upon microinjection with cRNA at pipette concentrations of (a) 20, (b) 2.0, (c) 0.2 and (d) 0.02 $\mu\text{g ml}^{-1}$. (e) Mean interspike interval of Ca^{2+} oscillations in mouse oocytes triggered by the various hPLC ζ cRNA concentrations. The number of microinjected oocytes is shown above each dose. The mean interspike interval differs significantly among doses (Student's unpaired *t* test; $P < 0.0001$) (20 $\mu\text{g ml}^{-1}$: 4.21 ± 1.79 ; 2.0 $\mu\text{g ml}^{-1}$: 9.26 ± 7.14 ; 0.2 $\mu\text{g ml}^{-1}$: 16.0 ± 6.40 ; 0.02 $\mu\text{g ml}^{-1}$: 24.34 ± 7.68).

stimulus (Swann, 1990; Saunders *et al.*, 2002). Even at pipette concentrations of 0.02 $\mu\text{g ml}^{-1}$, hPLC ζ cRNA could induce Ca^{2+} oscillations within 2 h of microinjection.

Although a wide range of cRNA concentrations (20 to 0.02 $\mu\text{g ml}^{-1}$) were used, the Ca^{2+} oscillations observed at each concentration lasted for a similar period of 3–4 h (Fig. 6a–d). The mean interspike interval data showing the dose–response relationship with hPLC ζ cRNA is summarized (Fig. 6e).

Embryo development with hPLC ζ

Microinjection of 20 $\mu\text{g ml}^{-1}$ hPLC ζ into mouse oocytes induces Ca^{2+} oscillations and development to the blastocyst stage at rates comparable to that observed in *in vitro* fertilization (Saunders *et al.*, 2002). Oocytes arrested at metaphase II were injected with 20, 2.0 and 0.2 $\mu\text{g ml}^{-1}$ hPLC ζ cRNA and monitored after 24 and 96 h to examine whether hPLC ζ is also able to support development, and what effect the oscillation frequency has on embryo development. All three concentrations were effective at activating the oocytes and enabling development to the two-cell stage (Fig. 7). Mouse embryo development to morula–blastocyst was 33.3 and 38.9% after injection with 2.0 and 0.2 $\mu\text{g ml}^{-1}$ hPLC ζ cRNA, respectively (Fig. 7a). These results are comparable to developmental rates observed in *in vivo* fertilization and parthenogenetic activation of 55–60% under the conditions described and using outbred mouse strains (Saunders *et al.*, 2002). However, the high Ca^{2+} oscillation frequency (low mean interspike interval) produced with 20 $\mu\text{g ml}^{-1}$ hPLC ζ was ineffective at supporting development to the morula–blastocyst stage (1.8% of oocytes reached the morula–blastocyst stage) and most embryos arrested at the two-cell stage.

Mouse embryos produced by microinjection of hPLC ζ cRNA are similar in morphology to those obtained after *in vivo* fertilization (Fig. 7b,c), although the number of blastocyst cells was not determined. These data indicate that microinjection of hPLC ζ cRNA into unfertilized eggs alone can trigger early embryonic development to the blastocyst stage in mouse embryos, but it appears that the high frequency of Ca^{2+} oscillations caused by higher doses of hPLC ζ is detrimental to development beyond the two-cell stage.

Triggering of Ca^{2+} oscillations in mouse oocytes by simian PLC ζ

The observations described above (Figs 6 and 7) and in a previous report (Saunders *et al.*, 2002) show that the human and mouse PLC ζ can cause fertilization-like Ca^{2+} oscillations that initiate activation and development of mouse oocytes. The identification of two related, testis-specific cDNA sequences of 2.3 kb from *M. fascicularis* (Fig. 1), and the high degree of similarity of their ORF with the human and mouse PLC ζ (Fig. 2), enabled the prediction that these sequences were simian PLC ζ homologues. Therefore, the ability of cRNA prepared from the two forms of sPLC ζ , designated s¹PLC ζ and s²PLC ζ (AB070108 and AB070109, respectively) to generate Ca^{2+} oscillations in mouse oocytes

Fig. 7. Embryonic development of mouse oocytes microinjected with human phospholipase C ζ (hPLC ζ) cRNA. (a) Mouse oocytes were microinjected with different hPLC ζ cRNA concentrations (20–0.2 $\mu\text{g ml}^{-1}$). The percentage of oocytes reaching (■) the two-cell stage after 24 h and (□) morula–blastocyst after 96 h were recorded. Photomicrographs showing development of mouse embryos at (b) the two-cell stage and (c) the blastocyst stage after microinjection of unfertilized oocytes with hPLC ζ cRNA (0.2 $\mu\text{g ml}^{-1}$).

was compared. Both forms of sPLC ζ were able to trigger Ca²⁺ oscillations and no functional difference was detected upon microinjecting either s¹PLC ζ or s²PLC ζ cRNA (data not shown). Therefore, s¹PLC ζ (AB070108) was used for all subsequent experiments. s¹PLC ζ cRNA triggered dose-dependent Ca²⁺ oscillations in mouse oocytes comparable to those seen with human and mouse PLC ζ , at each of the three doses tested (0.2, 0.02, 0.002 mg ml^{-1}) (Fig. 8a–c). Similar to the results obtained with human PLC ζ (Fig. 6a–d), the period over which Ca²⁺ oscillations occurred was 3–4 h for each of the three s¹PLC ζ cRNA concentrations microinjected. However, the frequency of Ca²⁺ spikes was different for each cRNA concentration, with the mean interspike

interval decreasing with increasing concentration of stimulus (Fig. 8d). These data indicate that PLC ζ , derived from the spermatozoa or testis of various mammals lacks species specificity and, once introduced by microinjection, is able to trigger Ca²⁺ oscillations in heterologous mammalian oocytes. This finding is fully consistent with earlier observations that sperm extracts derived from various sources, including non-mammalian species, each caused Ca²⁺ oscillations in different mammalian oocytes (Homa and Swann, 1994; Wu *et al.*, 1997; Tang *et al.*, 2000).

Mean interspike intervals for the three different mammalian forms of PLC ζ at various pipette concentrations of cRNA were compared (Fig. 9). Microinjecting cRNA for

Fig. 8. Ca^{2+} oscillations in mouse oocytes with simian phospholipase C ζ (sPLC ζ) cRNA. (a) Dose-dependent Ca^{2+} oscillations in mouse oocytes arrested at metaphase II after microinjection of sPLC ζ cRNA. Traces show the cytosolic Ca^{2+} oscillations observed upon microinjection with cRNA at pipette concentrations of (a) 200, (b) 20 and (c) 2 $\mu\text{g ml}^{-1}$. (d) Mean interspike interval of Ca^{2+} oscillations in mouse oocytes triggered by the various sPLC ζ cRNA concentrations. The number of microinjected oocytes is shown above each dose. The mean interspike interval differs significantly among doses (Student's unpaired *t* test; $P < 0.0001$) (200 $\mu\text{g ml}^{-1}$: 3.18 ± 0.55 ; 20 $\mu\text{g ml}^{-1}$: 7.35 ± 2.69 ; 2.0 $\mu\text{g ml}^{-1}$: 15.77 ± 5.20).

mPLC ζ , hPLC ζ or sPLC ζ all produced Ca^{2+} oscillations over a range of concentrations from 200 to 2 $\mu\text{g ml}^{-1}$. However, hPLC ζ was distinct in its ability to cause Ca^{2+} oscillations at the lower concentrations of 0.2–0.02 $\mu\text{g ml}^{-1}$

Fig. 9. Comparison of the mean interspike intervals of Ca^{2+} oscillations in mouse oocytes triggered by the three species of PLC ζ cRNA. (■) Human, (□) simian and (▨) mouse PLC ζ cRNAs each triggered Ca^{2+} oscillations within 2 h of microinjection of 200–2.0 $\mu\text{g PLC}\zeta \text{ cRNA ml}^{-1}$. Only hPLC ζ was effective at the lower doses of 0.2 and 0.02 $\mu\text{g ml}^{-1}$. The number of oocytes microinjected is shown above each dose. The mean interspike interval differs significantly at each dose for human, simian and mouse PLC ζ cRNA (Student's unpaired *t* test; $P < 0.005$).

(Fig. 9), indicating that, under the same experimental conditions, the human form of PLC ζ is more effective at generating Ca^{2+} oscillations in mouse oocytes than is the PLC ζ from mice and monkeys.

Discussion

There is mounting evidence that during fertilization, the spermatozoon delivers a cytosolic factor that diffuses into the ooplasm after gamete fusion and triggers the Ca^{2+} changes required for embryo development (Stricker, 1999; Swann *et al.*, 2001). The Ca^{2+} -releasing activity of a cytosolic sperm factor has been demonstrated in several mammalian species. In particular, microinjection of human sperm extracts is able to cause Ca^{2+} oscillations in both human and mouse oocytes (Homa and Swann, 1994; Palermo *et al.*, 1997). The ICSI technique can also lead to generation of the crucial Ca^{2+} oscillations that precede activation of the oocyte (Nakano *et al.*, 1997). As has been observed with sperm extract microinjection, the species of mammalian spermatozoa used for ICSI does not need to match the species of the oocyte, since ICSI with either human or monkey spermatozoa can activate mouse oocytes (Rybouchkin *et al.*, 1996; Ogonuki *et al.*, 2001). These reports indicate that the cytosolic sperm factor, when identified, would be present in humans and monkeys, and would be able to act across species.

A novel, sperm-specific enzyme, PLC ζ , has been identi-

fied as being synonymous with the mouse sperm factor responsible for the Ca²⁺-releasing activity of sperm extracts (Saunders *et al.*, 2002). The presence of a PLC ζ -like immunoreactive protein in hamster and boar sperm extracts has also been demonstrated (Saunders *et al.*, 2002). However, to the authors' knowledge, no other PLC ζ s have been cloned and functionally characterized in species other than the mouse. In the present study, the full-length sequences for both the human and simian PLC ζ were identified, the organizational structure of the human *plc ζ* gene was described and the reduced length of the X–Y linker sequence in the human PLC ζ was noted. Analysis of human tissues showed that hPLC ζ is expressed solely in testis tissue and is readily detected in human sperm extracts. The PLC ζ from humans and cynomolgus monkeys, like the PLC ζ of mice, lacks the amino-terminal PH domain found in other vertebrate somatic PLCs (Katan, 1998; Rebecchi and Pentyala, 2000; Saunders *et al.*, 2002). Because the human and simian PLC ζ s also possess the ability to trigger Ca²⁺ oscillations in oocytes, the lack of a PH domain appears to be an important feature of PLC ζ . Hence, the distinctive domain structure of the sperm PLC ζ is likely to be critical for the unique Ca²⁺-signalling properties exhibited by this isoform during fertilization (Saunders *et al.*, 2002). Furthermore, the high degree of conservation of the three identified PLC ζ protein sequences and their uniform Ca²⁺-mobilizing effects in oocytes are consistent with the conserved action of sperm extracts from a range of species in triggering Ca²⁺ oscillations in oocytes from various mammals.

In addition to demonstrating that hPLC ζ and sPLC ζ are able to cause Ca²⁺ oscillations in mouse oocytes, empirical evidence was obtained that hPLC ζ is more effective in causing Ca²⁺ oscillations than are sPLC ζ or mPLC ζ . The minimal amount of hPLC ζ cRNA required to trigger Ca²⁺ oscillations was one to two orders of magnitude lower (0.2–0.02 $\mu\text{g ml}^{-1}$) than the minimally effective dose of mouse or simian PLC ζ cRNA (2 $\mu\text{g ml}^{-1}$). These differences were observed as a consistent feature with different batches of cRNA that were each tested for expression *in vitro* (data not shown). Therefore, the superior potency of hPLC ζ cRNA is likely to represent a genuine feature of the hPLC ζ protein. This contention is supported indirectly by the linear correlation between amount of cRNA injected and protein expression observed in mouse oocytes with mPLC ζ (Saunders *et al.*, 2002). Thus, it could be predicted that there would be at least an order of magnitude difference between the sensitivity of mouse oocytes to hPLC ζ and their sensitivity to mPLC ζ . It is not clear why hPLC ζ exhibits greater effectiveness than mPLC ζ or sPLC ζ . One distinct structural difference in hPLC ζ compared with mPLC ζ or sPLC ζ that might be related to the higher potency is the shorter X–Y linker sequence of hPLC ζ , which reduces the number of acidic residues and results in a basic pI value of 9.3. It is possible that the sperm factor may need to be more vigorous in some species than in others. For example, dilution of the sperm factor is greater in humans, as the volume of the human oocyte is several times that of a mouse oocyte. Intrinsic

differences in the sperm factor sensitivity of oocytes from different species may also be important as, for example, mouse oocytes are more sensitive to sperm factor-induced Ca²⁺ release than are oocytes from some other species, such as hamsters (Parrington *et al.*, 1996). In addition, different amounts of PLC ζ may be present in the spermatozoa from individual species, and there may be a relationship between oocyte sensitivity and concentration of sperm PLC ζ .

Subsequent to stimulating Ca²⁺ oscillations in mouse oocytes, human PLC ζ was also able to trigger development of embryos to the blastocyst stage. This finding indicates that hPLC ζ is able to produce all of the normal events of oocyte activation. However, one feature of the greater efficacy of hPLC ζ is that high cRNA concentrations caused very high frequency Ca²⁺ oscillations in mouse oocytes. At concentrations of cRNA that resulted in Ca²⁺ oscillations of approximately one spike every 5 min, hPLC ζ was able to effect oocyte activation, but the embryos arrested at the two-cell stage. High frequency Ca²⁺ oscillations may lead either to apoptosis of oocytes or to developmental changes in post-implantation embryos (Gordo *et al.*, 2000; Ozil and Huneau, 2001). The present data provide the first indication that high frequency Ca²⁺ oscillations can also activate an oocyte, but this non-physiological stimulus leads to arrest during the early cleavage stages. The molecular mechanism causing this developmental block remains to be determined; however, it is possible that embryos arrest at the two-cell stage in these experiments as a result of failure of zygotic genome activation. The present data indicate that both the molecular properties and quantity of sperm PLC ζ introduced into an oocyte at fertilization are critical for successful embryonic development.

The discovery and characterization of PLC ζ from different species as an effective trigger for Ca²⁺ release and subsequent activation of mammalian oocytes will enable developmental biologists to study the mechanisms of early development using the physiological activating factors. Although the present experiments crossed species in studying the effect of human and monkey isoforms of PLC ζ in mouse oocytes, hPLC ζ and sPLC ζ would be expected to be similarly effective in stimulating Ca²⁺ oscillations in human and monkey oocytes, as has been reported at fertilization in these two species (Taylor *et al.*, 1993; Wu *et al.*, 1996). Identification of the human sperm PLC ζ and corresponding *plc ζ* gene structure enables detection, and possibly treatment, of male infertility resulting from a PLC ζ defect, in which otherwise normal spermatozoa would be predicted to be unable to activate the oocyte (Rybouchkin *et al.*, 1996; Battaglia *et al.*, 1997; Palermo *et al.*, 1997). Availability of hPLC ζ may also be pivotal to increasing oocyte activation rates in IVF, ICSI and nuclear transfer techniques in the treatment of human disease.

This work was supported by a SIF grant to F. A. Lai from the University of Wales College of Medicine. K. Swann holds a Wellcome Trust grant.

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Received 26 July 2002.

First decision 8 August 2002.

Revised manuscript received 15 August 2002.

Accepted 19 August 2002.