Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase C ζ

Akihiro Yoneda¹, Masashi Kashima¹, Shigeki Yoshida¹, Kei Terada¹, Shoma Nakagawa¹, Akiko Sakamoto¹, Koji Hayakawa¹, Keita Suzuki², Junji Ueda^{1,3} and Tomomasa Watanabe¹

¹Laboratory of Animal Breeding and Reproduction, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan, ²Experimental Farm, Field Science Center (FSC), Hokkaido University, Sapporo 060-0811, Japan and ³Department of Dairy Science, Rakuno Gakuen University, Bunkyo-dai-Midorimachi, Ebetsu, Hokkaido 069-8501, Japan

Correspondence should be addressed to T Watanabe; Email: watanabe@anim.agr.hokudai.ac.jp

Abstract

The molecular mechanism by which sperm triggers Ca²⁺ oscillation, oocyte activation, and early embryonic development has not been clarified. Recently, oocyte activation has been shown to be induced by sperm-specific phospholipase C ζ (PLC ζ). The ability of PLC ζ to induce oocyte activation is highly conserved across vertebrates. In the present study, porcine PLC ζ cDNA was identified and the nucleotide sequence was determined. The expression pattern of porcine PLC ζ mRNA during the period of postnatal testicular development was shown to be similar to that of mouse PLC ζ . PLC ζ mRNA expression in the pig and mouse was detected only in the testes when the elongated spermatids had differentiated, and was detected from day 96 after birth in the pig. Histological examination of porcine testis during the period of postnatal development revealed the presence of spermatozoa from day 110 after birth. These findings suggest that the synthesis of PLC ζ mRNA starts when spermiogenesis is initiated. Microinjection of porcine PLC ζ complementary RNA into porcine oocytes demonstrated that porcine PLC ζ has the ability to trigger repetitive Ca²⁺ transients in porcine oocytes similar to that observed during fertilization. It was also found that porcine PLC ζ cRNA has the potential to induce oocyte activation and initiate embryonic development up to the blastocyst stage.

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Introduction

The development of mammalian oocytes is arrested at the metaphase of the second meiosis until fertilization. During fertilization, activation of oocytes is induced by a repetitive rise of intracellular Ca²⁺ concentration, termed Ca²⁺ oscillation, triggered by sperm penetration (Miyazaki *et al.* 1993, Swann 1996, Stricker 1999). Oocytes activated by Ca²⁺ oscillation resume the second meiosis and extrude a second polar body, and form male and female pronuclei. It is known that this Ca²⁺ oscillation is derived from the inositol 1,4,5-triphosphate (IP₃)-signaling pathway in oocytes, which stimulates IP₃ receptors of the endoplasmic reticulum as the intracellular store of Ca²⁺ (Miyazaki *et al.* 1993, Brind *et al.* 2000, Jellerette *et al.* 2000). However, the molecular mechanism that results in the activation of the IP₃-signaling pathway followed by sperm penetration has not been elucidated.

One of the hypotheses to explain the Ca²⁺ oscillation observed during fertilization is that the sperm itself

contains a soluble activating factor that diffuses into the oocyte cytosol and stimulates the IP₃ pathway (Swann 1990, Stricker 1999). It has been reported that the injection of whole spermatozoa into the mouse oocytes elicits Ca²⁺ oscillation (Nakano *et al.* 1997). Furthermore, microinjection of sperm extracts or spermatogenic mRNA into the mouse oocytes is able to trigger Ca²⁺ oscillation similar to that observed during fertilization (Parrington *et al.* 2000). The Ca²⁺-releasing factor is thought to be a sperm-specific protein, because microinjection of other tissue extracts into mouse oocytes could not elicit Ca²⁺ oscillation (Swann 1990, Wu *et al.* 1997). On the other hand, since sperm extracts from various animals such as the hamster, human, pig, and cow have been reported to be able to trigger Ca²⁺ oscillation in mouse oocytes, the sperm factor is not species-specific (Homa & Swann 1994, Wu *et al.* 1997, Tang *et al.* 2000).

Recently, in the mouse, human, monkey, and chicken, phospholipase C ζ (PLC ζ) has been isolated as a

sperm-specific PLC (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). When PLC ζ cRNA was injected into the mouse oocytes at a concentration comparable to the content in a single sperm, Ca²⁺ oscillation was triggered in the oocytes (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). Furthermore, the injection of PLC ζ cRNA into the mouse oocytes induced embryonic development up to the blastocyst stage. However, Ca²⁺ oscillation was not triggered by microinjection of sperm extracts from which PLC ζ protein had been removed. Therefore, PLC ζ is thought to be the strongest candidate for a sperm factor that initiates oocyte activation and early embryonic development.

In the present study, we isolated PLC ζ cDNA homolog from porcine testis and examined the expression patterns of PLC ζ mRNA during the period of testicular postnatal development in the pig and mouse. Furthermore, we investigated whether porcine PLC ζ has the ability to trigger Ca²⁺ oscillation, induce oocyte activation, and initiate embryonic development up to the blastocyst stage, by injecting PLC ζ cRNA into porcine oocytes.

Materials and Methods

Isolation of porcine PLC ζ cDNA

Total RNA was isolated from testis of Landrace boars using an ISOGEN RNA extract reagent (Nippon Gene, Tokyo, Japan). Total RNA of 800 ng was reverse transcribed using a Reva-Tra Ace kit (Toyobo Bio, Osaka, Japan). Then PCR was performed using KOD-Plus DNA polymerase (Toyobo Bio, Osaka, Japan) with the following profile: one cycle of 95 °C for 2 min, 35 cycles of 95 °C for 0.5 min, 58 °C for 0.5 min and 68 °C for 2 min, and one cycle of 68 °C for 2 min. The oligonucleotide primers of 5'-GAAGGTTTTAAGAACTAGC and 5'-TTACTGTGATGTGTAGCTAA for reverse transcriptase (RT)-PCR were designed from nucleotide sequences showing high homology in 5'- and 3'-UTRs of mouse and human PLC ζ cDNA (Cox *et al.* 2002, Saunders *et al.* 2002). Then the oligonucleotide primers of 5'-GCCAGGAGAAACAGAACAGC (144–163) and 5'-AGCTAACGATATTCTGGCACT (2117–2096) for RT-PCR were designed with reference to the nucleotide sequences of DNA products that were amplified using the primers shown above. The RT-PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The nucleotide sequence of porcine PLC ζ cDNA was determined using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the sequence of porcine PLC ζ cDNA was registered in the GenBank with the accession number AB113581. Multiple sequence alignment with related PLCs was performed using ClustalW (www.clustalw.genome.ad.jp). The domain structure of porcine PLC ζ was investigated by RPS-Blast (www.ncbi.nlm.nih.gov/structure/cdd.shtml).

Expression of porcine and mouse PLC ζ mRNA and expression of porcine c-kit mRNA

Total RNA of 800 ng extracted from several tissues of Landrace boars and gilts as well as ICR (SLC Japan, Hamamatsu, Japan) mice was reverse-transcribed using a Reva-Tra Ace kit (Toyobo Bio). To check the expression of porcine and mouse PLC ζ mRNA, RT-PCR was performed using *Pfu* DNA polymerase (Promega) with the following profile: one cycle of 95 °C for 2 min, 35 cycles of 95 °C for 0.5 min, 58 °C for 0.5 min and 72 °C for 4 min, and one cycle of 72 °C for 4 min. The oligonucleotide primers of 5'-GGAACCTTAAAGGAACTCA (1080–1099) and 5'-CAACAAAACGTATTAATGCC (1926–1907) for porcine PLC ζ and of 5'-GACAAGCGGCCAGATCATG (170–189) and 5'-CTAACGCGTCAGTTACATGCG (2159–2139) for mouse PLC ζ cDNA were designed with reference to the nucleotide sequences of porcine (accession number AB113581) and mouse (AF435950) PLC ζ . As a control, RT-PCR for the expression of porcine c-kit mRNA was also carried out using KOD-Plus DNA polymerase (Toyobo Bio) with the following profile: one cycle of 94 °C for 2 min and 30 cycles of 94 °C for 0.5 min, 58 °C for 0.5 min and 68 °C for 1 min. The oligonucleotide primers of 5'-TCRTACATA-GAAAGAGAYGTGACT (2292–2316) and 5'-AGCCTT-CCTTGATCATCTTGTAAG (2714–2693) were designed with reference to the nucleotide sequence of porcine c-kit (accession number, AJ223228) and were used for porcine c-kit mRNA expression. In this study, as another control experiment, the expression of porcine β -actin mRNA and that of mouse β -actin mRNA were examined by RT-PCR using the primers of 5'-GCTGTCCCTGTAC-GCCTCT (34–52) and 5'-CATGATCGAGGTGAAGGTGGT (561–541) and of 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACCATTC-3' respectively. Their RT-PCR products were electrophoresed through 1% agarose (Nippon Gene) gel and visualized by ethidium bromide staining.

Histology of porcine testis

The testes obtained from Landrace boars at various postnatal stages from 13 to 217 days after birth were histologically examined. Briefly, their tissues were immersed in PBS (136 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄ 12H₂O, and 1.47 mM KH₂PO₄), quickly frozen in ornithine carbamyl transferase compound (Sakura, Tokyo, Japan), and stored in liquid nitrogen until use. Frozen testes were sectioned and mounted on poly-L-lysine-coated slides. For histological examination, the sections of testes were fixed with 10% formaldehyde and stained with hematoxylin and eosin (HE).

Collection of porcine oocytes and in vitro maturation

Porcine ovaries were collected at a local slaughterhouse and transported to the laboratory within 2 h in warm

0.9% saline solution. The oocytes were aspirated from follicles of 3–6 mm in diameter in the ovaries. Then the oocytes with evenly granulated cytoplasm surrounded by a compact cumulus mass (cumulus–oocyte complexes, COCs) were collected and washed with M2 medium (Quinn *et al.* 1982). These COCs were transferred into BSA-free NCSU23 medium (Petters & Wells 1993) supplemented with 0.57 mM cysteine, 10% porcine follicular fluid (pFF), 10 IU/ml eCG, and 10 IU/ml hCG, and were cultured in the same medium for 20 h at 38.5 °C in 5% CO₂ in air as the maturation period. After 20 h of maturation culture, COCs were washed with BSA-free NCSU23 medium supplemented with 0.57 mM cysteine and 10% pFF and cultured in the same medium for 24 h at 38.5 °C in 5% CO₂ in air. At the end of *in vitro* maturation, COCs were treated with 0.1% hyaluronidase in M2 medium to remove cumulus cells. Oocytes with a first polar body were selected and defined as matured oocytes. The matured oocytes were washed twice with M2 medium and then placed into Pig-FM medium (Suzuki *et al.* 2002) until *in vitro* fertilization.

In vitro fertilization

Frozen spermatozoa were thawed and washed twice with WS-PVA (Suzuki *et al.* 2002) by centrifugation at 600 *g* for 5 min. After washing, the sperm pellets were suspended in Pig-FM medium and diluted to the appropriate concentration of 1.0×10^6 sperm/ml. Aliquots of sperm suspension to give a final concentration of 1.0×10^5 sperm/ml were added to Pig-FM medium containing oocytes. Oocytes and spermatozoa were co-incubated for 6 h at 38.5 °C in 5% CO₂ in air. After *in vitro* insemination, the oocytes were washed and incubated in NCSU23 medium containing 4 mg/ml BSA for 144 h at 38.5 °C in 5% CO₂ in air.

Synthesis of porcine PLC ζ cRNA and its microinjection into porcine oocytes

The porcine PLC ζ cDNA encoding the open reading frame (ORF) was synthesized by RT-PCR against total RNA extracted from testes of Landrace boars as mentioned previously. The PLC ζ cDNA fragment was subcloned into the pT_NT expression vector (Promega). Then porcine PLC ζ cRNA was synthesized by using a Ribomax RNA synthesis system (Promega) after the linearization of PLC ζ cDNA inserted into the pT_NT vector by the digestion of EclHKL restriction enzyme. The synthesized porcine PLC ζ cRNA was diluted with an RNase-free injection buffer (120 mM KCl and 20 mM HEPES, pH 7.4) and injected into porcine oocytes matured *in vitro*. Porcine cRNA, 20 μ g/ml, was selected as the concentration for injection by reference to a

study on mouse PLC ζ cRNA by Saunders *et al.* (2002), and the amount of injected solution per oocyte was about 4 pl. The porcine oocytes injected with porcine PLC ζ cRNA were cultured in NCSU23 medium containing 4 mg/ml BSA for 144 h at 38.5 °C in 5% CO₂ in air.

Measurement of intracellular calcium

Measurement of intracellular Ca²⁺ rise in porcine oocytes was performed according to the method described by Amano (2004) with slight modification. The porcine oocytes were incubated in M2 medium containing 2 μ M Fura-2 AM (Dojindo, Kumamoto, Japan) at 39 °C for 30 min. The oocytes loaded with Fura-2 AM were transferred into M2 medium and set on the stage of an inverted microscope (NIKON Tokyo, Japan) heated at 39 °C. The measurement of intracellular Ca²⁺ level in porcine oocytes was carried out for an hour after the injection of porcine PLC ζ cRNA.

Argus 50 software program (Hamamatsu Photonics version 3.5 Shizuoka, Japan) was used for the recording of emission fluorescence at a wavelength of 510 nm to evaluate intracellular Ca²⁺. When Fura-2 AM couples Ca²⁺, the emission fluorescence at 510 nm is intensified by exposure to u.v. light at 340 nm (F340) but is not changed to u.v. light by exposure at 380 nm (F380). Intracellular calcium levels were evaluated from the F340/F380 ratio (R).

Results

Identification of porcine PLC ζ

Porcine PLC ζ cDNA was isolated by RT-PCR using the oligonucleotide primers designed with reference to the nucleotide sequences of mouse and human PLC ζ cDNA (Cox *et al.* 2002, Saunders *et al.* 2002). The determined nucleotide sequence of porcine PLC ζ cDNA had single ORF of 1911 bp encoding 636 amino acids of protein (accession number AB113581). The deduced amino acid sequence of porcine PLC ζ protein was compared to those of human, monkey, mouse, and chicken PLC ζ proteins using ClustalW (Fig. 1). The percentages of homology in the amino acid sequence of porcine PLC ζ protein to those of human, monkey, mouse and chicken were 80, 78, 71, and 57% respectively.

In the multiple sequence alignment with PLC δ 1, porcine PLC ζ protein as well as human, monkey, mouse and chicken PLC ζ lacked a plextrin-homology (PH) domain at the N-terminus, which is found in all other proteins of the PLC family (Fig. 2). On the other hand, other important domains such as the EF-hand region, X and Y catalytic domains and C2 domain were conserved in the porcine PLC ζ protein.

Pig	1	-----MENKWFLSMVRDDFKGGKINLEKAQKLEKLDIQCNTI
Human	1	-----MEMRWFLSKIQDDFRGGKINLEKTQRLLEKLDIRCSYI
Monkey	1	-----MEMKWFLSKIQDDFRGGKINLEKTQRLLEKLDIRCSYI
Mouse	1	-----MESQLHELAEARWFLSKVQDDFRGGKINVEITHKLEKLDPCFHA
Chicken	1	-----MAGMQEDLSLQDQGLRDGFMNGKIDFDSTVKLEKLEHMPFNLA
Pig	39	HVKCIFKD-NDRLKQGRITIEEFRTIYRIIAHREIEIEIFNAYPENRKILFERNLIDFLT
Human	39	HVKQIFKD-NDRLKQGRITIEEFRAIYRIITHREEIIEIFNTYSENRRKILLASNLACFLT
Monkey	39	HVKRIFKD-NDRLKQGRITIEEFRAIYRIILTHREEIIEIFNAYSENRRKILLNNLVQFLT
Mouse	47	HVKHIFKE-NDRNQGRITIEEFRAIYRCIVHREIEIEIFNTYTENRKILSENSLIEFLT
Chicken	44	HVKHVFKKTVDKRKIHTINIEFRAIYRAIVHRNEFIEIFCAYSENRRKNLADLTATFLK
Pig	98	QEQYSLDINRSIVYEITOKYEPIEEVKQAHQMSFEGFTIDMCSSECLLFNNECGSVYQDM
Human	98	QEQYAAEMSKATAFEITOKYEPIEEVRKAHQMSLEGFTIRYMDSRECLLFKNECRKVYQDM
Monkey	98	QEQYTTEMSKTIAFEITOKYEPIEEVRKARQMSLEGFTIRYMDSRECLLFKNECRKVYQDM
Mouse	106	QEQYEMEIDHSDSVEITNKYEPIEEVKGERQMSLEGFTIRYMDSRECLLFKNECKTVYQDM
Chicken	104	KEQFKTEGAETTALEVTOKYEPIEEVRRKRQMSLEGFTIRYMSSEDCITFKKEHRTVYQDM
Pig	158	THPLSDYFISSSHNTYILSDQILGPNLWGYVSALVKGCRCLEIDCDWGSQNEFVVYHGY
Human	158	THPLNDYFISSSHNTYILSDQILGPNLWGYVSALVKGCRCLEIDCDWGAQNEFVVYHGY
Monkey	158	THPLNDYFISSSHNTYILSDQILGPNLWGYVSALVKGCRCLEIDCDWGAQNEFVVYHGY
Mouse	166	NHPLSDYFISSSHNTYILSDQILGPNLWGYVSALVKGCRCLEIDCDWGSQNEFVVYHGY
Chicken	164	NHPLCDYFISSSHNTYILSDQILGPNLWGYVSALVKGCRCLEIDCDWGSQNEFVVYHGY
Pig	218	TLTSKLLFKTVIQAIHKYAFMITSDYPVVLSENHCSSLQOEVMADNLQSVFGDALLS-DV
Human	218	TLTSKLLFKTVIQAIHKYAFMITSDYPVVLSENHCSSLQOEVMADNLQATFGESLLS-DM
Monkey	218	TLTSKLLFKTVIQAIHKYAFMITSDYPVVLSENHCSSLQOEVMADNLQATFGESLLS-DM
Mouse	226	TLTSKLLFKTVQAIHKYAFVITSDYPVVLSENHCSSLQOEVMASILQSTFGESLLS-DM
Chicken	224	TLTSKILFCSVTHVVDKYAFAASDYPVVLSENHCSSLQOEVMASILQSTFGESLLS-DM
Pig	277	LDDCPDRLLPSPEALKFKLLVKNKKIGTLKETHERKGSFKHGVQVECEEEEE--BAEQEEE
Human	277	LDDFPDRLPSPEALKFKLLVKNKKIGTLKETHERKGSFKHGVQVECEEEEE--BAEQEEE
Monkey	277	LADFPDRLPSPEALKFKLLVKNKKIGTLKETHERKGSFKHGVQVECEEEEE--BAEQEEE
Mouse	285	LEEFPDRLPSPEALKFKLLVKNKKIGTLKETHERKGSFKHGVQVECEEEEE--BAEQEEE
Chicken	284	GDIEVTLPSPEALKFKLLVKNKKIGTLKETHERKGSFKHGVQVECEEEEE--BAEQEEE
Pig	334	ENEVRDSHILDILQDDLEKEE-LKRGVGIKFFKKK---KVKIATALSDLVIYTKVEKFRS
Human	318	-----DNQDKETGVKKLPGVMLFKKKKTRKLLKIALALSDLVIYTKAEKFKS
Monkey	337	EKFKESEIFESVLGENQDKETGVKKLPGVMLFKKKKTRKLLKIALALSDLVIYTKAEKFKS
Mouse	341	DSGMDPETWVDFLSRIKEERADPSTLSGIAGVKKRKKMKIAMALSDLVIYTKAEKFRN
Chicken	336	SSDEETDEKTPLYPKSGSSKRKSEGRSSPPPRKKAKVKMKIAMALSDLVIYTKSEKFS
Pig	390	FHYSRLYQOFNEINSIGETQARKLSKLRASEFTLHTRKFITRIYPKATRADSSNFPNQEF
Human	364	FOHSRLYQOFNEINSIGETQARKLSKLRASEFTLHTRKFITRIYPKATRADSSNFPNQEF
Monkey	397	FOHSRLYQOFNEINSIGETQARKLSKLRASEFTLHTRKFITRIYPKATRADSSNFPNQEF
Mouse	401	FQYSRLYQOFNEINSIGESRARKLSKLRASEFTLHTRKFITRIYPKATRADSSNFPNQEF
Chicken	396	FEHSLAHCKCYENNSIGELKAKQFVKHAANQFVSHTSRFTIRIYPKATRADSSNFPNQEF
Pig	450	WNIGCOMVALNFOTPGIPMDLQNGKFLNNGSGYILKPHFLRDGKSIFFNPKNAPINSNFI
Human	424	WNIGCOMVALNFOTPGIPMDLQNGKFLNNGSGYILKPHFLRESKSYFNPSNIKEG-MPI
Monkey	457	WNIGCOMVALNFOTPGIPMDLQNGKFLNNGSGYILKPHFLRESKSYFNPSDIKDS-MPI
Mouse	461	WNIGCOMVALNFOTPGIPMDLQNGKFLNNGSGYILKPHFLRESKSYFNPSDIKDS-MPI
Chicken	456	WNIGCOMVALNFOTPGIPMDLQNGKFLNNGSGYILKPHFLRESKSYFNPSDIKDS-MPI
Pig	510	TLTIRLISGIQLPPSYHSSSNKADTLVIEIEFGVPNDQMKQQTTRVIKKNAFSPRWNETFT
Human	483	TLTIRLISGIQLPLTHSSS-NKGDSTLVIEIEFGVPNDQMKQQTTRVIKKNAFSPRWNETFT
Monkey	516	TLTIRLISGIQLPLTHSSS-NKGDSTLVIEIEFGVPNDQMKQQTTRVIKKNAFSPRWNETFT
Mouse	521	TLTIRLISGIQLPVSSSSN--TPDIVLIEIEFGVPNDQMKQQTTRVIKKNAFSPRWNETFT
Chicken	516	SLTIRLISGHQLPPSNLSKSNKADPLVQLEIYGVPEQAKRKSSVIKSNALSPRWNETFT
Pig	570	FIIQVPELALIRFVVENQG-LITGNEFLGOYTLPLVLCMNKGYRRVPLFSKMGESLEPASL
Human	542	FIIHVPELALIRFVVEGQG-LIAGNEFLGOYTLPLVLCMNKGYRRVPLFSKMGESLEPASL
Monkey	575	FIIHVPELALIRFVVESQG-LIAGNEFLGOYTLPLVLCMNKGYRRVPLFSKMGESLEPASL
Mouse	579	FLIQVPELALIRFVVEQQLLSGNEFLGOYTLPLVLCMNKGYRRVPLFSKMGESLEPASL
Chicken	576	FTVQVPELALIRFCVQDEISLVAN-DELGOYTLPLVLCMNKGYRRVPLFSKMGESLEPASL
Pig	629	FVYVWYIR-
Human	601	FVYVWYVR-
Monkey	634	FVYVWYVR-
Mouse	639	FVYVWYFRE
Chicken	635	FVYVWYFY--

Figure 1 Comparison of the deduced amino acid sequences and the domain structures of porcine, human, monkey, mouse, and chicken PLC ζ . Alignment with the amino acid sequences of porcine (accession number AB113581), human (AF532185), monkey (AB070108), mouse (AF435950), and chicken (XM_416413) PLC ζ was performed using ClustalW. Conserved amino acids in all species are indicated by black shading. Identical amino acids over two species are shown in gray shading.

Figure 2 Schematic diagram of the domain structures of porcine, human, monkey, mouse, and chicken PLC ζ and also mouse PLC δ 1. aa, amino acid.

Tissue specificity and developmental features of porcine and mouse PLC ζ mRNA expression

Till date, expressions of PLC ζ mRNA and protein have been examined in only a few kinds of tissues from the mouse, human, monkey, and chicken (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). Therefore, in this study, the expression of PLC ζ mRNA was investigated in several tissues of a Landrace porcine breed and also an ICR mouse strain by RT-PCR, as shown in Fig. 3. A highly specific signal of PLC ζ mRNA was detected in the testis but not in any other tissues for both the pig and mouse.

In order to investigate the correlation between PLC ζ mRNA expression and spermatogenesis in the pig, we first ascertained the mRNA expression of c-kit tyrosine kinase receptor as a control by RT-PCR (Fig. 4A). It has been reported that a dominant white phenotype in the domestic pig is associated with *KIT* mutation (Marklund *et al.* 1998). This mutation includes a duplication of the *KIT* gene and a further G-to-A substitution at a duplicated gene in the first nucleotide of intron 17, which causes a skip of exon 17 as a splicing error. In the present study, the forward and reverse primers used for c-kit mRNA

Figure 3 PLC ζ mRNA expressions of several tissues in the pig and mouse. Porcine (A) and mouse (B) PLC ζ mRNA signals were detected only in the testis as 847 and 834 bp products respectively, by RT-PCR using oligonucleotide primers described in 'Materials and Methods'. β -Actin mRNA expressions of the pig and mouse were also examined as controls and observed as 528 and 578 bp RT-PCR products respectively, in all tissues examined. Abbreviations: B, brain; Lu, lung; H, heart; L, liver; K, kidney; P, pancreas; S, spleen; St, stomach; Si, small intestine; Li, large intestine; M, muscle; Sk, skin; O, ovary; and T, testis.

Figure 4 PLC ζ mRNA expressions during testicular postnatal development in the pig and mouse. Porcine (A) and mouse (B) PLC ζ mRNA signals during postnatal development were detected as 847 and 834 bp products respectively, by RT-PCR against total RNA extracted from the testes. Primers used in this experiment are described in 'Materials and Methods'. In the pig, c-kit expression was observed as a control and both 432 and 301 bp RT-PCR products were detected. β -Actin mRNA expressions in the mouse and pig were also examined as other controls. Mean days after birth given by figure above lane; A, adult.

expression by RT-PCR were designed in exons 16 and 19 respectively. Since the testes used in this study were from Landrace boars with a dominant white coat color, two bands were detected by RT-PCR using these primers (Fig. 4A). The signal of c-kit mRNA was detected throughout the period of postnatal development of the testis. Subsequently, PLC ζ mRNA expression was investigated during the period of postnatal development of the testis in both pig and mouse, as shown in Fig. 4A and B. The signal of porcine PLC ζ mRNA was not detected up to day 75 after birth and was clearly observed from day 96. On the other hand, the signal of mouse PLC ζ mRNA was not found up to day 18 after birth and was clearly observed from day 22.

The spermatogenesis in the pig has not been analyzed in detail, and we therefore performed histological examination of the testis throughout the period of postnatal development (Fig. 5A and B). Testes before day 96 after birth were immature with respect to the diameters of seminiferous tubules and the numbers of germ cells, and there were no spermatozoa (Fig. 5A and B). However, in the testes from day 110, both the diameters of the seminiferous tubules and the numbers of germ cells remarkably increased, and many spermatozoa were observed (Fig. 5C and D).

Repetitive calcium transients in porcine oocytes injected with porcine PLC ζ cRNA

The microinjection of mouse PLC ζ cRNA into the mouse oocytes has been reported to trigger Ca^{2+} oscillations

Figure 5 Histological sections of porcine testes at days (A) 75, (B) 96, (C) 110, and (D) 217 after birth. Spermatozoa were visible in the testes from day 110 and day 217. Scale bars indicate 50 µm in all pictures. SP, spermatozoa.

similar to that observed during fertilization (Saunders *et al.* 2002). We investigated whether porcine PLC ζ has the ability to initiate repetitive Ca^{2+} transients by means of the injection of porcine PLC ζ cRNA into porcine oocytes. A typical example of Ca^{2+} oscillations in porcine oocytes into, which three different concentrations of porcine PLC ζ cRNA were injected, is shown in Fig. 6. The frequency of repetitive Ca^{2+} transients in porcine oocytes injected with 4 pl of 2000 µg/ml porcine PLC ζ cRNA was higher than the frequencies of repetitive Ca^{2+} transients in porcine oocytes injected with 200 and 20 µg/ml porcine PLC ζ cRNA. In addition, the mean interval between Ca^{2+} transients in porcine oocytes injected with 2000 µg/ml (294.4 ± 15.6 s) was shorter than the intervals of Ca^{2+} transients in porcine oocytes injected with 200 (471.9 ± 26.8 s) and 20 µg/ml porcine PLC ζ cRNA (932.1 ± 66.2 s).

In vitro development of porcine oocytes injected with porcine PLC ζ cRNA

The ability of porcine PLC ζ protein to trigger oocyte activation would be identified by the development after injection of its cRNA into porcine oocytes. In the present study, the concentration of approximately 4 pl of 20 µg/ml porcine PLC ζ cRNA was used for microinjection into porcine oocytes, since the mean interval between Ca^{2+} transients in porcine oocytes injected with 20 µg/ml porcine PLC ζ cRNA was similar to that between porcine sperm-induced Ca^{2+} transients reported by Machaty *et al.* (1997), and also the same concentration of mouse PLC ζ cRNA microinjected into mouse oocytes, which is equivalent to the content of PLC ζ protein in a single sperm, which was reported to initiate Ca^{2+} oscillation similar to that observed during fertilization (Saunders *et al.* 2002). As shown

Figure 6 Repetitive calcium transients in porcine oocytes microinjected with different concentrations of porcine PLC ζ cRNA. Porcine oocytes were injected with 4 pl of (A) 2000, (B) 200, and (C) 20 µg/ml (C) PLC ζ cRNA and (D) buffer.

in Fig. 7, porcine oocytes injected with porcine PLC ζ cRNA showed higher developmental profiles, since 47.3% (52/110) of the oocytes developed to the cleavage stage and 16.4% (18/110) progressed to the blastocyst stage. These percentages were almost the same as those for oocytes derived from *in vitro* fertilization; 44.3% (51/115) to the cleavage stage and 20.0% (23/115) to the blastocyst stage.

Figure 7 Embryonic development of porcine oocytes injected with porcine PLC ζ cRNA. Porcine oocytes were microinjected with 4 pl of 20 μ g/ml porcine PLC ζ cRNA and cultured *in vitro* for 144 h at 38.5 °C in 5% CO₂ in air. The percentage of oocytes that developed to the two-cell stage at 48 h (■) and the blastocyst stage at 144 h (□) were calculated.

Discussion

A sperm-specific PLC isoform, PLC ζ , has been identified in the mouse, human, monkey, and chicken (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). Mouse oocytes into which PLC ζ had been injected acquired the ability to trigger Ca²⁺ oscillation and initiate embryonic development up to the blastocyst stage (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). Therefore, PLC ζ has been suggested to be the strongest candidate as a sperm factor that initiates oocyte activation and early embryonic development. In the present study, porcine PLC ζ cDNA was identified and the nucleotide sequence was determined. The deduced amino acid sequence of porcine PLC ζ was shown to have an EF-hand region, X and Y catalytic domains and a C2 domain, but lack of a PH domain that binds to phosphoinositides in the plasma membrane (Katan 1998, Rebecchi & Pentyla 2000, Saunders *et al.* 2002). Other members of the PLC family such as PLC β , γ , and δ have been shown to have a PH domain at the N-terminus.

PLC ζ mRNA in the pig as well as that in the mouse was expressed in the testis but not in other tissues. Mouse spermatogenesis is known to be initiated just after birth (Bellve *et al.* 1977, Rhee & Wolgemuth 1995). Then germ cells in the testis at day 7 after birth become mitotic spermatogonia, enter meiosis, and develop into spermatocytes at day 17 (Nebel *et al.* 1961). On day 20, round spermatids are observed, and thereafter differentiate into

elongated spermatids. Spermatozoa are found in the testis from day 35. It has been reported that the mouse elongated spermatids have the ability to initiate oocyte activation when they are microinjected into the mouse oocytes, but the round spermatids do not have such ability (Kimura & Yanagimachi 1995). In the present study, mouse PLC ζ mRNA was not expressed in the testis up to day 18 after birth but from day 22. Therefore, it has been demonstrated that the stage when spermatids acquire the ability to trigger Ca²⁺ oscillation and initiate embryonic development as a sperm factor, and the stage when a signal of PLC ζ mRNA is detected in the testis are the same in the mouse.

The porcine spermatogenesis during the postnatal development is not well understood because of the limited number of studies (Kosco *et al.* 1989, Franca *et al.* 2000, Goddard *et al.* 2001). *c-kit* is known to be a proto-oncogene encoding a tyrosine kinase receptor in the PDGF/CSF-1 receptor family (Yarden *et al.* 1987, Qiu *et al.* 1988). In the mouse, since the expression of *c-kit* protein is found in spermatogonia, its signal is observed in all the stages throughout the period of testicular postnatal development (Manova *et al.* 1990, 1993, Manova & Bachvarova 1991, Sorrentino *et al.* 1991, Rossi *et al.* 1992). In the pig, the expression of *c-kit* protein has also been detected in spermatogonia (Goddard *et al.* 2001). Therefore, the signal of porcine *c-kit* mRNA was utilized as a control in the present study, and was observed in the testes at all stages from day 13 to adult stage. Porcine male germ cells have been reported to proliferate continuously in seminiferous tubules during postnatal stages (Kosco *et al.* 1989, Franca *et al.* 2000). The diameter of seminiferous tubules and number of spermatogenic cells have been shown to increase gradually from day 94 to 122, and the onset of spermiogenesis has been observed at day 94 (Kosco *et al.* 1989). In the present study, histological examination of porcine testes throughout the period of postnatal development indicated that the diameter of seminiferous tubules and number of germ cells increased from day 96 to 110, and spermatozoa were observed in the testes at day 110. In addition, the signal of porcine PLC ζ mRNA was detected in the testes from day 96. These results suggest that the gene expression of porcine PLC ζ starts in elongated spermatids when spermiogenesis is initiated just after day 96.

Microinjection of porcine PLC ζ cRNA into porcine oocytes revealed that the porcine PLC ζ has the ability to trigger repetitive Ca²⁺ transients in oocytes. In the present study, the frequency of Ca²⁺ transients in porcine oocytes injected with 4 pl of 20 μ g/ml porcine PLC ζ cRNA was similar to the frequency of porcine sperm-induced Ca²⁺ transients reported by Machaty *et al.* (1997). Furthermore, microinjection of 4 pl of 20 μ g/ml porcine PLC ζ cRNA into porcine oocytes was able to induce oocyte activation and initiate embryonic development up to the blastocyst stage. The amounts of injected PLC ζ cRNA needed to induce oocyte activation

have been reported to be different in the mouse, human, monkey, and chicken (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). In the mouse, the concentration of 3–6 pl of 20 µg/ml PLCζ cRNA used for microinjection into oocytes corresponded to 44–75 fg of PLCζ protein, which is almost the same content of PLCζ contained in a single sperm (Saunders *et al.* 2002). On the other hand, the amount of human PLCζ cRNA that is needed for the injection to cause activation of mouse oocytes was smaller than the amounts of mouse and monkey PLCζ cRNA (Cox *et al.* 2002, Rogers *et al.* 2004). In the present study, the concentration of porcine PLCζ cRNA injected into porcine oocytes is the same as that of mouse PLCζ injected into mouse oocytes. Recently, Kurokawa *et al.* (2005) reported that a single porcine sperm contains upwards of 350 fg of porcine PLCζ protein. It is necessary in the future study to determine the minimum concentration of porcine PLCζ protein that can trigger oocyte activation and induce embryonic development.

The *in vitro* developmental capacity of porcine *in vitro* fertilized embryos is known to be much poorer than that of the embryos derived from other domestic animals (Abeydeera 2001). Furthermore, in the pig, a high frequency of polyspermy has been found in *in vitro*-fertilized oocytes (Funahashi & Day 1997). The utilization of porcine PLCζ would be useful in study of the protection against polyspermy during *in vitro* fertilization and the improvement of early embryonic development in porcine oocytes.

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