Species-Specific Differences in the Activity and Nuclear Localization of Murine and Bovine Phospholipase C Zeta 1[1]

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ABSTRACT

Injection of mammalian sperm extracts or cRNA of the sperm-specific phospholipase C zeta 1 (*PLCZ1*) has been shown to trigger repetitive oscillations in the concentration of free calcium ($[Ca^{2+}]_i$), leading to oocyte activation and embryo development in all mammals studied to date. While PLCZ1 has cross-species activity, it has also been observed that species-specific differences may exist in the frequency and pattern of the resulting $[Ca^{2+}]_i$ oscillations following *PLCZ1* cRNA injection into oocytes of different species. Accordingly, we used a crossover design strategy to directly investigate the activity of murine and bovine PLCZ1 in both murine and bovine oocytes. In murine oocytes, injection of murine *Plcz1* cRNA induced $[Ca^{2+}]_i$ oscillations at 10-fold lower concentrations than bovine *PLCZ1*, although in bovine oocytes bovine *PLCZ1* was more effective than murine *Plcz1* at inducing $[Ca^{2+}]_i$ oscillations. Investigation of ITPR1 (IP_3R1) down-regulation in bovine oocytes by *PLCZ1* cRNA also showed that bovine *PLCZ1* was more active in homologous oocytes. To determine whether these PLCZs exhibited similar cellular distribution, Venus-tagged *PLCZ1* cRNA was injected into oocytes, and PLCZ1 was overexpressed. Bovine PLCZ1 failed to accumulate in the pronucleus (PN) of bovine or murine zygotes, despite possessing a putative nuclear localization signal. Conversely, murine PLCZ1 accumulated in the PN of both murine and bovine zygotes. These results demonstrate that murine PLCZ1 and bovine PLCZ1 possess species-specific differences in activity and suggest potential differences in the mode of action of the protein between the two species. Variation in sperm PLCZ1 protein content among species, along with oocyte-specific differences in the localization and availability of PLCZ1 substrates, may further contribute to optimize the activation stimulus to enhance embryo development.

activation, calcium, fertilization, nuclear localization, ovum, phospholipase C zeta, species difference, sperm

INTRODUCTION

Phospholipase C zeta 1 (*Plcz1*) was first identified in mouse testis expressed sequence tags and sperm extracts [1] and has subsequently been shown to be capable of inducing free calcium ($[Ca^{2+}]_i$) oscillations similar to those seen at fertilization. As reviewed by Schultz and Kopf [2], these $[Ca^{2+}]_i$ oscillations are believed to be responsible for inducing all events of egg activation and the initiation of embryo development. Therefore, PLCZ1 is thought to represent the long sought-after sperm factor. As reviewed by Swann et al. [3], highly homologous *PLCZ1* sequences have now been identified in all mammalian species studied to date, including the human, monkey, rat, pig, cow, and chicken.

Like other PLC family members [4], PLCZ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol [5, 6]. Therefore, during mammalian fertilization, PLCZ1 drives the production of IP_3 and binding of IP_3 to its receptor, which is located on the endoplasmic reticulum (ER), the main Ca^{2+} reservoir of the cell. Binding of IP_3 promotes Ca^{2+} release from the ER and triggers $[Ca^{2+}]_i$ oscillations through a currently unknown mechanism [7]. Of the three known isoforms, mammalian oocytes express predominantly the type 1 IP_3 receptor ITPR1 (also known as IP_3R1) [8, 9], and bovine oocytes contain (on a volume-to-volume basis) approximately twice the receptor mass of mouse oocytes [10]. During oocyte maturation, the concentration of ITPR1 increases, and its cellular distribution changes to a largely cortical localization [11, 12]. These changes lead to the enhanced sensitivity of ITPR1 in MII oocytes, the stage at which fertilization takes place [13, 14]. During fertilization and due to the prolonged production of IP_3, ITPR1 undergoes progressive down-regulation, which is mediated by ubiquitination and proteasomal processing [15]. This receptor degradation results in the desensitization of the Ca^{2+} release system, which contributes to the eventual termination of the $[Ca^{2+}]_i$ oscillations [8, 16]. The importance of ITPR1-mediated Ca^{2+} release in the initiation of mammalian development is well established, as inhibition of ITPR1 function by injection of a blocking antibody leads to termination of $[Ca^{2+}]_i$ oscillations and prevents activation of mouse and hamster oocytes [17, 18].

Key to the identification of the molecular nature of the sperm factor was the ability of sperm extracts to induce $[Ca^{2+}]_i$ oscillations in a variety of oocytes from mammalian and nonmammalian species [19, 20]. This is consistent with findings that PLCZs from different species show widespread activity in mammalian oocytes. For example, injections of human, monkey, and chicken *PLCZ1* cRNAs have all been

shown to trigger $[Ca^{2+}]_i$ oscillations in mouse oocytes [21, 22]. Interestingly, despite this conserved ability to induce oscillations, the specific activity of PLCZs appears to be different among species. For example, lower concentrations of human than murine or simian *PLCZ1* cRNAs are required to initiate comparable oscillations in murine oocytes [21, 23]. This difference was also observed following injection of murine *Plcz1* into human oocytes [23]. Similarly, it appears that different concentrations of murine *Plcz1* cRNA and bovine *PLCZ1* cRNA are required to activate bovine oocytes, as lower concentrations of homologous cRNA were more effective at initiating $[Ca^{2+}]_i$ oscillations [24]. Nevertheless, it was not examined whether the cRNAs were translated at the same rate or whether the differences in activity were maintained when examined in murine oocytes. Besides the possible differences in specific activity, each species also appears to differ in the distinct amounts of PLCZ1 within the sperm, with porcine sperm reportedly containing approximately 7-fold greater amounts of PLCZ1 than mouse sperm [25]. Nevertheless, the precise concentration of PLCZ1 per sperm has thus far only been closely investigated in the mouse [1, 26], possibly due to the difficulty in producing abundant amounts of recombinant protein and due to the limited availability of species-specific antibodies.

Sequence analysis of PLCZ1 from all species studied to date demonstrates conservation of the characteristic PLC family domains, including the presence of a putative nuclear localization signal (NLS) in the linker region between the X and Y catalytic domains. Although different in length and precise sequence, the NLS among various species, including the bovine, shows overall conservation of a cluster of basic amino acids that are characteristic of a functional NLS [27, 28]. Consistent with this, in the mouse at 5–6 h after cRNA injection, the PLCZ1 protein was shown to accumulate within the formed pronucleus (PN) [29, 30, 31]. Given that in the mouse $[Ca^{2+}]_i$ oscillations cease around the time of PN formation, it was suggested that PLCZ1 sequestration may regulate the observed cell cycle entrainment of $[Ca^{2+}]_i$ oscillations in this species [12, 32, 33]. However, recent investigations using *PLCZ1* cRNAs from human, rat, and medaka fish suggest that PLCZs of these species do not share the ability of murine PLCZ1 to translocate to the PN [28]. Therefore, it remains unclear whether nuclear translocation of PLCZ1 in zygotes is a widespread phenomenon in mammals and what its physiological importance is.

To address the potential species-specific differences in activity of PLCZ1, this study compares $[Ca^{2+}]_i$ oscillations triggered by injection of bovine *PLCZ1* and mouse *Plcz1* cRNAs into oocytes of both species. We also investigated the ITPR1 down-regulation induced in bovine oocytes by injection of both cRNAs. To quantify the PLCZ1 content of a single bovine sperm, we used a recombinant bovine PLCZ1 protein. Last, to investigate the nuclear translocation ability in the two species, we injected murine and bovine oocytes with murine *Plcz1* and bovine *PLCZ1* cRNAs encoding the yellow fluorescent protein Venus.

MATERIALS AND METHODS

Most chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Other sources are stated in the text.

Animal Ethics

Experimental procedures were approved by the Monash University Animal Ethics Committee for animal experimentation and were conducted in accord with the Australian National Health and Medical Research Council guidelines ''Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'' (7th edition, 2004). Experimental procedures at the University of Massachusetts, Amherst, were performed according to protocols approved by the Institutional Animal Care and Use Committee of the university.

Construction of Venus-Tagged PLCZ1 Constructs

The cDNA encoding *PLCZ1* was amplified by Taq DNA polymerase from bovine testis cDNA synthesized using Superscript III (Invitrogen, Carlsbad, CA). The full-length cDNA sequence (634 amino acids [GenBank accession number AAI4837]) was then cloned into the pGEM Teasy vector (Promega, Madison, WI). The sequence encoding for Venus was a gift from Dr. Atushi Miyawaki (Riken, Japan) to Dr. J. Ito of the Fissore laboratory. This fluorescent protein variant [34] was subcloned into the pcDNA 6.1 vector (Invitrogen, Carlsbad, CA). *Eco*RI restriction sites were inserted by PCR into the noncoding 5′ region of bovine *PLCZ1* using the sense primer 5′-CCGGAATTCAC CATGGAGAACAAATGG-3′, and the stop codon of both was replaced with an *Xho*I restriction site by PCR using the antisense primer 5′-TCCGCTCGA GATTCTGATGTACCAAA-3′. For murine *Plcz1* (kindly provided by Dr. Kiyoko Fukami, Tokyo, Japan), an *Eco*RI restriction site was inserted by PCR into the noncoding 5′ region using the sense primer 5′-CCGGAATTCAC CATGGAAAGCCAACTTCAT-3′, and the stop codon was replaced with an *Xba*I restriction site by PCR using the antisense primer 5′-TGCTCTA GAATCTCTCTGAAGTACCAAAC-3′. The PCR products of each were digested with the respective restriction enzymes and, following purification, were ligated into a precut pcDNA 6.1 vector with cohesive overhangs to generate an inframe 5′-*Plcz1*-Venus-3′ fusion.

Preparation of PLCZ1 cRNA

Vectors containing either the full-length or tagged cDNA sequence of bovine *PLCZ1* and mouse *Plcz1* (GenBank accession numbers AF435950 and BC114836, respectively) were linearized at restriction sites just beyond the 3′ end of the coding sequence, and mRNA was transcribed in vitro from the linearized DNA using the T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions. This reaction results in the incorporation of a 7-methyl guanosine cap at the 5′ end of the mRNA. A poly A tail of greater than 150 bases was then added to the 3′ end of the transcript using the Poly(A) Tailing kit (Ambion). Capped and tailed mRNA was purified using the MEGAclear kit (Ambion), eluted in RNase-free water, and stored at −80°C in single-use aliquots.

His-Tagged Recombinant Bovine PLCZ1 Protein

Recombinant bovine PLCZ1 was produced by GenScript Corp. (Piscataway, NJ) using an *Escherichia coli* expression system. Two forms of recombinant PLCZ1 were produced tagged with either glutathione *S*-transferase (rbPLCZ-GST) or 6xHis (rbPLCZ-His). Using *Nde*I and *Xho*I restriction sites, bovine *PLCZ1* was cloned into the pCOLD IV expression vector (Takara Bio, Shiga, Japan), which contains an N-terminal 6xHis tag sequence. The vector was transformed into the *E. coli* strain Arctic Express (DE3) RP (Stratagene, La Jolla, CA), and expression was induced by 0.5 mM isopropyl-β-D-thiogalactoside overnight. Following affinity purification, the final concentration of the protein was 0.30 mg/ml in 20 mM Tris buffer (pH 8.0). Recombinant protein was stored at −80°C until use. The immunoreactivity of the recombinant protein was tested using an anti-bovine PLCZ1 antibody raised in rabbits against a C-terminal region of the PLCZ1 protein (GYRRVPLFSKMGESLE-PAS).

Collection of Gametes

Murine oocytes were collected from 6- to 8-wk-old superovulated CD-1 female mice (Jackson Labs, Bar Harbor, ME). Ovulation was induced by injection of 5 IU of human chorionic gonadotropin administered 46–48 h after injection of 5 IU of equine chorionic gonadotropin. Metaphase II (MII)-stage oocytes were collected from the oviducts 14–15 h after human chorionic gonadotropin administration in Hepes-buffered Tyrode lactate solution (TL-Hepes) containing 5% fetal calf serum (FCS) (Gibco BRL, Carlsbad, CA). Cumulus cells were removed by brief exposure to 0.1% bovine testis hyaluronidase, followed by washing in TL-Hepes.

Germinal vesicle-stage bovine oocytes were purchased from BOMED (Madison, WI). Oocytes were matured in TCM199 with Earle salts supplemented with 10% FCS (Hyclone, Logan, UT), 0.1 U/ml of luteinizing hormone (Sioux Biochemical, Sioux Center, IA), 1 μg/ml of estradiol (follicle-stimulating hormone [Sioux Biochemical]), 0.22 mM sodium pyruvate, and 25 μg/ml of gentamycin during overnight transportation at 39°C in a portable

TABLE 1. $[Ca^{2+}]_i$ oscillations triggered by *PLCZ1* cRNA injection into bovine oocytes.

PLCZ1 cRNA	Concentration (µg/µl)	No. injected	No. oscillating (%)	Average oscillation frequency[a]
Bovine	0.1	12	1/12 (8)	NA
Bovine	1.0	27	15/27 (55)	40 min
Mouse	0.1	9	1/9 (11)	NA
Mouse	1.0	11	6/11 (55)	45 min

[a] NA, not available.

incubator (Mini Tube of America, Verone, WI). At 22–24 h after maturation, oocytes were denuded of their cumulus cells by vortexing for 3 min in TL-Hepes supplemented with 0.2% hyaluronidase. Oocytes were held in potassium simplex optimized media (KSOM) [35] with 1 mg/ml of bovine serum albumin (BSA) until use.

Microinjections of PLCZ1 cRNA

The cRNA was thawed on ice, centrifuged at $12\,000 \times g$ at 4°C for 10 min, and injected at a final pipette concentration of 1 µg/µl. Microinjection procedures were as previously described [36]. Briefly, glass micropipettes were filled by suction from a microdrop containing cRNA, which was then delivered to the oocytes by pneumatic pressure (PLI-100 Pico-Injector; Harvard Apparatus, Cambridge, MA). Microinjections were performed using Narishige manipulators (Medical Systems Corp., Great Neck, NY) mounted on a Nikon Diaphot microscope (Nikon Inc., Garden City, NY). Before injection, bovine oocytes were centrifuged in TL-Hepes at $10\,000 \times g$ for 8 min to allow for easier visualization of the injection volume. Injection volumes were approximately 1%–5% of the total volume of the oocyte, and all manipulations were carried out at room temperature in drops of TL-Hepes with 5% FCS and 2.5% sucrose. After injection of mRNA, oocytes were returned to KSOM supplemented with 1 mg/ml of BSA at 37°C in 5% CO_2 in air. Injected oocytes were removed from KSOM at various time points for $[Ca^{2+}]_i$ measurement or fluorescent microscopy.

Ca^{2+} Monitoring

Bovine and murine oocytes were injected with the fluorescent dye Fura-2 dextran or loaded with 1 µM Fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) supplemented with 0.02% Pluronic F-127 (Molecular Probes) at room temperature for 20 min. Measurement of $[Ca^{2+}]_i$ was carried out as previously described [37]. Oocytes were placed in 50-µl drops of TL-Hepes on a glass coverslip sealed over an opening made in the bottom of a Petri dish and covered with mineral oil. Oocytes were observed using a 20× objective on a Nikon Diaphot inverted microscope with up to 15 murine oocytes or nine bovine oocytes monitored simultaneously. Fluorescence ratios were taken every 20–30 sec for various time points depending on the experiment and recorded as direct image files in Simple PCI software (Compix Imaging Systems, Sewickley, PA). Time-lapse image files were then transposed to numeric values reported as the ratios of 340:380-nm fluorescence. Files were imported to Microsoft Excel (Redmond, Washington), and representative calcium traces were graphed as a function of F340:F380 over time.

Confocal Microscopy

Confocal images were acquired using the Zeiss (Welwyn Garden City, UK) LSM510 confocal system with differential interference contrast for brightfield images and excitation at 488 nm with an argon laser and image capture through a 515-nm barrier filter for Venus. LSM510 images were saved as TIFF files using the Zeiss image browser, and image contrast was equally adjusted for all images using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Bull Sperm Preparation for Western Blot

Frozen bull spermatozoa were kindly donated by Dr. Marvin Pace (American Breeder Services, DeForest, WI). Live motile mature sperm were isolated by centrifugation at $400 \times g$ through a dual-density discontinuous gradient of 45% and 90% isotonic Percoll and washed twice with warmed Dulbecco PBS. The pellet was diluted in an appropriate concentration and kept at −80°C in 2× sample buffer [38] until use.

Western Blot

To investigate the down-regulation of the ITPR1 protein, bovine oocytes were collected 12 h after microinjection of *PLCZ1* cRNA. Five oocytes were added to 15 µl of 2× sample buffer as described previously [39], and samples were boiled for 3 min to denature the proteins and loaded onto NuPAGE Novex 3%–8% Tris-Acetate gels (Invitrogen). The proteins were separated by electrophoresis, followed by transfer to nitrocellulose membrane (Micron Separations, Westboro, MA). Membranes were blocked in PBS with 0.1% Tween 20 (T-PBS) supplemented with 6% nonfat dry milk for 2 h and incubated at 4°C overnight with Rbt03 polyclonal antibody raised against the C-terminal amino acids 2735–2749 of mouse ITPR1 [40]. Following several washes with T-PBS, a horseradish peroxidase-conjugated goat-anti-rabbit antibody (BioRad, Hercules, CA) was applied at a 1:3000 dilution for 1 h at 4°C. Membranes were washed and incubated for 1 min in chemiluminescence reagent (NEN Life Science Products, Boston, MA) and developed according to the manufacturer's instructions. Membranes were exposed using the Kodak Image Station 440CF (NEN Life Science Products), and images captured using the Kodak 1D Image Analysis software. The mean pixel intensity of the ITPR1 band was quantified using either the Kodak 1D Image Analysis software or Adobe Photoshop and plotted using Microsoft Excel as described previously [16]. The band from the MII control oocytes was used as a reference and was assigned the value of 100%.

To quantify the PLCZ1 content of a single bull sperm, recombinant full-length bovine PLCZ1 (rbPLCZ1) and bull sperm were prepared in various concentrations in 2× sample buffer and denatured at 95°C for 3 min. Samples were separated on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 6% skim milk dissolved in PBS containing 0.1% Tween 20 (PBST) at 4° for 2 h and probed with rabbit polyclonal PLCZ1 antibodies raised against either an N-terminal peptide sequence (MENKWFLSMVRDDFKGG-KI) of porcine PLCZ1 [25] or a C-terminal peptide sequence (GYRRVPLFSKMGESLEPAS) of bovine PLCZ1 at 4°C overnight. After several washes with PBST, the blot was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000; BioRad). Antibody binding was detected using a Kodak Image Station 440CF, and the band intensities were analyzed by Kodak 1D Image Analysis software version 3.5 (Eastman Kodak, Rochester, NY). The amount of PLCZ1 in a single sperm was quantified by standardizing to known concentrations of rbPLCZ1 and comparing with that of particular concentrations of bull sperm (1.0×10^5, 1.5×10^5, and 2.0×10^5).

RESULTS

Injection of Bovine PLCZ1 and Murine Plcz1 cRNAs Triggers $[Ca^{2+}]_i$ Oscillations with Different Efficacy in Bovine and Murine Oocytes

We reported in a previous study [24] that lower concentrations of bovine *PLCZ1* cRNA than murine *Plcz1* cRNA were required to initiate $[Ca^{2+}]_i$ oscillations in bovine oocytes. However, this study was based on a bovine *PLCZ1* sequence that differed from our own by five amino acids and was subsequently retracted from the GenBank database (accession number AY646356). The voluntary withdrawal suggests that possible PCR or sequencing errors were found within the original sequence; however, the replacement sequence in GenBank (accession number Q1RML2) is identical to the consensus sequence of the *PLCZ1* used in the present study (accession number BC114836). In the present study, we confirm and add to these results by performing a complete crossover design and by investigating the protein expression from these different constructs. Our results largely extend the previously published information in that injection of bovine *PLCZ1* cRNA in bovine oocytes initiated higher-frequency oscillations than murine *Plcz1* cRNA (Fig. 1, a–d, and Table 1), although injection of 0.1 µg/µl of either *PLCZ1* cRNA into bovine oocytes failed to initiate ongoing oscillations, unlike what was reported previously [24]. We estimate that these minor differences may relate to smaller volumes delivered by our microinjection procedures, leading to smaller relative intracellular concentrations of the *PLCZ1*. This suggestion is

Bovine oocytes

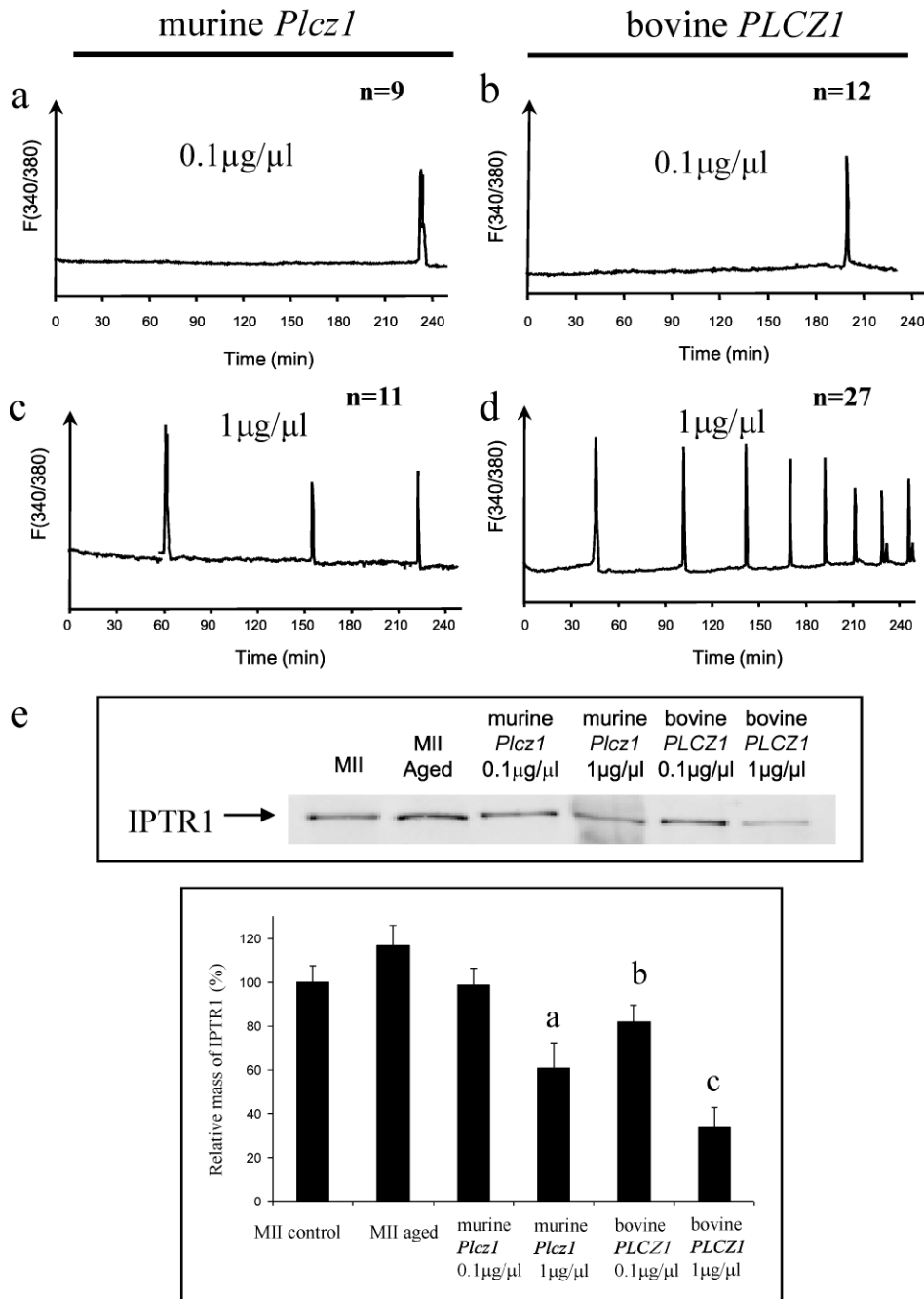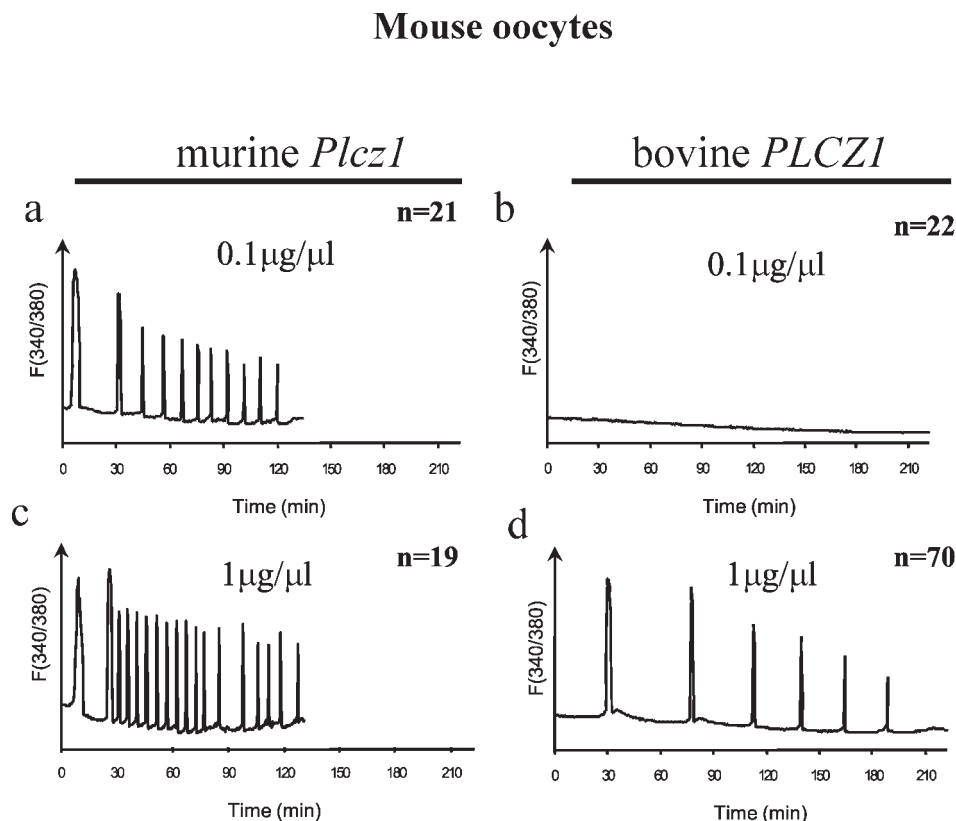### murine *Plcz1*

bovine *PLCZ1*

FIG. 1. Species-specific activity of PLCZ1 in triggering $[Ca^{2+}]_i$ oscillations in bovine oocytes. Representative $[Ca^{2+}]_i$ profiles of bovine oocytes injected with murine *Plcz1* or bovine *PLCZ1* cRNA at 0.1 µg/µl (**a** and **b**) or 1 µg/µl (**c** and **d**). **e**) Groups of five bovine oocytes were analyzed for ITPR1 down-regulation 12 h after injection of mouse *Plcz1* or bovine *PLCZ1* cRNA and expressed as the relative mass of ITPR1 compared with MII oocyte controls. Significantly higher rates of down-regulation were observed in injected oocytes compared with the controls. Levels of ITPR1 were significantly reduced following injection of bovine *PLCZ1* at 1 µg/µl. Error bars on the graph represent the mean ± SEM of four replications. Different letters indicate $P < 0.05$. b, bovine; m, murine.

supported by the finding that, although injection of 1.0 µg/µl of *PLCZ1* cRNAs initiated oscillations, the frequency was considerably lower than that reported by Ross et al. [24].

To further examine whether bovine *PLCZ1* cRNA was indeed more effective in bovine oocytes than murine *Plcz1* cRNA at engaging the signaling pathway that leads to Ca^{2+} release, we investigated their efficacy in inducing ITPR1 degradation. Given that ITPR1 down-regulation requires production and binding of IP_3 to ITPR1, we envisioned this approach as a means to confirm the data obtained from $[Ca^{2+}]_i$ monitoring. Accordingly, bovine oocytes were injected with murine and bovine *PLCZ1* cRNA as before, and ITPR1

degradation was examined by Western blot 12 h after injection (Fig. 1e). While ITPR1 concentrations did not change in noninjected MII control and MII-aged control eggs (which were left in culture for the same period of time), oocytes injected with either *PLCZ1* cRNA showed a dose-dependent down-regulation of ITPR1. Regardless of the concentration, the species of origin influenced the degree of degradation of ITPR1, as bovine *PLCZ1* induced greater down-regulation than murine *Plcz1* (Fig. 1e). Interestingly, bovine *PLCZ1* cRNA injected at 0.1 µg/µl induced marked ITPR1 down-regulation even though it barely managed to initiate $[Ca^{2+}]_i$ responses, suggesting that, while oscillations might have been present,

FIG. 2. Species-specific activity of PLCZ1 in triggering $[Ca^{2+}]_i$ oscillations in mouse oocytes. Representative $[Ca^{2+}]_i$ profiles of mouse oocytes injected with murine *Plcz1* or bovine *PLCZ1* cRNA at 0.1 µg/µl or 1 µg/µl. **a**) At 0.1 µg/µl, murine *Plcz1* triggered fertilization-like oscillations. **b**) Bovine *PLCZ1* at 0.1 µg/µl was not able to initiate $[Ca^{2+}]_i$ release. **c** and **d**) At 1 µg/µl, both *PLCZ1* and *Plcz1* resulted in ongoing $[Ca^{2+}]_i$ oscillations. b, bovine; m, murine.

Mouse oocytes

murine *Plcz1*   bovine *PLCZ1*

they occurred at a very low frequency, escaping our detection during the initial monitoring period. The Western blot showed extraneous bands at approximately 90 kDa and at less than 40 kDa in the group injected with 1 µg/µl of murine *Plcz1*. These bands clearly did not represent the 270-kDa band that corresponds to the ITPR1 and were most likely due to a nonspecific reaction or possible protein degradation of the particular sample. If this was due to protein degradation, these bands represented a small fraction of the total bands and, as such, were not included in the analysis. This was supported by replicate blots that did not show evidence of the extra bands in this particular treatment group (data not shown) but exhibited comparable down-regulation of ITPR1 in response to *PLCZ1*, as in previous investigations [24]. Altogether, our results confirm that in bovine oocytes the homologous *PLCZ1* cRNA is more efficient than the murine *Plcz1* cRNA at initiating $[Ca^{2+}]_i$ responses.

In order to examine whether this preference for the homologous cRNA extended to the murine system, both cRNAs were then injected into murine oocytes, followed by monitoring of the $[Ca^{2+}]_i$ responses. In murine oocytes, injection of 0.1 µg/µl of murine *Plcz1* cRNA induced persistent $[Ca^{2+}]_i$ oscillations (Fig. 2a and Table 2), and injections of 1.0

µg/µl of cRNA induced even higher-frequency oscillations (Fig. 2c and Table 2). These data are in agreement with results from several other publications [40–42]. Remarkably, the oscillations initiated by bovine *PLCZ1* cRNA even when injected at 1.0 µg/µl did not attain the frequency that was observed by injection of 0.1 µg/µl of murine *Plcz1* cRNA (Fig. 2d). The lower concentration of bovine *PLCZ1* cRNA failed to initiate ongoing oscillations in murine oocytes altogether (Fig. 2b). Collectively, these results indicate that murine and bovine oocytes show preference for their homologous PLCZ1 protein.

Quantification of PLCZ1 Content in Bovine Sperm

The presented results suggest that, while PLCZ1 activity is conserved among mammals, it might be more efficient in oocytes of the same species. Nevertheless, research shows that injection of sperm from different mammalian species into murine oocytes initiates highly frequent oscillations and, paradoxically, that injection of heterologous sperm into murine oocytes can demonstrate higher oscillatory activity. For example, injection of a single porcine sperm into murine oocytes initiates oscillations with higher frequency than bovine sperm, which in turn show higher oscillatory activity than murine sperm [38]. Although there are several possible explanations for these results, one possibility is that sperm from different species possess different quantities of PLCZ1. To date, the PLCZ1 content of a single sperm has been examined closely only in the mouse [1, 26]. Therefore, to determine the quantity of PLCZ1 per bovine sperm, we produced affinity-purified recombinant bovine PLCZ1 protein from an *E. coli* expression system and performed Western blot, along with extracts from known numbers of bovine sperm. The identity of the recombinant protein was first confirmed using two different anti-PLCZ1 antibodies, one raised against a C-terminal peptide of bovine PLCZ1 (Fig. 3a) and the other

TABLE 2. $[Ca^{2+}]_i$ oscillations triggered by *PLCZ1* cRNA injection into mouse oocytes.

PLCZ1 cRNA	Concentration (µg/µl)	No. injected	No. oscillating (%)	Average oscillation frequency[a]
Bovine	0.1	22	1/22 (4)	NA
Bovine	1.0	70	50/70 (71)	25 min
Mouse	0.1	21	17/21 (80)	18 min
Mouse	1.0	19	17/19 (89)	8 min

[a] NA, not available.

a

Recombinant bovine PLCZ1   **Bull sperm**

M   0.015µg 0.02µg 0.025µg 1.0×10^5 1.5×10^5 2.0×10^5
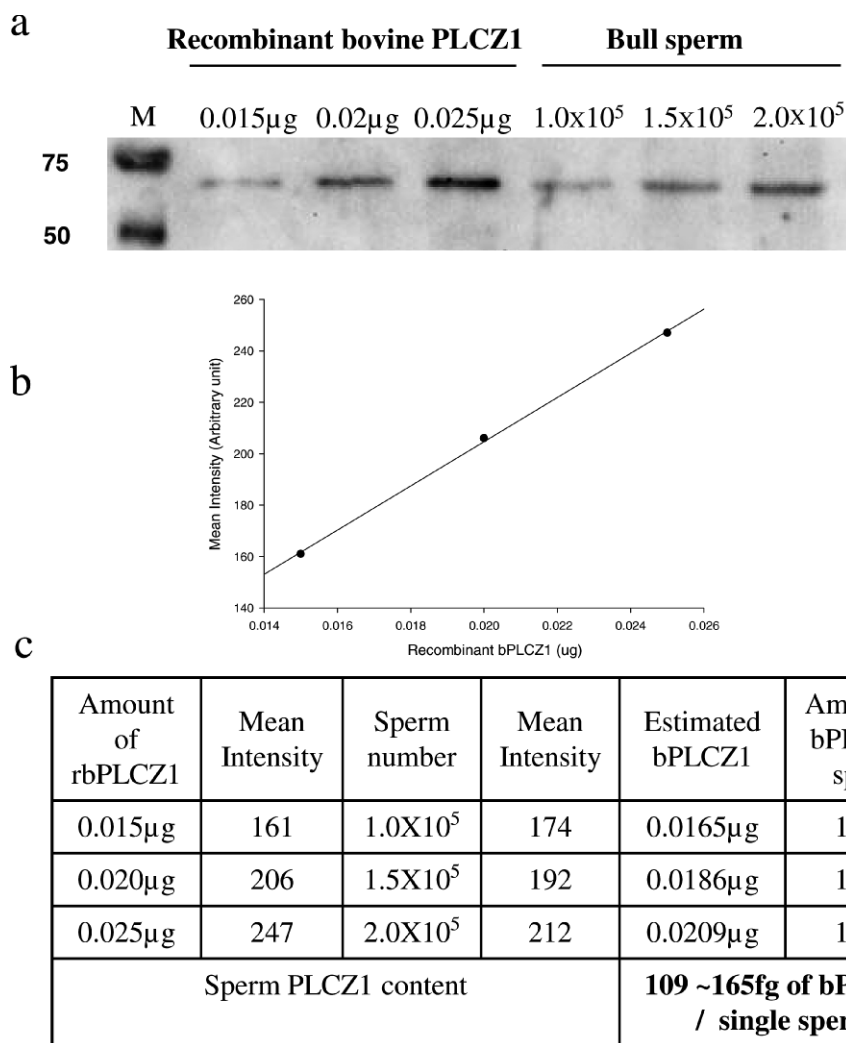
75

50

b

c

FIG. 3. Quantification of PLCZ1 content in a single bovine sperm. Recombinant bovine PLCZ1 protein (rbPLCZ1) was used to quantify the PLCZ1 content of bovine sperm. **a** and **b**) Western blot analysis of rbPLCZ1 using an anti-PLCZ1 antibody resulted in relative band intensities showing strong correlation to the concentrations of protein used. **c**) By plotting known concentrations of bovine sperm, the PLCZ1 content of a single bovine sperm was estimated to be 105–165 pg. b, bovine; M, molecular weight.

Amount of rbPLCZ1	Mean Intensity	Sperm number	Mean Intensity	Estimated bPLCZ1	Amount of bPLCZ1 / sperm
0.015µg	161	1.0×10^5	174	0.0165µg	165fg
0.020µg	206	1.5×10^5	192	0.0186µg	124fg
0.025µg	247	2.0×10^5	212	0.0209µg	109fg
Sperm PLCZ1 content			**109 ~165fg of bPLCZ1 / single sperm**		

raised against an N-terminal peptide from the porcine PLCZ1 sequence (data not shown); both antibodies recognized a band of approximately 72 kDa, which corresponds to the expected molecular weight of bovine PLCZ1. Different concentrations of the recombinant protein were then run on a 10% acrylamide gel, and the relative intensity of each concentration was evaluated and used to construct a standard curve (Fig. 3b). The standard curve was used to estimate the quantity of PLCZ1 in the adjacent bovine sperm samples (Fig. 3c). Our estimations, based on the comparisons of their relative mean intensities, reveal the PLCZ1 content of a single bovine sperm ranges from 105 to 165 fg, a content that is approximately 3–5-fold greater than the concentration of PLCZ1 present per murine sperm [1, 26].

To determine whether recombinant bovine PLCZ1 was able to induce $[Ca^{2+}]_i$ oscillations, we investigated the $[Ca^{2+}]_i$ responses induced by injection of the recombinant protein into bovine oocytes. Given that the concentrations of recombinant PLCZ1 protein required to induce $[Ca^{2+}]_i$ oscillations in murine oocytes are reportedly greater than the amount of PLCZ1 present in a single murine sperm, we first injected amounts of recombinant bovine PLCZ1 that exceeded those present in a single bovine sperm. Surprisingly, despite injecting 1 pg and 10 pg of recombinant bovine PLCZ1 protein, which represent approximately 10–100-fold the amount of protein per sperm, $[Ca^{2+}]_i$ oscillations were not observed in any bovine

oocyte (zero of nine and zero of three, respectively). Similarly, nine of nine mouse oocytes injected with 10 pg of bovine PLCZ1 failed to show $[Ca^{2+}]_i$ responses. Therefore, although recombinant bovine PLCZ1 was useful to ascertain the concentration of protein per bovine sperm, it was functionally inactive.

Nuclear Localization Ability of Bovine PLCZ1

Previous studies [29, 30] have shown that mouse PLCZ1 is able to translocate into the PN of one-cell mouse embryos, which is the time when sperm-initiated oscillations cease in this species. This sequestration has therefore been proposed to have a role in the termination of the $[Ca^{2+}]_i$ oscillations. Notably, further research has shown that human and rat PLCZ1 does not seem to translocate to the PN in mouse zygotes, suggesting species-specific differences in the cellular distributions of PLCZ1 [28]. Thus, to determine whether bovine PLCZ1 underwent nuclear translocation, a sequence encoding for the fluorescent protein Venus was added to the 5′ end of the sequence encoding for bovine PLCZ1. Following in vitro transcription, this Venus-*PLCZ1* cRNA (0.5 µg/µl) was injected into bovine and murine oocytes. In addition to monitoring of the cellular distribution of PLCZ1, we used the presence of Venus fluorescence to quantify expression of bovine PLCZ1 in these oocytes. A similarly labeled mouse *Plcz1* cRNA (0.5 µg/µl) was used as a positive control. To

a

Mouse oocytes

(bars at 2hr and 4hr; y-axis: Fluorescence Intensity (Arbitrary Units), 0–40)

■ bovine *PLCZ1*
□ murine *Plcz1*

b

Bovine oocytes

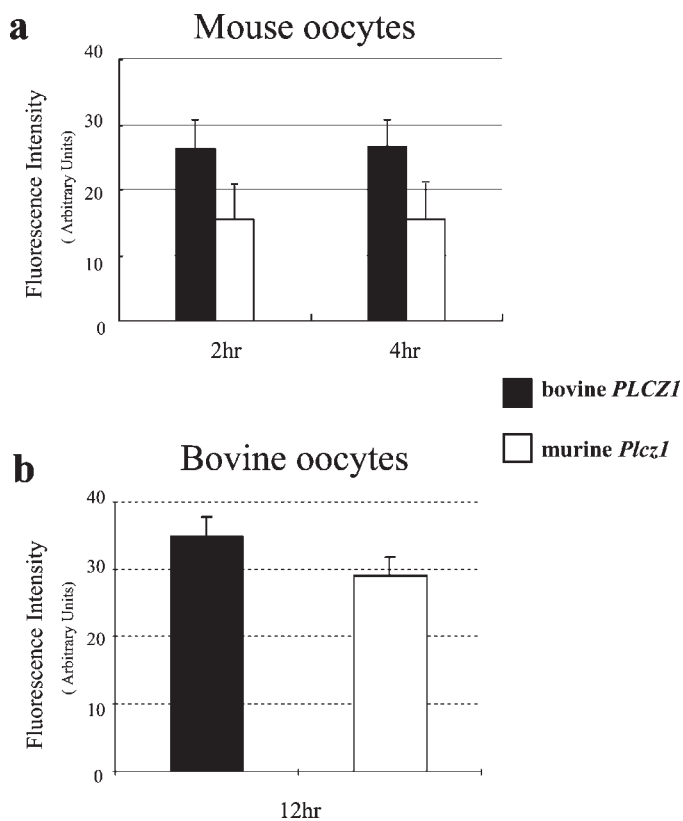(bar at 12hr; y-axis: Fluorescence Intensity (Arbitrary Units), 0–40)

FIG. 4. Quantification of PLCZ1 expression in mouse and bovine oocytes. Venus fluorescence was measured and used to quantify the expression of PLCZ1 protein in mouse (**a**) and bovine (**b**) oocytes injected with *Plcz1* or *PLCZ1* cRNA. Mouse oocytes were imaged at 2 and 4 h after injection and bovine oocytes at 12 h after injection. Error bars represent the mean ± SEM. b, bovine; m, murine.

quantify PLCZ1 expression in murine oocytes, fluorescence images were taken at 2 and 4 h after injection, given that in these oocytes oscillations begin within 20 min of injection and last approximately 4 h. As shown in Figure 4a, the expression of murine and bovine PLCZ1 in murine oocytes was comparable (n = 4 and n = 14, respectively). Quantification of expression in bovine oocytes was performed later at 12 h after injection, during the time of PN formation, as oscillations still occur at this stage [10, 36, 43]. In bovine oocytes, both cRNAs appear to reach comparable levels of expression (Fig. 4b; n = 4 for both cRNAs). Therefore, the specific activity of these cRNAs does not appear to be directly due to differential protein expression.

To evaluate the cellular distribution of the expressed PLCZ1-Venus proteins, bovine and murine zygotes were subjected to confocal microscopy at around the time of PN formation. High overexpression of PLCZ1-Venus was necessary for detection of fluorescence by confocal microscopy due to the high lipid content of bovine oocytes. For this reason, standard immunofluorescence using the bovine-specific PLCZ1 antibody was found to be unsuitable for investigating PLCZ1 localization in the fertilized oocyte (data not shown). Bovine oocytes injected with bovine PLCZ1-Venus cRNA showed widespread fluorescence in the ooplasm, although patches of higher fluorescence were clearly observed throughout the cytoplasm (Fig. 5d, arrows). Importantly, PLCZ1-Venus did not accumulate into the PN of bovine zygotes (Fig. 5d; dark area shows the PN of the zygote without fluorescence) or murine zygotes (inset). In contrast, and as previously
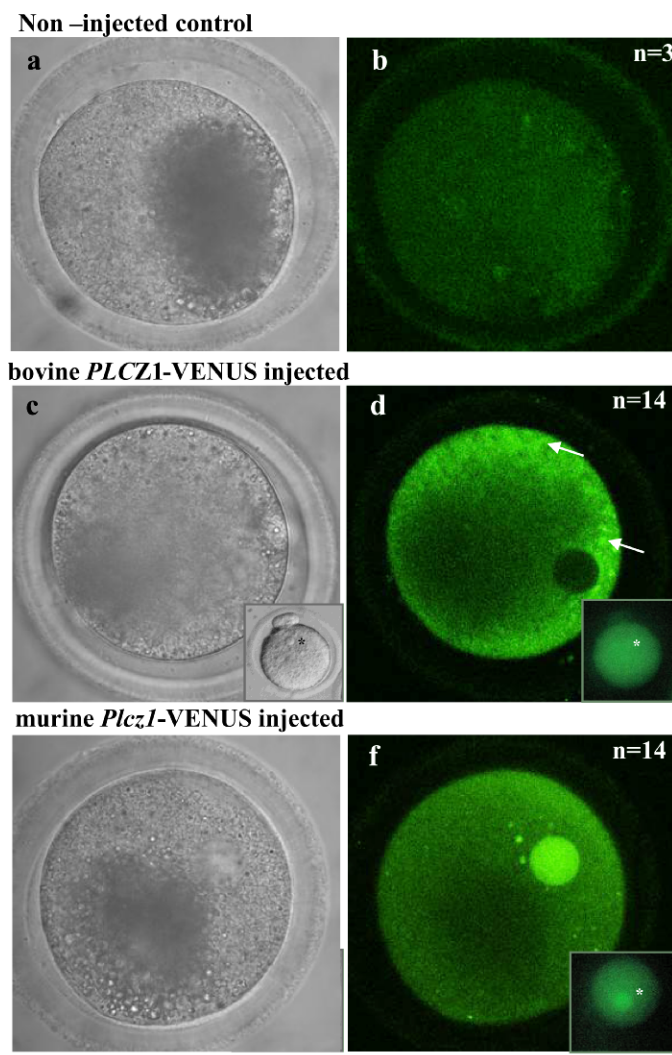
Non –injected control

bovine *PLCZ1*-VENUS injected

murine *Plcz1*-VENUS injected

FIG. 5. Cellular distribution of expressed PLCZ1-Venus proteins at the time of PN formation. Representative confocal fluorescent (**a–c**) and brightfield (**d–f**) images of bovine oocytes 14 h after injection of *Plcz1*-Venus or *PLCZ1*-Venus cRNA. **c** and **d**) Bovine PLCZ1-Venus did not show nuclear localization in any of the oocytes investigated, while patches of intense fluorescence were seen throughout the cytoplasm (arrows). **e** and **f**) Mouse PLCZ1-Venus showed strong staining of the pronucleus, suggesting nuclear translocation of the protein in bovine oocytes. (**c–f**) Asterisks indicate the localization of the pronucleus in similarly treated mouse oocytes (insets). b, bovine; m, murine; bar = 20 μm.

described for murine oocytes, murine PLCZ1 translocated into the PN of the bovine zygote (Fig. 5f), while the remaining fluorescence in the ooplasm appeared very uniform. As expected from previous murine findings, murine PLCZ1 also accumulated in the PN of murine zygotes (Fig. 5d, inset). Noninjected control bovine oocytes were devoid of background fluorescence and lacked PN formation (Fig. 5, a and b). Together, our results demonstrate that bovine PLCZ1 does not undergo PN accumulation in bovine and murine zygotes.

DISCUSSION

The discovery of the sperm-specific PLCZ1 in mammals, as well as the subsequent demonstration that it might represent the factor responsible for the initiation of $[Ca^{2+}]_i$ oscillations that lead to egg activation, has greatly advanced our understanding of fertilization and has set the stage for more precise studies on the functional characteristics of PLCZ1. While there is

evidence that PLCZ1 possesses cross-species activity [10, 21, 23, 24, 28], as seen by the widespread activity of sperm extracts to induce $[Ca^{2+}]_i$ transients in oocytes of mammalian and nonmammalian species [19, 20, 44, 45], it remains unclear how the activity of PLCZ1 is affected when expressed in oocytes of heterologous species. To address this question, we herein used a crossover design whereby bovine PLCZ1 and murine PLCZ1 were reciprocally expressed in oocytes of both species. Our results show that the homologous protein more effectively induced $[Ca^{2+}]_i$ oscillations in both species. We also found that the endogenous PLCZ1 content of bovine sperm is seemingly greater than that reported to be present in murine sperm [1, 26]. Last, we report that bovine PLCZ1 fails to undergo translocation to the PN of bovine and murine zygotes, although bovine zygotes are capable of incorporating murine PLCZ1.

Crossover Analysis to Study Species Specificity of Murine and Bovine PLCZ1

Comparison of the activity of murine and bovine PLCZ1 in oocytes of both species revealed that the ability of each PLCZ1 to initiate oscillations was influenced by the source of oocytes. For example, approximately 10-fold less murine *Plcz1* cRNA was required to initiate oscillations in murine oocytes than in bovine oocytes. In contrast, while similar quantities of bovine *PLCZ1* cRNA were needed to induce oscillations in oocytes of both species, the lower concentration of bovine *PLCZ1* cRNA failed altogether to induce $[Ca^{2+}]_i$ responses in murine oocytes. Moreover, the higher activity of bovine PLCZ1 in bovine oocytes was confirmed by the greater down-regulation of ITPR1. Therefore, we interpret the results to mean that bovine PLCZ1 and murine PLCZ1 are more efficient at inducing $[Ca^{2+}]_i$ oscillations in oocytes of homologous species. These results therefore extend previous findings [28] and suggest that the function of PLCZ1 may be optimized for the natural host oocyte. While this conclusion seems obvious, previous investigations have shown that the injection of *PLCZ1* cRNAs from several species into murine oocytes produces some unexpected results. For example, of the four species examined (human, murine, medaka, and rat), nearly 40-fold less human cRNA was needed to induce oscillations than the homologous murine *Plcz1* cRNA [28]. In contrast, many-fold higher concentrations of rat *Plcz1* cRNA were needed to induce oscillations similar to those induced by murine *Plcz1* cRNA [28]. Remarkably, lower concentrations of human cRNA are seemingly required to induce $[Ca^{2+}]_i$ oscillations in murine oocytes than in human oocytes [21], which suggests that the oscillation pattern triggered during fertilization depends not only on the specific activity of the enzyme but also on the PLCZ1 content of the sperm, as well as unknown oocyte factors. Regarding the latter, it is well established that the sensitivity of ITPR1 in bovine oocytes is significantly lower than in murine oocytes [46]. It would therefore be expected that ITPR1, or other cellular components that regulate production of IP_3 by PLCZ1, might be less sensitive or accessible in human oocytes than in murine oocytes, whereas the opposite might happen in rat oocytes, which are known to undergo high rates of spontaneous activation [47], despite the low $[Ca^{2+}]_i$ oscillatory activity shown by rat PLCZ1 [28].

Translational differences can often account for variations in the expression of proteins following cRNA injection, therefore influencing the total enzyme activity in the egg. This was unlikely to underlie the differences in PLCZ1 oscillatory responses observed in our studies between murine and bovine oocytes. First, murine PLCZ1 and bovine PLCZ1 were

produced using the same DNA constructs, which consisted of the complete *PLCZ1* coding sequence and to whose 3′ end, after in vitro transcription, a poly A tail greater than 150 bases was added. Second, equal volumes of each cRNA were injected into the oocytes of each species. Third, our quantification of the Venus-labeled cRNAs revealed approximately equal expression of both cRNAs in oocytes of both species. Together, our results support the view that, while mammalian PLCZ1 exhibits widespread activity among mammalian oocytes, its activities seemed optimized to perform in the natural host oocyte. Thus, cross-species studies such as the ones performed herein should prove useful to elucidate the egg factors that regulate the function of PLCZ1.

Expression and Analysis of Recombinant Bovine PLCZ1

Progress in the functional analysis of PLCZ1 would be greatly facilitated by production and purification of recombinant forms of the protein. To this end, we herein obtained recombinant bovine PLCZ1 from a bacterial expression system, although its production proved difficult in terms of low levels of expression and poor yields. This may explain at least in part the lack of reports demonstrating $[Ca^{2+}]_i$ oscillations following injection of recombinant proteins. To date, only a single study [41] using murine recombinant PLCZ1 has proven successful, with recombinant PLCZ1 protein synthesized using a baculovirus/Sf9 cell expression system. Unfortunately in the present study, recombinant bovine PLCZ1 failed in all instances to trigger $[Ca^{2+}]_i$ oscillations in bovine oocytes even when injected at the highest concentrations, which easily exceeded the normal amounts of PLCZ1 present in a single sperm. Importantly, our recombinant protein showed high immunoreactivity when probed with antibodies directed to both N-terminal and C-terminal peptides of the molecule, suggesting that the molecule was intact. Nonetheless, the lack of in vivo activity of the PLCZ1 protein suggests possible inactivation during protein purification or the absence of or critical posttranslational modifications or protein folding produced by the *E. coli* expression system. In the future, these problems may be overcome by using mammalian expression systems or by better understanding of the molecular determinants of PLCZ1 function.

While not functional, the immunoreactive properties of our recombinant protein allowed quantification of the PLCZ1 content of bovine sperm. According to our estimation, the PLCZ1 content of each bovine spermatozoan ranged from 105 to 165 fg, which is significantly greater than the published PLCZ1 content of murine sperm of between approximately 20 and 50 fg [1, 26], although less than the reported concentration of the enzyme in porcine sperm of approximately 350 fg per sperm [25]. While these estimations were not performed in a single study and using a common antibody, this apparent variation in the content of PLCZ1 in the sperm of these species is in agreement with the $[Ca^{2+}]_i$ oscillation-inducing ability differences observed following injection of their sperm into murine oocytes [39]. Importantly, species-specific differences in PLCZ1 concentration and activity may have arisen to ensure that the correct "dose" of the protein is delivered into the oocyte during fertilization, thereby initiating a pattern of $[Ca^{2+}]_i$ oscillations that will lead to normal embryo development.

Interphase Localization of Bovine PLCZ1

All species studied to date possess a presumptive NLS within the X-Y linker region of the PLCZ1 sequence. This NLS

is shown to result in the translocation of murine PLCZ1 to the PN of the one-cell zygote [29, 30, 42]. Importantly, other PLC isoforms including beta, gamma, and delta, are also found to possess this nuclear translocating activity [48]. The putative NLS is marked by a cluster of basic amino acids, which confer transport of the protein into the nucleus via the importin pathway [49]. Notably, the actual sequence of the PLCZ1 NLS is marked by substitution of important basic residues or insertion of additional amino acids, which may modify the ability of PLCZ1 from different species to undergo PN localization [29]. For instance, of all mammalian species, the mouse appears to possess the most traditional NLS, with six consecutive basic amino acids, whereas the bovine sequence is missing three of these residues. Therefore, we investigated whether bovine PLCZ1 could translocate to the PN of bovine and murine zygotes using a PLCZ1 construct tagged with the fluorescent protein Venus. Although the high overexpression of PLCZ1-Venus that was required for detection of fluorescence would result in abnormal $[Ca^{2+}]_i$ oscillations, it is unlikely to have detrimental effects on the localization of PLCZ1 within the zygote, as the termination of oscillations does not occur at the PN stage in the bovine. Similar techniques have been previously described in both mouse and rat oocytes in which the localization of PLCZ1-Venus was not disturbed by the overexpression of the protein [29, 42]. Direct immunofluorescence using PLCZ1 antibodies would be ideal for following the localization of PLCZ1 throughout early embryo development without the need for protein overexpression; however, to date this technique remains unsuccessful, which is most likely due to the high lipid content of the bovine oocyte.

After injection of *PLCZ1*-Venus cRNA, we found that bovine PLCZ1 was unable to translocate to the PN of either bovine or murine zygotes and remained dispersed throughout the cytoplasm. Conversely, Venus-labeled murine PLCZ1 was transported to the PN of both species. Therefore, bovine PLCZ1 appears similar to the PLCZs of rat, human, and medaka fish in its inability to translocate to the PN when expressed in murine oocytes [28] even though we show that bovine zygotes are equipped with an active nuclear transport system.

Therefore, it remains unclear why bovine PLCZ1, along with PLCZ1 from most other species, is not transported into the PN and what the exact physiological role of murine PLCZ1 is within the nucleus. It could be envisioned that the absence of three basic residues may incapacitate the bovine PLCZ1 NLS, although this is unlikely to be the case for the rat, given that its NLS differs from that of the mouse by only a single amino acid [28]. Thus, it is possible that, in addition to the NLS, several other residues of murine PLCZ1 may contribute to its PN translocation ability. For instance, the EF-hand domain of PLCs, which among other functions binds Ca^{2+} ions, has been shown to confer nuclear translocation ability of both PLCD1 and PLCZ1 [30, 49, 50]. Therefore, future studies should establish the precise residues and spatial configuration that make murine PLCZ1 the only PLCZ1 protein capable of undergoing translocation.

The physiological implications of PLCZ1 translocation to the PN remain uncertain. In the mouse, since $[Ca^{2+}]_i$ oscillations terminate around the time of PN formation, it is easy to associate both events [31]. This function however seems less than universal because, while termination of oscillations occurs in rat oocytes, it is unrelated to PN accumulation [28]. Moreover, in other species such as rabbits and bovine, fertilization oscillations persist well beyond PN formation. Whether or not the pattern of oscillations changes

after PN formation has not been closely scrutinized [37, 43, 51]. Importantly, the fact that $[Ca^{2+}]_i$ oscillations in bovine oocytes induced by injection of murine *Plcz1* or bovine *PLCZ1* cRNAs persist beyond PN formation [24], combined with the finding that only murine PLCZ1 protein undergoes PN accumulation, should allow the design of studies to discriminate how PN accumulation of PLCZ1 affects the pattern of $[Ca^{2+}]_i$ oscillations.

In summary, our results demonstrate species-specific differences in the activity and nuclear translocation ability of both bovine and murine PLCZ1. PLCZ1 was seen to be more efficient in triggering $[Ca^{2+}]_i$ oscillations in homologous oocytes. In addition, we show that bull sperm contain greater amounts of PLCZ1 than murine sperm. Therefore, we propose that the activity and concentration of PLCZ1 have been optimized in each of the mammalian species such that the sperm delivers PLCZ1 that is necessary to achieve the precise $[Ca^{2+}]_i$ signaling required for normal oocyte activation and embryo development.

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