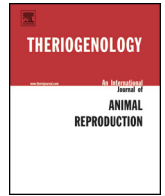




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Identification of phospholipase C zeta in normospermic and teratospermic domestic cat sperm

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ABSTRACT

In mammalian species, oocyte activation is initiated by oscillations in the intracellular concentration of free calcium ($[Ca^{2+}]_i$), which are also essential to allow embryonic development. To date, evidence supporting the hypothesis that a sperm factor is responsible for initiating oocyte activation has been presented in various mammalian species. Among the possible candidates to be the active sperm factor is the novel sperm-specific phospholipase C ζ (PLC ζ), which besides its testis-specific expression is capable of initiating $[Ca^{2+}]_i$ oscillations. In this study, we investigated the presence of PLC ζ in the sperm of the domestic cat and whether normospermic and teratospermic cats differ in their PLC ζ expression. Immunoblotting with anti-PLC ζ antibodies confirmed the presence of an immunoreactive band of ~ 70 kDa in whole sperm lysates of domestic cat as well as in both soluble and “insoluble” fractions from this sperm. Additional immunoreactive bands, probably C- and N-terminal truncated versions of PLC ζ , were also visualized in the soluble sperm fractions. Interestingly, immunoreactivity of PLC ζ was detectable in teratospermic sperm, although with slightly less intensity than in normospermic sperm. In conclusion, domestic cat sperm express PLC ζ in both cytosolic and high-pH fractions, which is consistent with data in other mammals. Sperm from teratospermic cats also express PLC ζ , albeit at reduced concentrations, which may affect the fertility of these males.

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1. Introduction

It is well known that during mammalian fertilization, the sperm induces in oocytes oscillatory changes in the intracellular concentration of free calcium ($[Ca^{2+}]_i$), which is essential for initiating activation and embryonic development [1–3]. For many years, the mechanism whereby the sperm trigger the oscillations and oocyte activation has been the subject of controversy [4,5]. However, recent evidence has reinforced the hypothesis that a sperm factor

(SF) might be involved in this process [6,7]. It is proposed that after gamete fusion, the fertilizing sperm delivers into the ooplasm a Ca^{2+} -active product, the so-called SF, which initiates the $[Ca^{2+}]_i$ oscillations.

In an attempt to characterize the active component of the SF, a novel sperm-specific phospholipase C (PLC) with a molecular weight (MW) of ~ 70 kDa was discovered and termed PLC ζ [8]. Besides having all the properties of other PLC isoforms, PLC ζ also has a high sensitivity to Ca^{2+} [9,10], which makes it ideally suited to be the initiator of $[Ca^{2+}]_i$ oscillations. Furthermore, although PLC ζ had initially been identified in soluble sperm extracts (SEs) [8,11,12], it was later found in “insoluble” sperm fractions, which correspond to the cytosol and perinuclear theca (PT) compartments,

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respectively [13]. To date, PLC ζ and its function have already been identified in several mammalian species, including mouse [8,14], rat [14], human [14,15], cynomolgus monkey [15], bovine [16,17], swine [18,19], and equine [20]. However, despite all studies reported in the last years addressing PLC ζ structure and function [6,7], the precise molecular mechanism(s) and site of action by which this testis-specific PLC triggers $[Ca^{2+}]_i$ oscillations remains to be fully elucidated.

Recent investigations in human patients who have experienced difficulties in conceiving after intracytoplasmic sperm injection (ICSI) have ascribed certain types of human infertility to the occurrence of absent or abnormally low PLC ζ expression and/or the presence of mutated forms of the enzyme, which undermines the ability of these sperm to initiate $[Ca^{2+}]_i$ oscillations [21–23]. Interestingly, in most cases these patients also presented high percentage of spermatozoa with abnormal morphology [21,24]. In felid species, the presence of high percentage of morphologically abnormal sperm (>60%), termed as teratospermia, is a common finding [25,26] and is implicated in cases of low fertility as a consequence of various structural and functional defects, even in morphologically normal spermatozoa [27].

It is well established that the phenomenon of teratospermia can impair *in vitro* fertilization outcomes in felids [28,29]. In this case, the application of ICSI could overcome this infertility problem and, thus, improve the success of assisted reproduction programs for endangered felid species. However, the impact of teratospermia on ICSI rates in felids is still uncertain. In domestic cats, a previous study has indicated that the percentage of fertilization and morulae formation was not affected by teratospermia, although blastocyst development was not assessed [30]. Nevertheless, it is noteworthy that in cats although similar percentages of embryo cleavage and morulae development rates were reported following ICSI and *in vitro* fertilization, ICSI had always lower rates of blastocyst formation [31,32] and high rates of embryos arrested at the 8- to 16-cell stage [33]. Thus, the ICSI procedure has not been optimized in felids. Further, previous studies have indicated that the pattern of $[Ca^{2+}]_i$ oscillations can exert a marked effect not only on oocyte activation but also on pre- and postimplantation embryo development [1,34]. For this reason, among the factors that can affect ICSI results in felids such as oocyte quality [31], perhaps a poor oocyte activation signal related to the SF may also be involved.

In this context, the study of PLC ζ expression in the sperm cells of the domestic cat, a suitable experimental model for felids, could contribute to a better understanding of the physiological process involved in oocyte activation in this species, allowing the use of this knowledge to improve ICSI and nuclear transfer outcomes. Therefore, we have examined whether or not PLC ζ is expressed in different fractions of domestic cat spermatozoa and whether a differential PLC ζ expression exists between normospermic and teratospermic animals.

2. Material and methods

2.1. Reagents and antibodies

All reagents were of high purity grade and, unless stated otherwise, they were purchased from GE Healthcare

(Uppsala, Sweden) or Sigma (St. Louis, MO, USA). Two different anti-PLC ζ rabbit sera were used to detect cPLC ζ : one against a 19-mer sequence (GYRRVPLFSKSGANLEPSS) at the C-terminus of mouse (m) PLC ζ (accession no. NP_473407; α PLC ζ -CT) [8] and the other against a 19-mer sequence (MENKWFLSMVRDDFKGGKI) at the N-terminus of porcine (p) PLC ζ (accession no. BAC78817; α PLC ζ -NT) [18]. Anti- α -tubulin monoclonal antibody (cat. no. T9026) were purchased from Sigma.

2.2. Animals and semen collection

The experiment was approved by the Ethical Committee for Experimental Animal Uses of the College of Veterinary Medicine and Animal Science, UNESP, Botucatu, Brazil (protocol no. 22/2007-CEEA).

Six adult mixed-breed male cats, 4 to 7 years old, from the Veterinary School research cattery, were used as semen donors. The animals were kept in rooms measuring 5 \times 2 m, exposed to >12 hours of natural light per day and provided with dry commercial cat food (FIT 32, Royal Canin, Descalvado, Brazil) and water *ad libitum*. Tomcats were previously classified as normospermic ($n = 4$; >60% of normal sperm morphology) or teratospermic ($n = 2$; <40% of normal sperm morphology) on the basis of repeated sperm analysis [25]. Sperm morphology from 200 cells per sample was assessed after staining with 1% Fast Green FCF/1% Rose Bengal [35].

Tomcats were collected using an artificial vagina and with at least a 2-day interval between collections. Semen collection was performed throughout the year, until an appropriate sperm quantity per cat to allow protein extraction was achieved.

2.3. Preparation of SEs

After semen collection, samples were diluted in Dulbecco PBS (DPBS; Nutricell, Campinas, SP, Brazil) and sperm concentration was determined. All subsequent steps were performed at 0 $^{\circ}$ C to 4 $^{\circ}$ C. Spermatozoa were washed twice with DPBS and the pellet resuspended in sperm buffer (75 mM KCl, 20 mM HEPES, 1 mM EDTA, 10 mM glycerophosphate, 1 mM DTT, 200 μ M PMSF, 10 μ g/mL pepstatin, 10 μ g/mL leupeptin, pH 7.0). Samples were frozen by immersion in liquid nitrogen and stored at -80° C until protein extraction.

Cytosolic cat sperm extracts (cSE^C) were prepared by following the procedure used by Wu et al. [12], with some modifications. Samples from the same tomcat were thawed, pooled, and sperm concentration assessed. A total of 1×10^9 to 2×10^9 spermatozoa were used in protein extraction for each cat. Sperm suspension was sonicated using ten 5-s bursts (60% output; S-250D, Branson Digital Sonifier, Danbury, CT, USA) at 4 $^{\circ}$ C, yielding >95% of sperm decapitation and completely demembrated sperm heads when evaluated with contrast microscopy. The lysate was centrifuged twice at $10,000 \times g$. Supernatant was then collected for ultracentrifugation at $100,000 \times g$ during 1 hour, whereas the pellet was resuspended in sperm buffer and stored at -80° C until extraction of high-pH soluble sperm proteins. The clear supernatant, obtained from ultracentrifugation, was washed

with buffer medium (75 mM KCl and 20 mM HEPES, pH 7.0) and concentrated using ultrafiltration membranes (cut-off 30 kDa; Millipore Co., Bedford, MA, USA). The extract was mixed for 30 minutes at 4 °C with ammonium sulfate at 50% final saturation, the precipitate collected by centrifugation (12,000 × g for 15 minutes), and the pellet kept at –20 °C. The pellets were then resuspended, washed at least three times using buffer medium to remove all traces of ammonium sulfate, and reconcentrated with ultrafiltration membranes (cut-off 10 kDa; Millipore Co.). Final protein concentration was assessed using a bicinchoninic acid protein assay kit (BCA protein assay kit; Pierce, Rockford, IL, USA). Samples were aliquoted and frozen at –80 °C.

High-pH soluble cat sperm extracts (cSE^{PH}) were prepared as previously described [18]. In brief, sperm cells after extraction of cSE^C were thawed and used as pool for normospermic and teratospermic animals, because this protocol resulted in lower amount of recovered protein. Pooled samples were centrifuged and the pellet was washed twice with sperm buffer, followed by a 30-minute incubation in a washing buffer containing high salts (1 M KCl, 10 mM Tris, 200 μM PMSF, pH 7.4) at 4 °C. The pellet was washed with sperm buffer and further submitted to alkaline carbonate extraction (100 mM Na₂CO₃, pH 11.5) for 10 minutes at 4 °C. Sperm suspension was then neutralized (0.5 M Tris and 12 mM DTT, pH 3.0), centrifuged twice, ultracentrifuged (110,000 × g) for 1 hour, concentrated, precipitated in ammonium sulfate, washed, and reconcentrated as described for cSE^C. Protein concentration was determined, and the samples were aliquoted and stored at –80 °C.

2.4. Transmission electron microscopy analysis

To evaluate the protocols for cSE^C and cSE^{PH} recovery, sperm cells were examined after collection, freezing-thawing, sonication, and alkaline carbonate extraction using transmission electron microscopy (TEM). Spermatozoa were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 5 °C for at least 24 hours. Sperm cells were then post-fixed during the 2 hours in 1% osmium tetroxide, stained en bloc for 2 hours with 0.5% uranyl acetate, dehydrated through a graded series of acetone (50%, 70%, 90%, and 100%), and embedded in epoxy resin. Ultrathin sections were cut on an ultramicrotome and double stained with uranyl acetate and lead citrate. Samples were then analyzed and photographed with a TEM (CM 100, Philips).

2.5. Electrophoresis analysis

Two-dimensional SDS-PAGE of cSE^C and cSE^{PH} samples was performed by following the methods as described in the Instructions Manual from Bio-Rad Laboratories (Hercules, CA, USA). For first dimension, a total of 224 μg of protein diluted in rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lytes, 0.001% bromophenol blue) was used to rehydrate 7-cm immobilized pH gradient strips (pH 3–10; linear; GE Healthcare) during 12 hours at 20 °C to 25 °C. Afterward, the strips were focused at 20 °C using the following program: conditioning step (0–250 V; 15 minutes), voltage ramping (rapid ramp; 250–4000 V; 2 hours), final focusing (4000–20,000 Vh), and hold step (500 V). Strips were then equilibrated, placed on

top of a 12% SDS-polyacrylamide gel, and proteins were separated in a vertical system (GE Healthcare) using 15 mA/gel for 100 to 130 minutes. Protein standards for reference (12 × 225 kDa) were always included on outside lanes. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 dye, 45% methanol, and 10% acetic acid. Images from gels were acquired using ImageScanner II (GE Healthcare) and analyses were performed with ImageMaster 2D Platinum v7.0 (GE Healthcare).

2.6. Immunoblotting analysis

Normospermic and teratospermic sperm extracts (cSE^C and cSE^{PH}), whole sperm cells, and demembrated sperm (devoid of cytosolic and acrosomal contents after sonication) were submitted to Western blot analysis as previously described [18]. Spermatozoa were washed five times with DPBS, counted, diluted 1:1 at 0.15 × 10⁵ spermatozoa/μL in 2× sample buffer (Laemmli), and stored at –20 °C until use. Sperm samples were placed at a final concentration of 0.15 × 10⁶ to 0.2 × 10⁶ per lane, whereas 18 μg of protein was loaded to each lane for SEs analysis. Spermatozoa and protein loading per lane was standardized using tubulin. Protein samples were separated by SDS-PAGE and, thereafter, transferred onto polyvinylidene difluoride membranes (Millipore Immobilon-P transfer membrane, Millipore Co.) for 1 hour at 4 °C. After incubation with 6% non-fat milk in PBS-0.1% Tween (TPBS), the membranes were probed with a primary antibody (1:2000) for 16 hours and then a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:3000; Bio-Rad Laboratories) was used. Immunoreactivity was detected using chemiluminescence reagents (NEN Life Science Products, MA, USA) and the Kodak Image Station 440CF (NEN Life Science Products). Three replicas from each sample were made following this procedure. Mouse sperm were used as positive control.

To confirm the binding specificity of the αPLCζ-NT antibody to cPLCζ, a peptide competition assay was run by preincubating the antibodies (1 μL) with the N-terminal antigenic peptides (5 μL) in TPBS for 2 hours at room temperature.

2.7. ICSI procedure in mouse eggs and [Ca²⁺]_i monitoring

Metaphase II (MII)-arrested eggs were obtained from B6D2F1 (C57BL/6J × DBA/2J) female mice (6–8 weeks old) superovulated by a single injection of 5 IU eCG followed 46 to 48 hours later by 5 UI hCG. Eggs were collected from the oviducts 14 to 15 hours post-hCG in HEPES-buffered Tyrode's lactate solution (TL-HEPES) supplemented with 5% heat-treated fetal calf serum (Gibco; Invitrogen, Carlsbad, CA, USA). Cumulus cells were removed by a brief treatment with 0.1% bovine testes hyaluronidase.

Cat sperm were collected from the cauda epididymis of a male cat, washed with microinjection buffer (75 mM KCl and 20 mM HEPES, pH 7.0), and mixed 1:1 with microinjection buffer containing 12% polyvinylpyrrolidone (PVP) prior to ICSI. MII eggs were injected with a single cat spermatozoon using a piezo micropipette-driven unit (PiezoDrill; Burleigh Instruments Inc., Rochester, NY, USA)

as previously described [36,37]. ICSI was performed in HEPES-buffered CZB medium [38] supplemented with 0.1% polyvinyl alcohol. Different pulse intensities were used to penetrate the zona pellucida and plasma membrane.

$[Ca^{2+}]_i$ monitoring was carried out as described by Kurokawa and Fissore [37]. Mouse eggs were loaded with 1 μ M fura-2-acetoxymethyl ester (Molecular Probes; Invitrogen, Carlsbad, CA, USA) supplemented with 0.02% pluronic acid (Molecular Probes; Invitrogen) for 20 minutes at room temperature. Immediately after ICSI procedure, the eggs were transferred into TL-HEPES microdrops placed on a monitoring glass bottom dish (Mat-Tek Corp., Ashland, MA, USA) and under mineral oil. Eggs were monitored using $\times 20$ objective on an inverted microscope (Nikon) outfitted for fluorescence measurements and with a temperature-controlled stage. The excitation wavelength was alternated between 340 and 380 nm using a filter wheel (Ludl Electronic Products Ltd., Hawthorne, NY, USA), and fluorescence ratios were taken every 20 seconds. After passing through a 510-nm barrier filter, the emitted light was collected by a CoolSNAPES digital camera (Roper Scientific, Tucson, AZ, USA). Images were analyzed by SimplePCI software (Hamamatsu, Sewickley, PA, USA). $[Ca^{2+}]_i$ values are reported as the ratio of 340/380 nm fluorescence in the whole egg.

2.8. Statistical analysis

Results for total protein amount in cSE^C and cSE^{PH} of normospermic and teratospermic cats were used to calculate 95% confidence interval, and animal group and extraction method were compared. The relative immunoreactivity of PLC ζ on cat sperm immunoblotting (IB) was compared between normospermic and teratospermic animals by one-way ANOVA followed by *t*-test using the SPSS Statistics 17.0 software. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Efficiency of sperm protein extraction

On the basis of TEM analysis (Fig. 1A–C), the efficiency of the protocol used to extract PT proteins from cat sperm was confirmed. The sonication procedure used to prepare the soluble fraction clearly removed great part of the acrosome, therefore exposing the PT (Fig. 1A and B). Following high-pH incubation, the entire PT was solubilized, and moderate chromatin decondensation was observed in the majority of the sperm cells (Fig. 1C).

Mean amount of protein retrieved from both normospermic and teratospermic cat PT (0.123 ± 0.001 μ g/million sperm) was approximately 3.3 times lower when compared with the results obtained from cytosol extracts (0.403 ± 0.019 μ g/million sperm). Furthermore, the quantity of recovered protein was not different between normospermic and teratospermic cats ($P > 0.05$).

3.2. Identification of PLC ζ expression in cat sperm

Immunoblot analyses of cat sperm using two anti-PLC ζ antibodies raised against the C- and N-terminal sequences

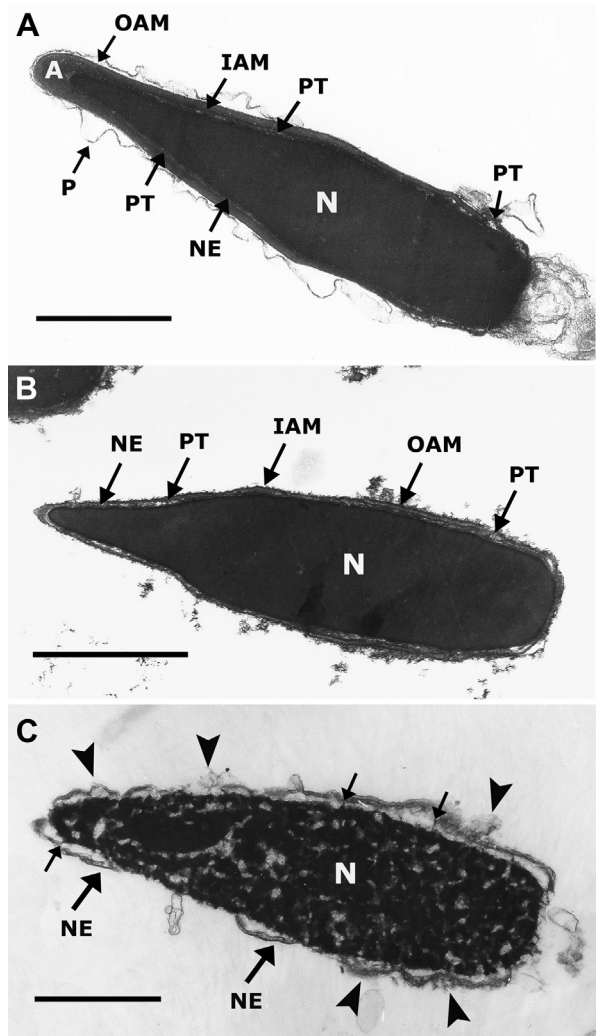


Fig. 1. Electron micrograph of cat sperm heads. (A) Intact spermatozoon and (B) sonicated spermatozoon. Note that the plasma membrane (P) and the apical part of the outer acrosomal membrane (OAM) have been solubilized, leaving the PT surrounding the condensed sperm nucleus (N). (C) Spermatozoon after high pH extraction. Note that PT has been completely solubilized and that a distinct layer of material is now surrounding the nucleus (arrowheads) showing an open chromatin configuration. Remnants of the nuclear envelope (NE) are seen lying outside this layer, which appears to be connected with the nuclear material (arrows). IAM, inner acrosomal membrane. Bar = 1 μ m.

of mPLC ζ (α PLC ζ -CT) and pPLC ζ (α PLC ζ -NT), respectively, identified an immunoreactive band of ~ 70 kDa, presumably the full-length cPLC ζ (Fig. 2A). Interestingly, the immunoreactive signal of cPLC ζ appeared to be higher when using α PLC ζ -NT compared with α PLC ζ -CT (Fig. 2A). As expected, mPLC ζ was only detected by α PLC ζ -CT (Fig. 2A). Furthermore, preincubation of both antibodies with the antigenic peptide used to raise the N-terminal antibody was only capable of blocking the signal from the α PLC ζ -NT antibody (Fig. 2B), strongly suggesting that the antibodies correctly recognized cPLC ζ in these extracts.

After sonication, sperm cells still presented immunoreactive PLC ζ , although in lower intensity compared with

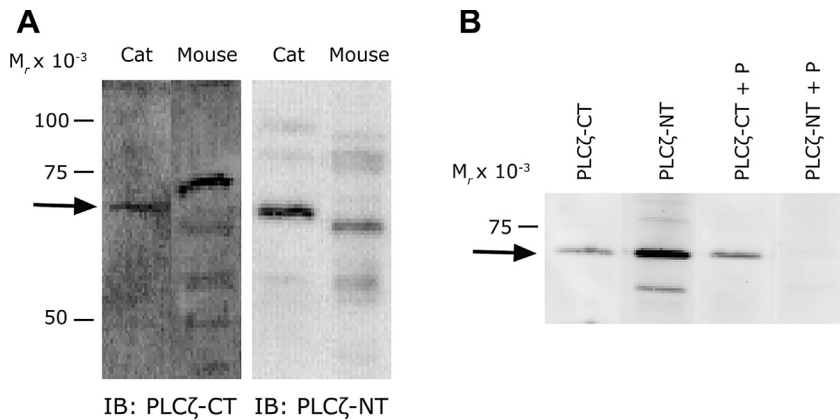


Fig. 2. Expression of PLC ζ by IB in cat spermatozoa. (A) Both anti-PLC ζ antibodies, α PLC ζ -CT (left panel) and α PLC ζ -NT (right panel), identified one protein band of ~ 70 kDa (arrow) in cat sperm (2×10^5 sperm/lane; normospermic sample), whereas in mouse sperm (2×10^5 sperm/lane) a 74-kDa band was detected only with α PLC ζ -CT (left panel) and a faint 66-kDa band was visualized using both antibodies. (B) Peptide competition assay showed that preincubation with the N-terminal peptide (P) only blocked immunoreactivity of the α PLC ζ -NT antibody.

intact sperm (Fig. 3A). Likewise, full-length cPLC ζ was found in both cSE^C and cSE^{PH}, with immunoreactivity varying among animals (Fig. 3B). Thus, it seems reasonable to assume that cPLC ζ is not only exclusively found in soluble sperm fractions (cytosol) but also in the “insoluble” fractions (PT).

Besides full-length PLC ζ , both anti-PLC ζ antibodies also detected in normospermic and teratospermic cSE^C one additional immunoreactive band with different MW (Fig. 3B). Whereas α PLC ζ -CT recognized a 48-kDa band, α PLC ζ -NT detected a 59-kDa band with immunoreactivity similar to the 70-kDa band.

For both normospermic and teratospermic cats, two-dimensional electrophoretic analysis of the cSE^C and cSE^{PH} samples showed a total of eight and two protein spots, respectively, ranging from 68 to 73 kDa (Fig. 4). These protein spots, showing MW close to the value found for the full-length cPLC ζ , were concentrated in the isoelectric point (pI) interval of 5.2 to 6.6 (Fig. 4).

Injection of cat epididymal sperm resulted in the initiation and maintenance of consistent $[Ca^{2+}]_i$ oscillations in all mouse oocytes successfully injected ($n = 12$) (Fig. 5). Regardless of using sperm samples from the same animal, variations in $[Ca^{2+}]_i$ oscillation profiles were observed.

3.3. Expression of PLC ζ in normospermic versus teratospermic cats

Because α PLC ζ -NT provided better detection of cPLC ζ than α PLC ζ -CT, it was used to determine the relative immunoreactivity of the 70-kDa full-length PLC ζ in normospermic and teratospermic cat sperm. IB of α -tubulin was used as a loading control and exhibited no difference between groups ($P > 0.05$). Data showed that, in average, normospermic cat sperm presented slightly higher ($P < 0.05$) cPLC ζ immunoreactivity compared with its counterpart (Fig. 6).

4. Discussion

Since the first report on the existence of a novel type of PLC as the putative SF responsible for triggering oocyte

$[Ca^{2+}]_i$ oscillations during fertilization [8], this testis-specific PLC, termed PLC ζ , has been found in a number of species [14,15,17–20] although not reported for the domestic cat. In the present study, we have found for the first time the presence of PLC ζ in cat sperm using IB techniques. Full-length native cPLC ζ showed to have ~ 70 kDa, being within the range reported for the species studied so far (70–75 kDa) [7,14,15,17–20]. Furthermore, the pI value for cPLC ζ seemed to be within 5.2 to 6.6, which is consistent with pI values reported for other species, except humans and cynomolgus monkeys [7].

In the present study, injection of cat sperm into mouse eggs found to efficiently induce $[Ca^{2+}]_i$ oscillations resembling the ones observed during fertilization in mouse [39]. This finding shows a cross-species activity for cat SF, which is a characteristic also described for the PLC ζ from other species [7,14,18]. In addition, this observation corroborates with a previous report in which a normal pronuclear formation, metaphase entry, and cleavage were obtained after ICSI of cat sperm into mouse eggs [40]. The long-lasting and high-frequency $[Ca^{2+}]_i$ oscillations observed in this study demonstrate the capacity of cat sperm to properly induce oocyte activation when ICSI technique is used.

The protocols used in this study to recover proteins from the cytosol and PT of domestic cat sperm were successful, although we have obtained lower amounts of total protein in cSE^C and cSE^{PH} than those reported in mouse and porcine sperm after extraction [12,18]. This result may be due to the well-known fact that cat spermatozoon is smaller in size than mouse and porcine sperm cells [41]. Nevertheless, the proportion of total protein present in cSE^C: cSE^{PH} was similar to the one reported for porcine sperm when using similar protocols [18].

Initially, the existence of PLC ζ has only been demonstrated in soluble sperm fractions [8]. However, because sperm heads after sonication and/or freeze/thaw procedures still contained significant amounts of $[Ca^{2+}]_i$ oscillation-inducing activity [37,42], the existence of an “insoluble” active SF possibly associated with PT was suggested. Later, tandem mass spectrometry analysis revealed

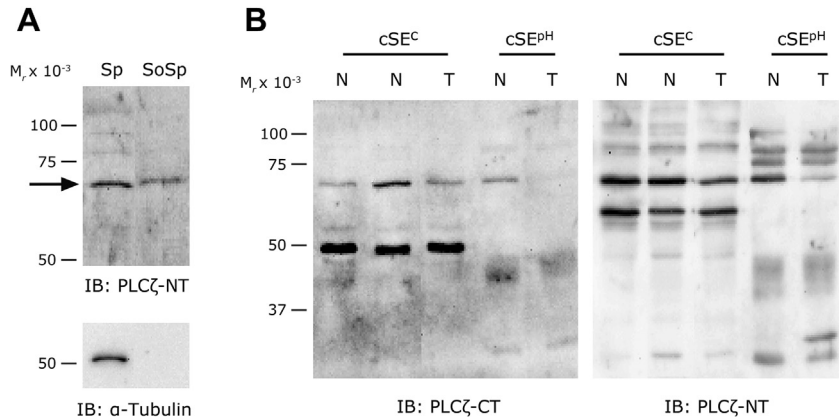


Fig. 3. IB of cat spermatozoa and SEs. (A) Compared with whole cat sperm (Sp) (1.5×10^5 sperm/lane; normospermic sample), sonicated sperm (SoSp) (1.5×10^5 sperm/lane; normospermic sample) presented lower immunoreactivity for the 70-kDa band (arrow) using α PLC ζ -NT antibody. Tubulin was presented only in whole sperm (Sp) sample. (B) Normospermic (N) and teratospermic (T) cytosolic and high-pH cat sperm extracts (cSEC and cSEP^H, respectively) (18 μ g/lane) were immunoblotted with either α PLC ζ -CT (left panel) or α PLC ζ -NT (right panel). The 70-kDa protein band (arrow), the predicted full-length PLC ζ , is presented in both extracts, although with different intensities among animals even within normospermic animals (lanes 1–2; left and right panels). In cSEC, a strong immunoreactive band of ~ 48 kDa was detected in all animals using α PLC ζ -CT antibody (left panel), whereas α PLC ζ -NT (right panel) identified a band of ~ 59 kDa with similar intensity to the 70-kDa band.

that the active component of the insoluble SF was indeed PLC ζ [13], which had been previously located in soluble fractions. In this study, full-length 70-kDa cPLC ζ was found in both SEs, following sonication (cSEC) and then high-pH extraction (cSEP^H). Likewise, the high-pH extraction protocol efficiently recovered the remaining SF activity in porcine sperm PT [18]. It is important to highlight that cSEC and cSEP^H were found to be derived from distinct sperm regions, as their two-dimensional electrophoresis protein profile greatly diverged (data not shown) and α -tubulin was only found in cSEC. Taken together, these results indicate that PLC ζ may be distributed in different compartments of the domestic cat sperm, soluble and insoluble fractions, as reported in other species [13,18].

Apart from the full-length 70-kDa PLC ζ , two protein bands with 48 and 59 kDa were recognized by the C- and

N-terminal PLC ζ antibodies, respectively, in all cytosolic extracts. Interestingly, IB analysis of whole sperm did not detect these additional immunoreactive bands. Thus, we can speculate that these C- and N-terminal truncated versions may in fact be proteolytic fragments of cPLC ζ , which could have been originated during SEs preparation. In support of this conclusion, N- and C-terminal fragments with different MWs have also been reported in porcine and equine SEs using the same antibodies [18,20]. Likewise, an additional 52-kDa band was also found in human sperm [21,23,43], whose intensity was higher than the full-length PLC ζ when sperm samples were previously submitted to freeze/thaw procedures [21]. It is important to note that to obtain enough material, multiple cat ejaculates were collected and kept frozen until the time of extraction. Therefore, this procedure may have increased the rate of fragmentation in the samples analyzed here. Additionally, although less likely, the existence of a splicing variant of PLC ζ , as reported in mouse sperm [9,13,18], cannot be discarded.

In the present study, we investigated whether teratospermic cats, aside from the already reported morphological and functional defects [27], also present an abnormal expression of PLC ζ . On the basis of our findings that the 70-kDa PLC ζ immunoreactivity was slightly higher in normospermic cats than in their abnormal counterparts, a reduction in PLC ζ expression may be associated with teratospermia in domestic cats. Nevertheless, future studies should examine whether or not there is individual variation in the expression of PLC ζ among the sperm of teratospermic cats.

Cases of infertility or subfertility associated with reduced/absent PLC ζ expression or the presence of a nonfunctional mutant form have been reported in other species [21–23,44]. In transgenic mice, PLC ζ expression of about half of normal level have shown to cause premature termination of [Ca²⁺]_i oscillations and litter size reduction

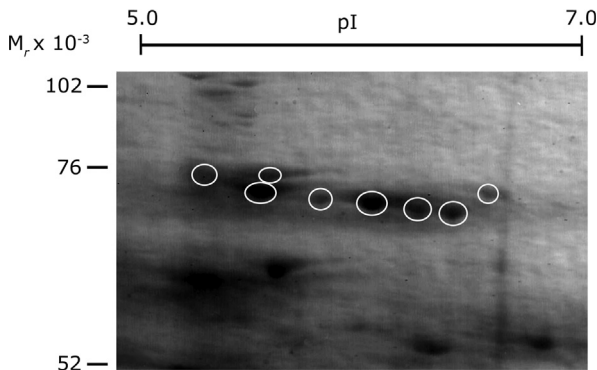


Fig. 4. Two-dimensional 12% SDS-PAGE gel of domestic cat cytosolic SEs stained with Coomassie Brilliant Blue. Isoelectric point (pI) of the first-dimensional gel is indicated at the top of the figure. MW standards (M_r × 10³) are to the left margin. Encircled protein spots presented mean MW and pI ranging from 68 to 73 kDa and 5.2 to 6.6, respectively.

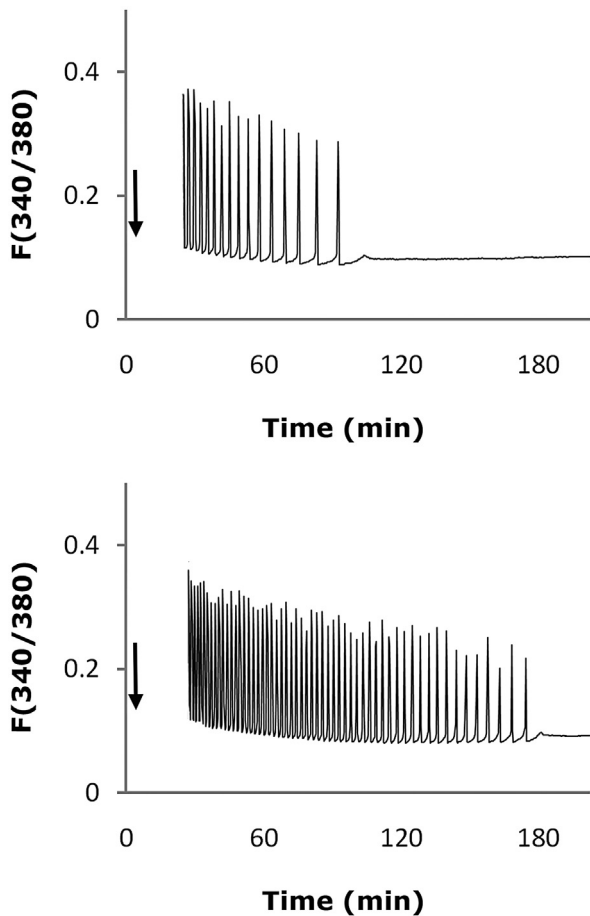


Fig. 5. Representative graphs indicating the capacity of domestic cat sperm to induce $[Ca^{2+}]_i$ oscillations when injected into mouse eggs. Both profiles are from the same tomcat. Arrows indicate the time of sperm injection (ICSI). $[Ca^{2+}]_i$ monitoring started after 25 minutes of sperm injection.

[45]. Indeed, the existence of a negative effect on embryo development due to disturbance of $[Ca^{2+}]_i$ oscillations pattern has already been demonstrated [34]. In this context, because teratospermic cats only show a modest reduction in PLC ζ expression, it is unknown whether a negative effect on fertility exists, and if it does, it is likely to be subtle and possibly manifested as impairment of embryo development rather than overt oocyte activation failures. This idea is consistent with the observation that using ICSI procedure for normospermic and teratospermic cats, similar fertilization rates were observed [30], although rates of blastocyst formation or embryo development to term were not provided.

In summary, our results indicated the expression of PLC ζ in domestic cat sperm, which is localized to different sperm compartments. Furthermore, expression of PLC ζ was lower in sperm of teratospermic cats than normospermic cats, although it is presently unknown if this affects the fertility of teratospermic cats. Future studies should be aimed to clone and sequence the PLC ζ gene in felids, examine its expression throughout spermatogenesis and test its specific activity.

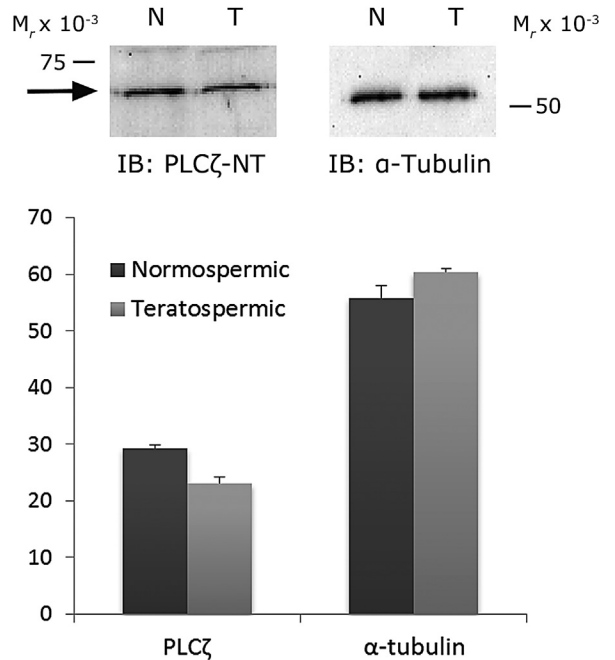


Fig. 6. IB of normospermic (N) and teratospermic (T) cat sperm (1.5×10^5 sperm/lane) using α PLC ζ -NT and α -tubulin antibodies. Relative immunoreactivity of the full-length 70-kDa PLC ζ (arrow) showed to be slightly different between groups, whereas α -tubulin exhibited equal loading per lane.

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