

During capacitation in bull spermatozoa, actin and PLC- ζ undergo dynamic interactions

Itzayana Mejía-Flores², Natalia Chiquete-Félix³, Icela Palma-Lara⁴, Salvador Uribe-Carvajal³
and María de Lourdes Juárez-Mosqueda¹

Departamento de Morfología, Facultad de Medicina Veterinaria y Zootecnia, UNAM, México; Instituto de Fisiología Celular, UNAM, México; and Escuela Superior de Medicina, IPN, Col. Santo Tomás, CDMX, México.

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Summary

The migration pattern of sperm-specific phospholipase C- ζ (PLC- ζ) was followed and the role of this migration in actin cytoskeleton dynamics was determined. We investigated whether PLC- ζ exits sperm, opening the possibility that PLC- ζ is the ‘spermatozoidal activator factor’ (SOAF). As capacitation progresses, the highly dynamic actin cytoskeleton bound different proteins to regulate their location and activity. PLC- ζ participation at the start of fertilization was established. In non-capacitated spermatozoa, PLC- ζ is in the perinuclear theca (PT) and in the flagellum, therefore it was decided to determine whether bovine sperm actin interacts with PLC- ζ to direct its relocation as it progresses from non-capacitated (NC) to capacitated (C) and to acrosome-reacted (AR) spermatozoa. PLC- ζ interacted with actin in NC spermatozoa (100%), PLC- ζ levels decreased in C spermatozoa to 32% and in AR spermatozoa to 57% ($P < 0.001$). The level of actin/PLC- ζ interaction was twice as high in G-actin ($P < 0.001$) that reflected an increase in affinity. Upon reaching the AR spermatozoa, PLC- ζ was partially released from the cell. It was concluded that actin cytoskeleton dynamics control the migration of PLC- ζ during capacitation and leads to its partial release at AR spermatozoa. It is suggested that liberated PLC- ζ could reach the egg and favour fertilization.

Keywords: Actin, Bull sperm capacitation, PLC- ζ , Protein–protein interaction, SOAF

Introduction

Phospholipase C- ζ (PLC- ζ or PLCz) is expressed specifically in sperm and testis in many species (Young *et al.*, 2009). Sperm penetration triggers a response in the oocyte that is mediated by a ‘spermatozoidal oocyte-activating factor’ (SOAF). PLC- ζ has been tentatively identified as SOAF (Saunders *et al.*, 2002; Swann *et al.*, 2004; Yoneda *et al.*, 2006) as the absence

of PLC- ζ or PLC- ζ mutation results in lack of oocyte activation (Yoon *et al.*, 2008; Nomikos *et al.*, 2011). PLC- ζ hydrolyzes phosphatidyl inositol 4,5-bisphosphate (PIP₂) to generate two products: inositol 1,4,5-trisphosphate (IP₃) that increases cytoplasmic calcium; and diacylglycerol (DAG), an activator of protein kinase C (PKC) (Yeste *et al.*, 2016). In addition, PLC- ζ coordinates oocyte movement shortly after gamete fusion (Swann *et al.*, 2012). Despite these processes, the exact process by which PLC- ζ is prepared for its role as SOAF is still unknown. (Nomikos *et al.*, 2005).

In hamster and mouse sperm undergoing capacitation and the acrosome reaction, PLC- ζ migrates while undergoing post-translational modifications (Young *et al.*, 2009). As capacitation advances, actin concentrates several proteins within the perinuclear theca (PT) (Howes *et al.*, 2001). In addition, actin is involved in trafficking during the AR (Howes *et al.*, 2001), therefore it was decided to determine whether, during bull sperm capacitation, actin cytoskeleton

¹All correspondence to: María de Lourdes Juárez-Mosqueda. Departamento de Morfología, Facultad de Medicina Veterinaria y Zootecnia, UNAM, C.U., CDMX, 04510, México. Tel./Fax: +52 55 56225910. E-mail: luerfco@unam.mx

²Departamento de Morfología, Facultad de Medicina Veterinaria y Zootecnia, UNAM

³Instituto de Fisiología Celular, UNAM, C.U., CDMX, 04510, México.

⁴Escuela Superior de Medicina, IPN, Plan de San Luis y Díaz Mirón, Col. Santo Tomás, CDMX, 11340, México.

dynamics lead to PLC- ζ migration. PLC- ζ was relocated during capacitation, in addition PLC- ζ exhibited a higher affinity for monomeric actin than for filamentous, polymeric actin. Most importantly, PLC- ζ was partially liberated from capacitated or AR sperm, possibly to interact with the oocyte. Therefore, it is suggested that during capacitation changes in PLC- ζ -actin interactions are important for egg release and egg activation promoted by this phospholipase.

Materials and methods

Sperm preparation and sample collection

Fresh semen samples were obtained from three healthy bulls (*Bos taurus*, Jersey) from the Center for Practical Learning and Research on Animal Production and Health (CEPIPSA, UNAM). Three semen ejaculates from each bull were collected using an artificial vagina and kept at 35°C in triladyl-20% egg yolk. Sperm cells were washed three times at 3500 g for 8 min in NKM buffer (110 mM NaCl, 5 mM KCl, and 20 mM 3-*N*-morpholino propanesulfonic acid, pH 7.4). Only ejaculates that were determined to have normal semen parameters (80% viable and motile) were selected for the study.

Sperm capacitation and acrosome reaction

Spermatozoa (100 million/ml) were centrifuged at 800 g for 10 min at room temperature. The pellet was resuspended in TALP modified capacitation medium (TMCM: 100 mM NaCl, 3.1 mM KCl, 1.5 mM MgCl₂, 0.92 mM KH₂PO₄, 25 mM NaHCO₃, 20 mM HEPES, 0.1 mM sodium pyruvate, 6.21 mM sodium lactate, 50 mg/ml gentamycin, 1 mg/ml BSA, 20 µg/ml heparin and 2 mM CaCl₂, pH 7.4), and then incubated for 4 h at 39°C in a 5% CO₂ atmosphere and 100% relative humidity. The sample was divided into three groups: non-capacitated (NC), capacitated (C) and capacitated with AR (AR). The NC spermatozoa were taken at time 0, C spermatozoa were taken at 4 h and AR spermatozoa were taken at 4 h and incubated for 1 h with 1 µM calcium ionophore (Sigma, A23187). When indicated, actin polymerization was inhibited with 25 µM cytochalasin-D (CD) (Chiquete-Félix *et al.*, 2009) or 1 µM latrunculin (Azamar *et al.*, 2007). Capacitation was evaluated by CTC-Hoechst 33258 staining (Fraser *et al.*, 1995). All the experiments were made in triplicate.

Antibody specificity and validation

The specificity of the anti-PLC- ζ antibody was tested by immunoblotting against PLC- ζ , molecular weight (MW) 73.7 kDa (UniProt, 2016) (see later Fig. 3A)

and the antibody against actin was tested against actin (MW 43 kDa; Flaherty *et al.* 1988) (see Fig. 3A). The PVDF membranes were incubated overnight at 4°C with anti-PLC- ζ (1:500) diluted in blocking solution. Samples were incubated for 1 h with horseradish peroxidase (HRP) secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA).

Immunocytochemistry

Sperm samples were spread on microscope slides, dried and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated in Universal Blocking BioGenex HK085-5K (Milmont Drive, Fremont, CA, USA). The slides were washed with PBS. Endogenous peroxidase was inactivated (DakoCytomation, Glostrup, Denmark). Samples were incubated overnight at 4°C with primary antibodies: PLC- ζ (C-12) or Actin (H-6) (Santa Cruz Biotechnol, Inc.). Antigen-antibody complexes were detected using the avidin-biotin peroxidase method with 3,3-diaminobenzidine tetrahydrochloride as a chromogenic substrate and a Dako kit (cat. no. KO679 LSAB+Sys/HRP, DakoCytomation). Parallel samples were processed similarly without the primary antibody. Immunocytochemistry was conducted using an Olympus BX51 microscope.

Sperm protein extracts

To solubilize membranes and obtain sperm protein, we used protocols reported by Takiguchi *et al.* (2011), Felipe-Perez *et al.* (2012) and de Lourdes Juárez-Mosqueda & Mujica (1999). Briefly, sperm were resuspended in PBS at 100 × 10⁶ cells/500 µl. Then, 3.75 mM dithiothreitol (DTT) was added and incubated on ice for 15 min. Next, an extraction buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.75% SDS and 0.1 mM EDTA), plus protease inhibitor (Complete[®]) and phosphatase inhibitor (Phosphostop[®]) was added to the sperm suspension and then the mix was further incubated on ice for 5 min. The sample was adjusted to 1 ml and centrifuged at 11,140 g for 5 min, the supernatant was recovered and Phosphostop[®] and Compleat[®] were added. Supernatants were concentrated in Amicon Ultra-10K Centrifugal filter units, Millipore[™] and centrifuged at 4°C at 2264 g for 30 min. The protein concentration was determined (Bradford, 1976) and run by SDS-PAGE and western blotting (Okó & Maravei, 1994).

Immunoprecipitation

Protein A agarose (10 µl) was incubated with 10 µl anti-PLC- ζ (E-13; Santa Cruz Biotechnol, Inc.) 15 min at 4°C and 300 µg sperm protein extract was added.

The mixture was agitated overnight at 4°C. Then, samples were washed twice at 1940 g for 6 min in PBS plus 1 mM PMSF and 0.1% Tween-20. Pellets were resuspended and boiled in 50 µl Laemmli's buffer (Laemmli, 1970) and used for SDS-PAGE or western blots.

SDS-PAGE and western blotting

Samples were subjected to SDS-PAGE in 8% polyacrylamide (Moreno-Fierros *et al.*, 1992). Samples were incubated overnight at 4°C with anti-PLC-ζ (1:500) diluted in blocking solution. Samples were incubated 1 h with HRP secondary antibody (Jackson Immuno Research, West Grove, PA, USA). Membranes were stripped for 20 min in (15 g glycine, 1 g SDS, 10 ml Tween-20/l, pH 2.2) and subjected to western blotting using anti-actin antibody (JLA20, Millipore, Billerica, MA, USA). HRP was revealed with an ECL kit from Amersham (Arlington Heights, IL, USA) (Chiquete-Felix *et al.*, 2009). Brain extract was used as a positive control (Katan & Parker, 1987).

Quantification of F-actin

Samples were fixed in 1% paraformaldehyde for 30 min, washed twice by centrifugation at 2040 g for 5 min and resuspended in PBS. Five million cells were stained with 1.5 µM rhodaminated phalloidin. Fluorescence was measured at 540–590 nm in a BMG LABTECH POLARstar Omega spectrometer.

Far-western blotting

A Far-western blot assay was used to determine the interaction between PLC-ζ and actin. PLC-ζ peptide (sc-131 753-Pz; Santa Cruz Biotechnology; product now discontinued) was used as prey, F/G-actin as bait (Saba *et al.*, 2011) and controls were testicle extract and sperm extract. F/G-actin was obtained (Pardee & Spudich 1982; Sato *et al.*, 2011). Samples were run on SDS-PAGE 8% and transferred to PVDF membranes. PVDF membranes were processed as described previously (Moreno-Fierros *et al.*, 1992). Membranes were incubated for 12 h in blocking solution. The membrane was washed twice with TBS-T for 5 min, and was incubated for 3 h with 200 µg of F-actin or actin-G respectively. Washing was repeated and samples were incubated for 3 h with the appropriate primary antibody (actin or PLC-ζ) in 1% skimmed milk in TBS-T and then with the HRP-labeled appropriate secondary antibody. The interaction was revealed using a chemiluminescence ECL kit. The intensity of the obtained bands was analysed by densitometry.

Affinity chromatography for PLC-ζ

Cyanogen bromide-activated Sepharose beads was used following standard procedures. F-actin 6.9 mg was dissolved in coupling buffer (0.1 M NaHCO₃ and 0.5 M NaCl, pH 8.3–8.5) and covalently attached to a cyanogen bromide-activated Sepharose column. Eluted proteins were pooled and concentrated in Amicon Ultra centrifugal filter units and centrifuged at 2264 g at 4°C, for 30 min. PLC-ζ was detected by western blot, and a silver-stained gel was used as loading control. Band intensity was analysed by densitometry. This procedure was realized for each experimental group.

Densitometry

Band intensity was quantified using a scanner and the Image J software version 1.46 (NIH, Bethesda, MD, USA) was performed to compare the differences between the bands for NC, C or AR; non-treated (NT) or treated with CD, F-actin and actin-G, as indicated. Differences were evaluated using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance (ANOVA) and Dunnett multiple comparison test.

Statistics

Data are the mean ± standard error of the mean (SEM) of at least three independent experiments. One-way ANOVA (multiple comparison test Dunnett) using GraphPad Prism Version 5 (GraphPad software, Inc., La Jolla, CA, USA) was used to compare the localization pattern of PLC-ζ /actin and the differences in PLC-ζ–actin concentrations at NC, C and RA and also in non-treated (NT) samples versus samples treated with CD.

Results and Discussion

PLC-ζ and actin localization in NC, C and AR sperm

In NC sperm PLC-ζ was detected in the equatorial region (83.3 ± 0.9%) (Fig. 1A and Table 1); while in C sperm PLC-ζ migrated to the post-acrosomal region (86.6 ± 0.7%) (Fig. 1B and Table 1) and in the AR sperm PLC-ζ remained in the final region and was also detected at the base of the neck (88.3 ± 0.7%) (Fig. 1C and Table 1). In addition, in all three stages PLC-ζ was present in the middle piece of the tail (Fig. 1A–C). The negative control for anti-PLC-ζ is shown in Fig. 1D. These results agree with those reported by Young *et al.* (2009), who observed the migration of PLC-ζ in both mouse and hamster sperm during sperm capacitation and AR.

Table 1 PLC- ζ distribution in non-capacitated, capacitated and capacitated with acrosome reaction bull sperm

Location	Sperm capacitation stage		
	Non-capacitated %	Capacitated %	Capacitated with acrosome reaction %
Acrosomal	1.3 \pm 0.3 ^a	0	0
Equatorial	83.3 \pm 0.9 ^b	13.3 \pm 0.7 ^c	2.3 \pm 0.5 ^a
Post-acrosome	15.3 \pm 0.8 ^c	86.6 \pm 0.7 ^b	9.0 \pm 1.0 ^c
Post-acrosomal with less intensity and base of the neck	0	0	88.3 \pm 0.7 ^b

Values are the mean \pm standard error of the mean (SEM) ($n = 9$).

^{a-c}Means with different superscripts indicate significant difference with $P < 0.001$.

Table 2 Actin distribution in non-capacitated, capacitated and capacitated with acrosome reaction bull sperm

Location	Sperm capacitation stage		
	Non-capacitated %	Capacitated %	Capacitated with acrosome reaction %
P _{al} , neck and middle piece	89.0 \pm 0.6 ^a	9.44 \pm 0.7 ^b	0
P _{Am} , middle piece	11 \pm 0.6 ^b	90.0 \pm 0.7 ^a	10.0 \pm 0.7 ^a
Neck, middle piece and head	0	0	90.0 \pm 0.7 ^b

P_{al}, less intense mark in post-acrosome; P_{Am}, more intense mark in post-acrosome.

Values are the mean \pm standard error of the mean (SEM) ($n = 9$).

^{a-c}Means with different superscript indicates significant difference with $P < 0.001$.

Actin migration occurs during capacitation and AR as a result of cytoskeletal dynamics (Breitbart *et al.*, 2005; Kierszenbaum *et al.*, 2007; Jagan Mohanarao & Atreja, 2011). In different species, in parallel to sperm capacitation, there is an increase in formation of F-actin, first in the mid-piece and later in the sperm head (Brener *et al.*, 2003). We observed that actin migrated as follows: NC sperm exhibited labeling at the post-acrosomal region, in the base of the neck and middle piece of the tail (89 \pm 0.6%) (Fig. 2A and Table 2), in C sperm a more intense mark on the post-acrosomal region and middle piece of the tail was observed (90 \pm 0.1%) (Fig. 2B and Table 2). Nonetheless, in AR sperm a faint mark was observed at the base of the neck, in the middle piece of the tail and in the head (90 \pm 0.7%) (Fig. 2C). As the negative control, samples were processed in parallel without the primary antibody versus actin (Fig. 2D). Thus, during capacitation both PLC- ζ and actin were localized in the middle piece of the tail, while in C sperm both proteins were found in the post-acrosomal region.

Actin dynamics and PLC- ζ interactions

Immunocytochemistry data suggested that PLC- ζ and actin migrate together during capacitation, therefore it was decided to determine by immunoprecipitation if there was a physical interaction between PLC- ζ and actin in NC, C and AR spermatozoa. As seen in Fig. 3, PLC- ζ interacts with actin in all three capacitation stages. By densitometry it was determined that in

NC PLC- ζ -actin interaction was high (100%), reaching only 68% in C spermatozoa and gradually decreasing as capacitation progressed, reaching 42% at AR spermatozoa.

Once the actin/PLC- ζ interaction was demonstrated, we decided to determine the percentages of F-actin in the different physiological states of the sperm and then whether actin dynamics affects PLC- ζ association. We found that in capacitation F-actin increased to 18.25%, while in AR it decreased to 11.5% in comparison with NC (Fig. 4). These results suggest that PLC- ζ has less affinity for F-actin.

To demonstrate whether actin dynamics affect PLC- ζ association, we used actin depolymerizing agents such as CD or latrunculin during capacitation. First we measured F-actin concentrations (Fig. 5), which decreased to 23% when treated with CD and to 20.25% with latrunculin. In regard to the actin/PLC- ζ association, it increased to 52.33% in CD-treated samples (Fig. 6). These results suggest that PLC- ζ interacts with G-actin rather than F-actin.

In order to further confirm that PLC- ζ has a high affinity for G-actin, globular or filamentous actin and PLC- ζ peptide were subjected to a far-western assay. The PLC- ζ interaction was twice as high with G-actin (201.66%) than with F-actin (100%) (Fig. 7). Before capacitation, different proteins co-localize with G-actin (Breitbart & Finkelstein, 2015) and relocation is necessary for capacitation. The high PLC- ζ /G-actin affinity we observed indicated that PLC- ζ is among

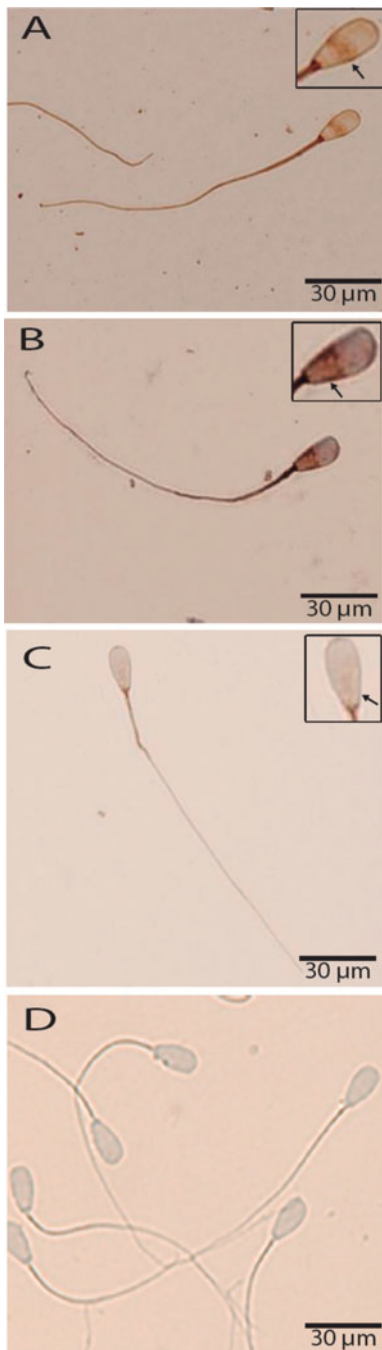


Figure 1 PLC- ζ migration in bull sperm in non-capacitated (NC), capacitated (C), and capacitated with acrosome reaction (AR). Immunocytochemistry showing the localization pattern of PLC- ζ (arrow in insets) during capacitation. (A) Non-capacitated spermatozoa (NC), PLC- ζ is found in the equatorial region. (B) Capacitated spermatozoa (C), PLC- ζ is found in the post-acrosomal region. (C) Capacitated with acrosome reaction spermatozoa (AR), PLC- ζ levels are decreased in the post-acrosomal region, increasing at the base of the neck. In all three states PLC- ζ exhibited an intense mark in the middle piece of the tail. (D) Negative control samples were processed without the primary antibody.

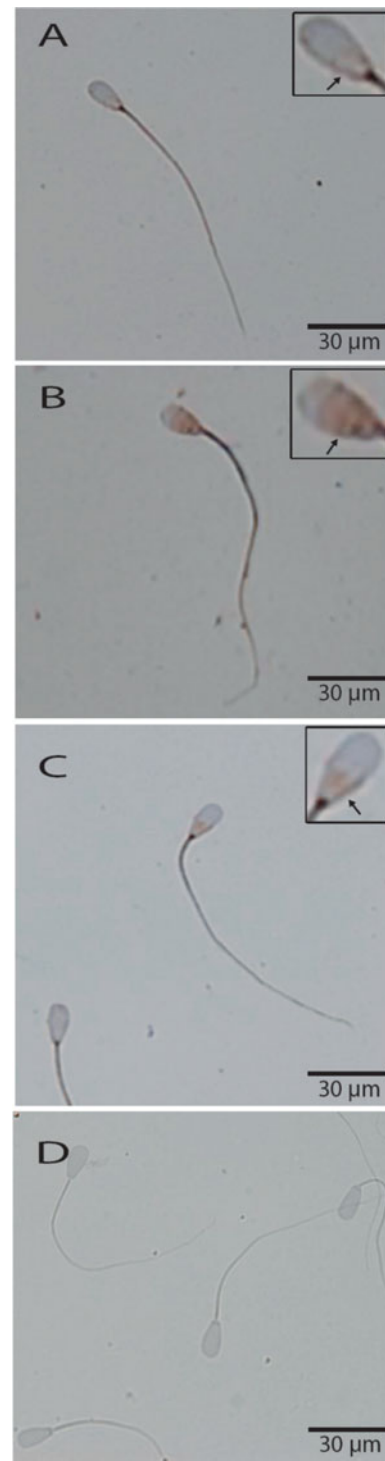


Figure 2 Actin localization in non-capacitated (NC), capacitated (C), and capacitated with acrosome reaction (AR) bull sperm. Using immunocytochemistry it was detected that actin migrated as follows. (A) NC, actin was detected at the post-acrosomal region (arrow in insert), the base of the neck and the middle piece of the tail. (B) C, actin was in the post-acrosome (arrow in insert) and middle piece of the tail. (C) AR, actin decreased in the post-acrosomal region and increased at the base of the neck (arrow in insets) and the middle piece of the tail. (D) As negative control, samples are processed without the primary antibody versus actin.

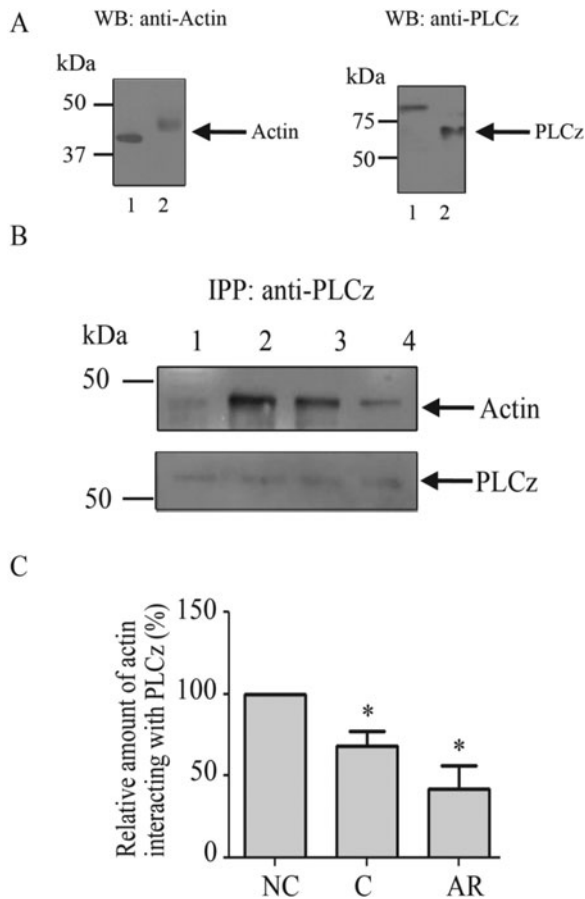


Figure 3 Antibody specificity and validation, immunoprecipitation and densitometry of PLC- ζ -actin. (A) Antibody specificity by western blot identifying actin and PLC- ζ . In both blots, lane 1 shows testis extract and lane 2 is sperm extract. (B) Samples were immunoprecipitated using PLC- ζ antibodies. Lanes: (1) control sperm extract (100 μ g); (2) non-capacitated (NC), (3) capacitated (C); and (4) capacitated with acrosome reaction (AR) spermatozoa. As indicated, blotting was made with actin and PLC ζ antibodies. (C) Densitometric analysis of the bands in (B). *Significant difference with $P < 0.001$.

these proteins and this interaction may be important for capacitation and the AR.

Release of PLC- ζ to the supernatant

If PLC- ζ is SOAF, then it would have to exit sperm in order to interact with the egg. In this regard, the existence of two populations of PLC- ζ has been proposed, in which one might be released during capacitation and AR (Fujimoto *et al.*, 2004). Furthermore, PLC- ζ is found in both soluble and insoluble fractions in the sperm head of hamster and mouse, in which the ability to induce Ca^{2+} oscillations in the oocyte depends on solubility (Kashir *et al.*, 2014). It was observed that G-actin decreased during capacitation, so it was decided to test whether

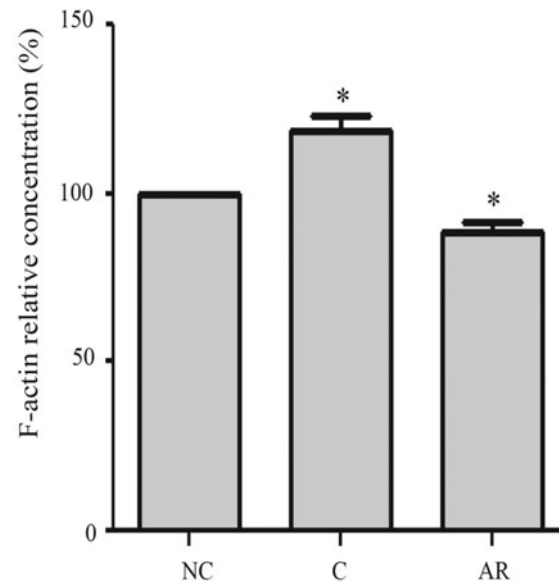


Figure 4 Percentage of relative concentration of F-actin in non-capacitated (NC), capacitated (C), and capacitated with acrosome reaction (AR) sperm. Experimental conditions as in Fig. 2. *Significant difference with $P < 0.001$.

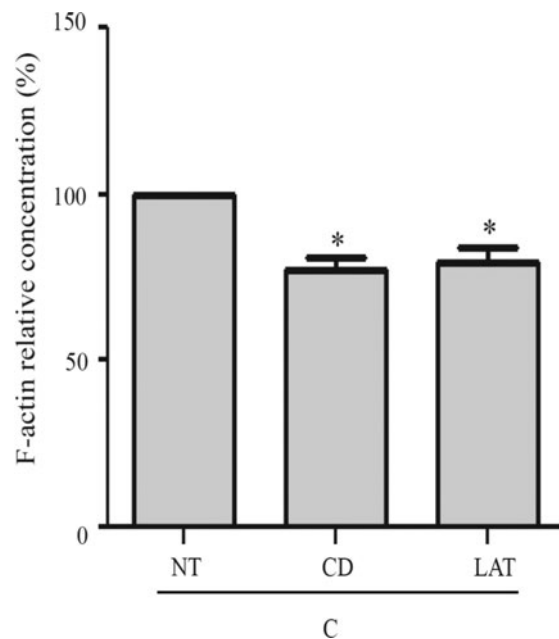


Figure 5 Percentage of F-actin concentration in C sperm. NT, untreated sperm; CD, sperm treated with cytochalasin-D; and LAT, sperm treated with latrunculin. *Significant difference with $P < 0.001$.

PLC- ζ exits the cell at any capacitation stage. As capacitation progressed, extracellular PLC- ζ increased in the supernatant (Fig. 8), as follows: in NC it was 100% and then in C it was 158%, and in AR it was 186.5%. In Fig. 8, most lanes show the band at the expected weight for PLC ζ plus a slightly heavier band

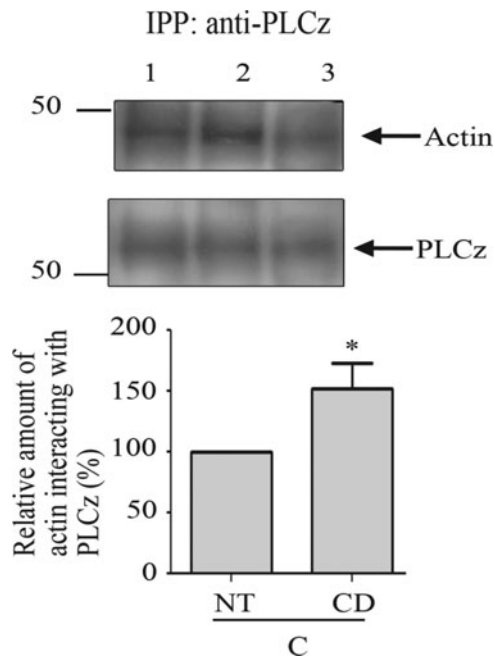


Figure 6 Detection of actin in the sperm extracts that were immunoprecipitated with anti-PLC- ζ antibody. Extracts obtained from capacitated (C) sperm with or without treatment with cytochalasin-D. (Top) Lane 1: C without cytochalasin-D; lane 2: C with cytochalasin-D; and lane 3: sperm extract. (Bottom) Densitometry showing the relative concentration of actin interacting with PLC- ζ with actin in C sperm. NT, non-treated control group; CD, treated with cytochalasin-D. *Significant difference with $P < 0.001$.

that may be a phosphorylation product as suggested previously by others (Bernabó *et al.*, 2011). Thus, upon capacitation and AR it was demonstrated that while PLC ζ decreases in the pellet (Fig. 3) it increases in the supernatant (Fig. 8).

Co-migration of PLC- ζ and actin during capacitation was demonstrated. In NC sperm, the affinity is the highest, although each protein seems to be in a different compartment, except for the middle piece of the flagellum, in which both proteins remain at all times. Then, in C sperm both proteins are found in the post-acrosomal region and then they decrease in affinity as AR is reached. Furthermore, in this late capacitation stage, PLC- ζ is liberated from sperm, probably as a strategy to reach the egg. In regard to the identity of SOAF, we cannot discard other proteins such as the post-acrosomal-WWP-domain-binding protein (PAWP), which participates in the interaction between sperm and the oocyte (Aarabi *et al.*, 2014). PAWP injection to oocytes from different species activates meiosis (Aarabi *et al.*, 2010, 2014; Kennedy *et al.*, 2014).

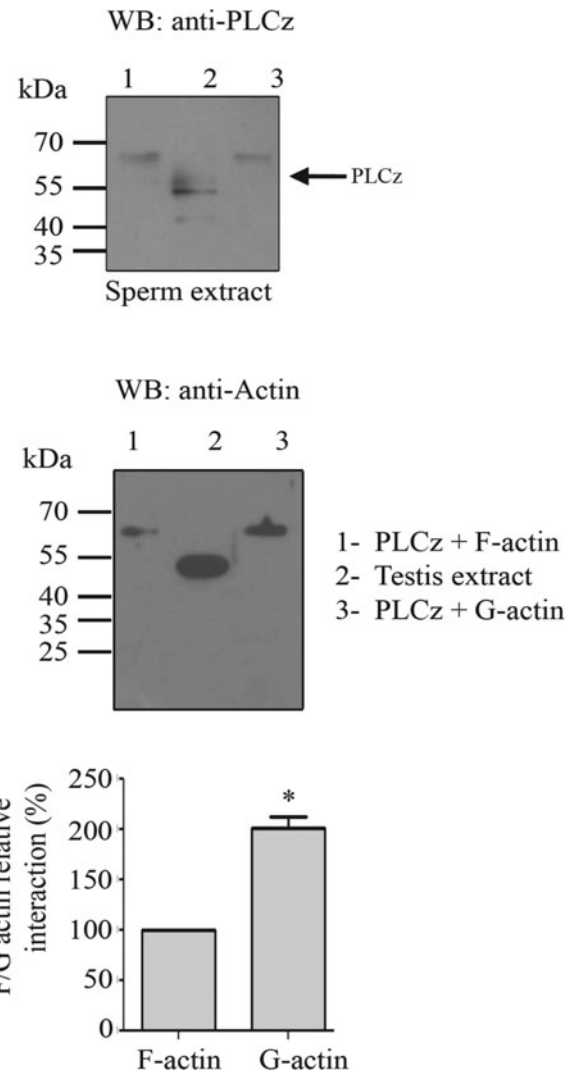


Figure 7 Far-western blot of PLC- ζ revealed with PLC ζ and actin. (Top/Middle) Western blot shows the sperm extract control used for PLC- ζ . Lane 1: peptide of PLC- ζ with F-actin; lane 2: control extract; and lane 3: PLC- ζ peptide with G-actin. (Bottom) Graph depicts densitometry showing the relative interaction of PLC- ζ with F-actin or G-actin. *Significant difference with $P < 0.001$.

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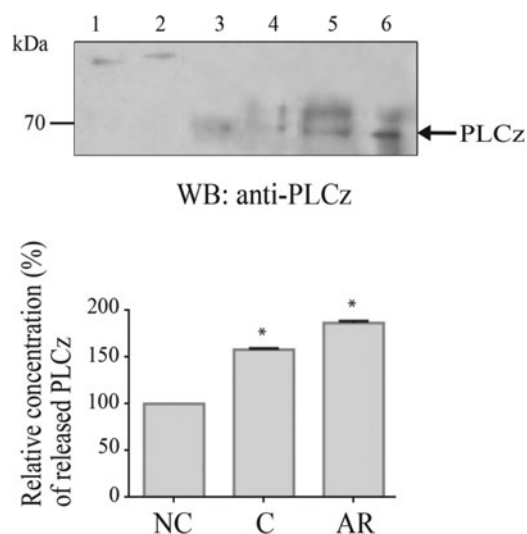


Figure 8 Western blot identifying PLC- ζ in the supernatants after the isolation procedure by affinity chromatography. (Top) Western blot shows the different controls for PLC- ζ : (1) brain extract, (2) testis extract, (3) supernatant non-capacitated sperm, (4) supernatant capacitated sperm, (5) supernatant capacitated sperm with acrosome reaction, and (6) sperm extract. (Bottom) Densitometry showing the percentage of relative PLC- ζ released. Lane 3 was defined as 100% and thus lane 4 was 154% and lane 5 was 186%. *Significant difference with $P < 0.001$.

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Statement of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical standards

The authors assert that all procedures handling animals in this study comply with the ethical standards of the 'Committee for Care and Use of Experimental Animals' at FMVZ/UNAM (CICUA), based on NOM-062-ZOO-1999.

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