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Identification of phospholipase activity in *Rhinella arenarum* sperm extract capable of inducing oocyte activation

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Summary

Egg activation, which includes cortical granule exocytosis, resumption and completion of meiosis and pronuclear formation culminates in the first mitotic cleavage. However, the mechanism through which the fertilizing sperm induces this phenomenon is still controversial. We investigated the effect of the microinjection of homologous sperm soluble fractions obtained by fast protein liquid chromatography (FPLC) from reacted sperm (without acrosome) and non-reacted sperm on the activation of *Rhinella arenarum* oocytes matured *in vitro*. The FPLC-purified sperm fraction obtained from reacted or non-reacted sperm is able to induce oocyte activation when it is microinjected.

This fraction has a 24 kDa protein and showed phospholipase C (PLC) activity *in vitro*, which was inhibited by D-609 but not by n-butanol or neomycin, suggesting that it is a PLC that is specific for phosphatidylcholine (PC-PLC). The assays conducted using inhibitors of inositol triphosphate (IP₃) and ryanodine receptors (RyRs) indicate that the fraction with biological activity would act mainly through the cADPr (cyclic ADP ribose) pathway. Moreover, protein kinase C (PKC) inhibition blocks the activation produced by the same fraction. Immunocytochemical studies indicate that this PC-PLC can be found throughout the sperm head.

Keywords: (???), Oocyte activation, PC-PLC, DAG *Rhinella arenarum*, Sperm factor

Introduction

An essential feature of fertilization is the activation of the oocyte, the process by which it can be transformed into an embryo. The earliest signalling event in the activation of an egg by a spermatozoon is a large transient increase in intracellular free calcium ion concentration (Runft *et al.*, 2002). In

many non-mammalian species, such as sea urchin and amphibian, the observed Ca²⁺ increase in the egg comprises a single transient rise but in mammals there is a series of repetitive Ca²⁺ oscillations (Swann *et al.*, 2004, Miyazaki & Ito, 2006).

In amphibians, this Ca²⁺ signal is sufficient to trigger the events associated with egg activation, including a depolarization of the egg membrane and the exocytosis of cortical granules (Talevi *et al.*, 1985; Bement & Capco, 1989; Oterino *et al.*, 2001; Runft *et al.*, 2004). This signal takes the form of a single wave, which passes across the egg from the site of sperm fusion (Jones, 1998).

In echinoderms, ascidians and some nematodes, such as *Caenorhabditis elegans*, egg activation is governed by similar mechanisms (Runft *et al.*, 2004; Bembenek *et al.*, 2007).

The Ca²⁺ increase at fertilization in frog eggs is thought to be mediated by inositol triphosphate (IP₃) receptors in the egg endoplasmic reticulum (Nuccitelli

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et al., 1993; Stith *et al.*, 1993; Snow *et al.*, 1996). This Ca^{2+} release is necessary and sufficient for the re-entry of the egg into the meiotic cell cycle (Douglas & Kline, 1992) and the start of embryonic development (Whitaker & Swann, 1993; Stricker, 1999).

The mechanism whereby the sperm triggers the release of Ca^{2+} in the egg has been a subject of controversy with opinions divided as to whether egg activation is initiated by a surface interaction between a sperm ligand and an egg receptor (Gould & Stephano, 1991; Mizote *et al.*, 1999; Dale & DeFelice, 2011) or by a cytosolic sperm factor that is released into the egg during gamete fusion (Dale *et al.*, 1985; Wilding *et al.*, 1997; Evans & Kopf, 1998; Wilding & Dale, 1998; Wolny *et al.*, 1999; Machaty *et al.*, 2000; Saunders *et al.*, 2002; Kurokawa *et al.*, 2004). In mammals, injection of the sperm extract into the unfertilized eggs causes intracellular Ca^{2+} release and Ca^{2+} oscillations (Parrington *et al.*, 1996, 1999). In intact eggs and egg homogenates, mammalian sperm extract triggers Ca^{2+} release by stimulating IP_3 production (Jones, 1998; Jones *et al.*, 1998, 2000; Rice *et al.*, 2000; Wu *et al.*, 2001; Saunders *et al.*, 2002), indicating the involvement of a phospholipase C (PLC) in the signal transduction mechanism (Sato *et al.*, 2000; Mehlmann *et al.*, 2001).

Different isoenzymes of PLC are capable of catalyzing the hydrolysis of phosphatidylinositol 2,5-bisphosphate (PIP_2), generating IP_3 and diacylglycerol (DAG). IP_3 regulates the release of Ca^{2+} from the endoplasmic reticulum (Singer *et al.*, 1997; Dale & DeFelice, 2011), while DAG is the physiological activator of PKC (Nishizuka, 1984; Bement, 1992). As the natural substrate used by PLC are phosphatidylinositol (PI) and phosphatidylcholine (PC), they can be classified into PI-PLC (specific for PI) and PC-PLC (specific for PC) (Hinkovska-Galchev & Srivastava, 1992; Szumilo & Rahden-Staroń, 2008). At present there is abundant knowledge of the properties and activation of PI-PLC, while there is scarce information concerning PC-PLC (Ramoni *et al.*, 2001, 2004).

Among the phospholipases that hydrolyze phosphatidylinositol we find $\text{PLC}\gamma$, which is activated by tyrosine phosphorylation (Runft & Jaffe, 2000) and $\text{PLC}\zeta$, which has been identified in mice, humans, monkeys, bovine and porcine (Cox *et al.*, 2002; Saunders *et al.*, 2002; Fujimoto *et al.*, 2004; Coward *et al.*, 2011).

Microinjection with $\text{PLC}\zeta$ recombinant or its RNA in mouse, bovine and human oocytes was capable of releasing Ca^{2+} from its reservoirs with pronucleus formation (Cox *et al.*, 2002; Saunders *et al.*, 2002; Fujimoto *et al.*, 2004; Larman *et al.*, 2004; Rogers *et al.*, 2004; Malcuit *et al.*, 2005).

In *Xenopus* oocytes, $\text{PLC}\gamma$ activation induced cortical granule exocytosis (Sato *et al.*, 2000, 2006; Carroll *et al.*, 1997).

The species-specific ryanodine receptor (RyR), which takes part in Ca^{2+} -induced Ca^{2+} release (CICR), is involved in the Ca^{2+} release from intracellular stores. Functional RyR/channel complexes seem to be present in the eggs of some species such as sea urchin, mice, bovine and humans (Ayabe *et al.*, 1995; Galione & Summerhill, 1995; Yue *et al.*, 1995), even if their role in Ca^{2+} signalling at fertilization is unclear (Galione *et al.*, 1993; Ayabe *et al.*, 1995; Berridge, 1997). RyR agonists include exogenous ryanodine and caffeine, and endogenous cyclic ADP ribose (c-ADP), a CICR modulator that has been proposed as second messenger (Galione *et al.*, 1993). In this sense another Ca^{2+} mobilizing messenger, cADPR, has also been shown to play a role during egg activation (Parrington *et al.*, 2007).

The participation of inositol trisphosphate and RyRs in *Rhinella arenarum* oocyte activation was demonstrated previously by Ajmat *et al.* (2011, 2013).

When sperm extract from chicken or *Xenopus laevis* is injected into mouse oocytes, it triggers Ca^{2+} oscillations similar to those found during fertilization (Busa *et al.*, 1985; Dong *et al.*, 2000). However, to date, there have been no reports that sperm extract from amphibians such as *Rhinella arenarum* can trigger Ca^{2+} release in homologous oocytes. In addition, it remains far from clear whether such sperm factor activities are functions of a PLC or are caused by a different signalling protein.

In *Rhinella arenarum*, the mechanism by which the oocytes are activated has not been elucidated yet. However, Bonilla *et al.* (2008) demonstrated the presence in sperm extract of a 24 kDa protein able to induce activation when it was microinjected into the oocytes.

The purpose of this work was to isolate and characterize the components of *Rhinella arenarum* sperm extract that have the capacity to induce activation when microinjected into homologous oocytes, the participation of IP_3R , RyR and PKC in the process and the location of the active factor in the sperm.

Materials and methods

Animals

Sexually mature *Rhinella arenarum* males and females were collected in the northwestern area of Argentina and kept at 15 °C until use, which generally took place 15 days after collection.

Sperm extract preparation

Sperm suspensions were obtained by gently disrupting the testes in 4 ml Ringer solution (AR) and centrifuging at 1085 g for 10 min; then, the pellet was

resuspended in AR. A swim-up procedure was used for the selection of motile sperm. The high quality sperm suspension was centrifuged at 1085 g for 10 min. Sperm were resuspended in calcium-free Tris-saline buffer (7.59 g NaCl/l, 2.40 g Tris/l-HCl, pH 7.4). The number of sperm was adjusted to 1×10^9 sperm/ml. The sample was separated into two aliquots. The first was processed immediately. The second was placed in 10% AR, incubated for 4 h to induce the acrosome reaction (Raisman *et al.*, 1980; Martinez & Cabada, 1996; Krapf *et al.* 2009) and washed in Tris-saline solution.

Both samples were lysed by three cycles of freezing (-196°C) and thawing (25°C). The lysates were centrifuged for 30 min at 16,000 g at 6°C and the supernatant was collected as crude sperm extract. The extracts were observed under a microscope to check that intact sperm or sperm heads were not present. The protein concentration of the samples was adjusted to $9.0 \mu\text{g}/\mu\text{l}$.

The sperm extract obtained from intact and reacted sperm was run through size exclusion chromatography using a Bio-Gel P-60 chromatography column. The column was washed with calcium-free Tris-saline buffer and the 27 fractions obtained were stored at -20°C until use.

The biological activity of all fractions was assayed according to Bonilla *et al.* (2008).

Fast protein liquid chromatography (FPLC)-mono Q column

The fraction with biological activity (fraction 18 from size exclusion chromatography) was submitted to a FPLC-Mono Q column. The chromatographic columns were washed with Ca^{2+} -free buffer. The proteins were eluted with elution buffer (Tris 20 mM, NaCl 1 M and HCl pH 7) at a flow rate of 0.1 ml/min and detected at OD_{280} .

The sample obtained from FPLC was dialyzed and concentrated by evaporation of solvents in SAVANT SpeedVac® Concentrators. The protein concentration of the samples was adjusted to $0.1 \mu\text{g}/\mu\text{l}$ with Tris-saline and tested for biological activity.

Oocyte isolation and maturation

Denuded oocytes were obtained according to Lin & Schuetz (1985). Follicle cells were removed by incubation of defolliculated oocytes in AR (6.6 g NaCl/l, 0.15 g CaCl_2 /l, 0.15 g KCl/l) containing penicillin G-sodium (30 mg/l) and streptomycin sulphate (50 mg/l), pH 7.4, for 5 min with gentle shaking (100 oscillations/min). Denuded oocytes were kept in AR until use.

Maturation was induced by progesterone treatment ($2.5 \mu\text{M}$) and scored for the presence of a transient white spot in the animal pole.

In vitro cultures were carried out at room temperature ($22-25^\circ\text{C}$) using plastic multiwell culture dishes (Costar 3524, Cambridge, MA, USA). Randomized samples of 20 freshly denuded oocytes were distributed into separate wells containing 2 ml of AR; the reagents were added ($5 \mu\text{l}$) directly to the culture medium. Two well duplicates were routinely run in each experimental group.

Microinjection

The different fractions from the sperm extract were microinjected into *in vitro* matured oocytes to test for biological activity using intracytoplasmic sperm injection (ICSI) micropipettes (Humagen™ Fertility Diagnostics) attached to a micromanipulator (Leitz). The injection volume was 30 nl. The microinjections were carried out in calcium-free Tris-buffered saline (7.59 g NaCl/l, 2.40 g Tris/l-HCl, pH 7.4) at 20°C . Following injection, the oocytes were kept in Tris-buffered saline. Injection of Tris-buffered saline alone was used as control.

The injection volume was approximately 30 nl corresponding to the 0.10% of the oocyte volume. The amount of protein microinjected per oocyte was $3.0 \mu\text{g}$. In all the experiments, the oocytes were injected into the animal hemisphere. Activation was scored 20 min after injection.

We considered as activation parameters the disappearance of the white spot, the elevation of the vitelline envelope and the exocytosis of the cortical granules.

For pronuclear observation, oocytes were fixed 120 min after injection.

Cytological preparations

The oocytes were fixed in Ancel and Vintemberger's solution (10% formol; 0.5% acetic acid and 0.5% NaCl), embedded in paraffin and sliced into $7\text{-}\mu\text{m}$ thick sections that were then stained with Ehrlich hematoxylin and eosin. This method allowed us easily to observe the pronucleus (Bühler *et al.*, 1994).

Determination of enzymatic activity of F_1

Phospholipase activity was determined by the effect of F_1 ($0.1 \mu\text{g}/\mu\text{l}$) on PC ($0.30 \mu\text{M}$) and phosphatidylinositol (PI) ($0.50 \mu\text{M}$).

PC and PI were incubated with F_1 for 60 min at 37°C . The lipids obtained were extracted and subjected to thin layer chromatography (TLC).

Thin layer chromatography (TLC)

In total, $20 \mu\text{l}$ of the fraction with biological activity obtained with the FPLC (Fraction F1) was incubated in $300 \mu\text{l}$ Dulbecco's phosphate-buffered saline (PBS; Irvine Scientific®) with PC ($0.26 \mu\text{mol}$) and PI

(0.50 μmol) at 37 °C for 60 min. The products generated during the reaction were studied using TLC. Extractions of lipids from the products were performed with the solvent combination chloroform:methanol (2:1) using the Folch technique (Folch *et al.*, 1957). The sample was shaken and equilibrated. The mixture partitioned into two layers, the lower one containing virtually all the lipids. This layer was treated with nitrogen vapors to dryness and resuspended with chloroform.

The chloroform fraction was put on silica gel plates that were then placed in a TLC tank. The phospholipids were separated by TLC. The plate for polar lipids was developed in one dimension with chloroform/methanol/acetic acid. Lipids patterns are chromatographed in adjacent lanes.

The presence of lipids was revealed using iodine vapours (Minahk *et al.*, 2008).

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of sperm fractions was performed according to Laemmli (1970) with 7.5% running gels. Proteins were stained with silver staining (Richert *et al.*, 2004).

Hydrolysis of egg yolk

In order to test the phospholipase activity of Fraction F₁, 20 μl of different dilutions of F₁ were seeded in 2 mm thick wells on an egg yolk agar plate at 1% (2 mm thick). Serial dilutions were made from the F₁ fraction (protein concentration 0.1 $\mu\text{g}/\mu\text{l}$). The Petri plate was kept at 37 °C for 60 min. At the end of this period, the halos caused by the hydrolysis were measured. As a positive control we used PC-PLC from *Bacillus cereus* (final concentration 0.1 $\mu\text{g}/\mu\text{l}$) and Tris-saline as a negative control.

Effect of phospholipase inhibitors on F₁ activity

Fraction F₁ was pre-treated with different phospholipase inhibitors for 30 min and then incubated with commercial PC for 60 min. The lipids produced were extracted and TLC for neutral lipids was performed. The following were used as inhibitors: n-butanol (50 $\mu\text{g}/\text{ml}$), neomycin (5 mM) and D-609 (50 $\mu\text{g}/\text{ml}$).

Participation of IP₃R, RyR and PKC on F₁ activity

The assays were carried out by microinjecting the inhibitors into the mature oocyte. Ten min after the injection of the inhibitor the activation of the oocytes was induced by injecting the biologically active fraction (F₁) at a concentration of 0.01 mg/ml. Activation was assessed after 20 min.

Immunocytochemical studies

The cellular location of phospholipase with biological activity was studied using for this purpose a polyclonal antibody specific for phosphatidylcholine-specific PLC. An aliquot of the reacted sperm suspension was extended for processing glass slides and another aliquot was kept overnight in methanol to facilitate permeabilization of the sperm (Sheikhnejad & Srivastava, 1986).

Smears from both samples were washed in Dulbecco's PBS and then incubated in blocking solution (PBS-2% BSA) for 30 min. The first incubation was performed with the primary antibody anti-PC-PLC obtained from *Bacillus cereus* at a 1:100 dilution of serum from immunized rabbits (New Zealand variety). Antibodies (IgG) were isolated and purified from antiserum by column chromatography on an Affi-Prep Protein A matrix.

As the negative control we incubated 12-h smears processed in the same way but without the primary antibody. Mouse embryo fibroblasts were used as the positive control (data not shown). Smears were washed with PBS. The second incubation was performed with a secondary antibody (anti-rabbit IgG) at 1:2000 dilution.

After washing with PBS the samples were incubated with streptavidin-alkaline phosphatase for 30 min. at 25 °C. We performed a final wash with PBS and nitroblue tetrazolium (NBT) was added prepared from 0.023 g/ml. The reaction was stopped with the addition of distilled water.

Reagent

Progesterone, n-butanol, neomycin, phospholipids, ruthenium red, heparin, 1,5-isoquinolinesulfonyl-2-methylpiperazine (H7) and PC-PLC from *Bacillus cereus* were provided by Sigma and D-609 by Calbiochem.

Anti-PC-PLC antibody was obtained from rabbits immunized with PC-PLC from *Bacillus cereus* kindly provided by Dr H. Borsetti, University of Houston, USA.

Results

Size exclusion chromatography of sperm extract

Crude sperm extract of intact sperm and acrosome-reacted sperm, obtained according to Materials and Methods, were subjected to chromatography. Twenty-seven fractions were obtained. As shown in Fig. 1, the chromatogram exhibited differences in peak I in the extract obtained from intact sperm or from acrosome-reacted sperm, the other peaks being similar. All

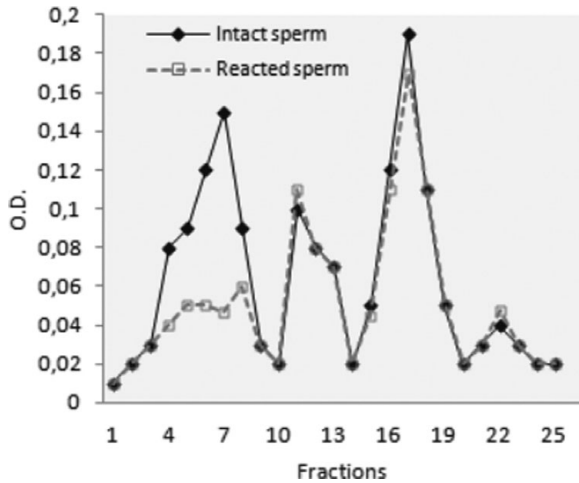


Figure 1 Size exclusion chromatography of intact sperm extract and acrosome-reacted sperm extract. The solid line indicates intact sperm extract (—) and the broken line indicates acrosome-reacted sperm extract (---). The protein was detected at OD₂₈₀. Aliquots of all the fractions were injected into oocytes matured *in vitro*. Only fraction 18, corresponding to peak III of the reacted and non-reacted sperm, was able to induce activation when it was microinjected into the oocytes matured *in vitro* (+).

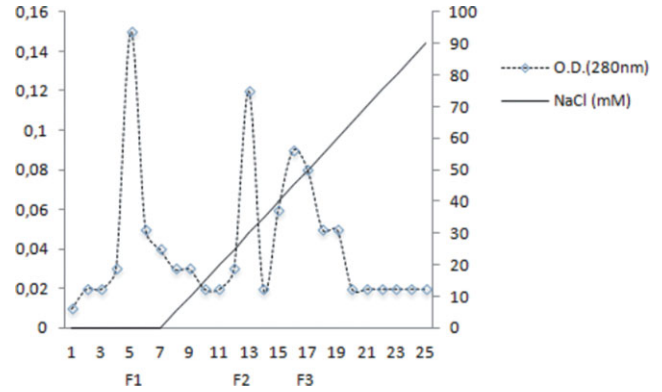


Figure 2 Mono Q column using an fast protein liquid chromatography (FPLC) system of fraction 18 from size exclusion chromatography of sperm extract. Fractions (0.30 ml) from each peak were collected (F₁, F₂, F₃) and concentrated to give a final protein concentration of 0.1 µg/µl.

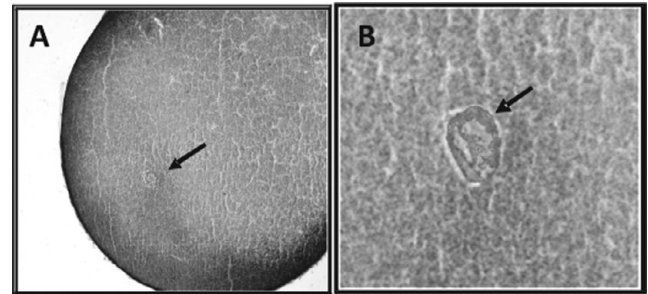


Figure 3 Cytological preparations. (A) Formation of female pronucleus 120 min post-microinjection of F₁ (magnification ×400). (B) Hematoxylin-eosin (magnification ×1000). arrow

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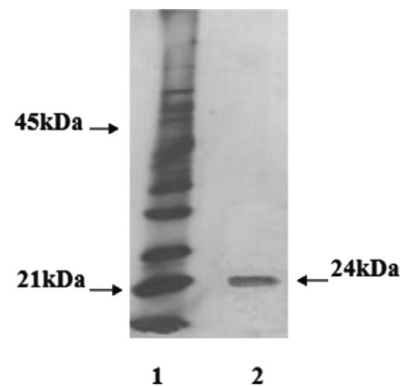


Figure 4 Electrophoresis of F₁. Lane 1, Molecular weight markers. Lane 2, F₁ from the Mono Q column.

fractions were microinjected into mature oocytes to test for biological activity.

Only fraction 18, corresponding to peak III of reacted and non-reacted sperm, was able to induce activation when injected into mature oocytes.

FPLC of fraction 18

Fraction 18 (with biological activity) obtained from size exclusion chromatography was subjected to a Mono Q column using an FPLC system. As shown in Fig. 2, three peaks (F₁, F₂, F₃) were obtained with this procedure. The first peak corresponds to washing the column. The fractions belonging to the three peaks were injected to determine their ability to activate mature oocytes. Activation was scored 20 min after injection and pronuclear development was observed after 120 min of culture (Fig. 3). Only the first peak (F₁) of the Mono Q column using FPLC showed biological activity when it was microinjected. The activity of F₁ disappeared when it was heated for 3 min at 90 °C. Microinjections with Tris-saline had no effect on oocyte activation.

Biochemical characterization of F₁

Electrophoretic analysis of the active sperm fractions F₁
F₁ from FPLC, which exhibited highest biological activity, were analyzed by SDS-PAGE.

Electrophoretic profiles obtained by SDS-PAGE (Fig. 4) indicate that the fraction with biological activity (F₁) is a protein of approximately 24 kDa and isoelectric point (IEP) of 8.8 (Fig. 5).

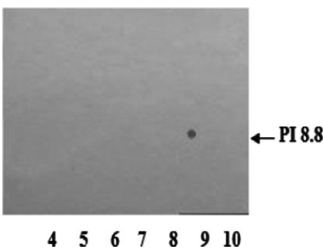


Figure 5 Isoelectric point of F_1 . F_1 was subjected to bidimensional electrophoresis. Silver staining.

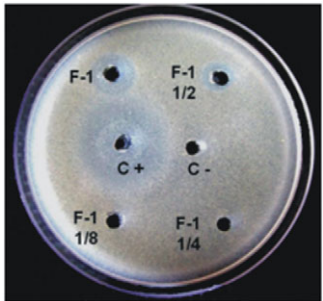


Figure 6 Effect of different F_1 concentrations on egg lipids. F_1 was seeded on egg yolk agar. The halo produced by F_1 was measured at 60 min.

Determination of phospholipase activity of F_1
In order to determine if F_1 had phospholipase activity, different concentrations of F_1 were seeded in a Petri dish with egg yolk agar at 1%.

As shown in Fig. 6, the halo around the wells indicates hydrolysis on the phospholipids present in the egg yolk in a dose-dependent manner, suggesting the presence in F_1 of a protein with phospholipase activity.

Effect of F_1 on different phospholipids
TLC from hydrolytic products from F_1 on PC and PI are shown in Fig. 7. Results indicated that F_1 hydrolyzed only phosphatidylcholine with neutral lipids formation without generating lyso-PC. In addition, F_1 was unable to hydrolyze PI. These results indicate that F_1 would not have PLA₂ activity.

The analysis of the neutral lipids generated by the effect of F_1 on PC (Fig. 8) showed that F_1 was able to hydrolyze PC with DAG production, which would indicate that it has PLC activity.

Treatment of fraction F_1 with phospholipase inhibitors
The ability of F_1 to hydrolyze PC in the presence of different inhibitors was analyzed. Fraction F_1 was pretreated for 30 min with different phospholipase inhibitors: neomycin (5 mM), a specific inhibitor of PI-PLC, D-609 (50 μ g/ml), a specific inhibitor of PC-

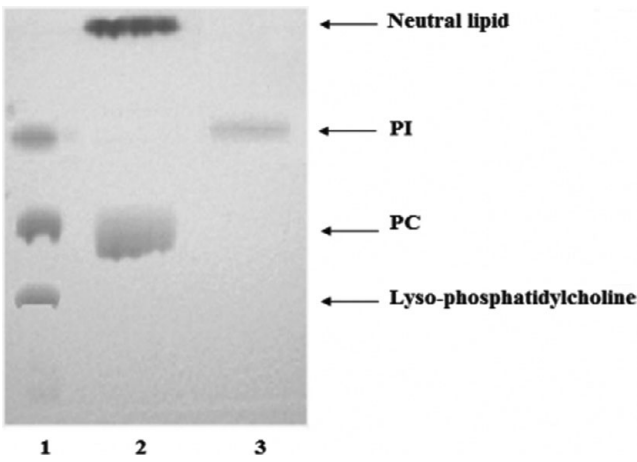


Figure 7 Thin layer chromatography (TLC) of hydrolysis products from phosphatidylcholine (PC) and phosphatidylinositol (PI). PC and PI were exposed to F_1 for 60 min at 37°C. The lipids obtained were recovered in chloroform//methanol and processed with thin layer chromatography. Lane 1, markers (lyso-PC, PC and PI). Lane 2, product of hydrolysis from PC. Lane 3, product of hydrolysis from PI.

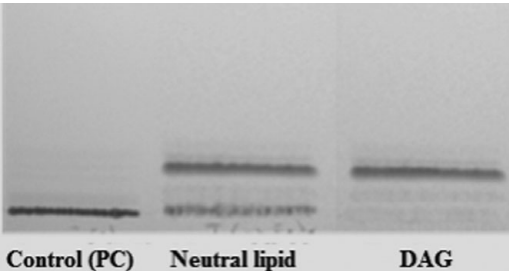


Figure 8 Thin layer chromatography (TLC) of neutral lipids formed in the incubation of phosphatidylcholine (PC) with F_1 . The neutral lipids formed in the incubation of PC with F_1 were subjected to thin layer chromatography. TLC revealed the presence of 1,2 diacylglycerol. Commercial diacylglycerol (DAG; Sigma) was used as a control.

PLC, and n-butanol (50 μ g/ml), a specific inhibitor of phospholipase D (PLD).

The results in Fig. 9 show that the activity of F_1 was inhibited by D-609 but not by neomycin or n-butanol.

The lack of inhibition by n-butanol or neomycin suggests that F_1 is not a PLD or a PLC specific for PI. The fact that the action of F_1 was inhibited only by D-609 suggests that it would be a PLC specific for PC.

Effect of PC-PLC from Bacillus cereus on oocytes matured in vitro

In order to determine whether a commercial PC-PLC has the same effect as the F_1 fraction, the oocytes

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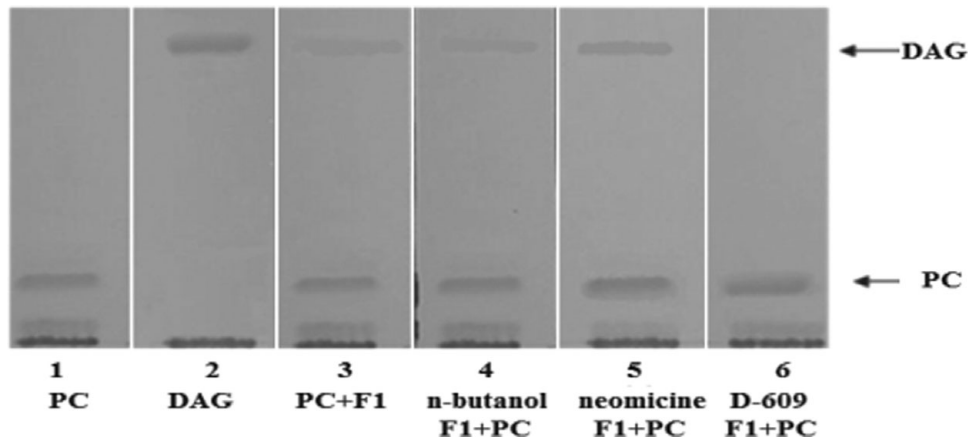


Figure 9 Thin layer chromatography (TLC) of the products resulting from the effect of F_1 on PC in the presence of inhibitors. 1, phosphatidylcholine; 2, diacylglycerol (DAG) as control; 3, hydrolysis products of phosphatidylcholine by F_1 ; 4, phosphatidylcholine, n-butanol and F_1 ; 5, phosphatidylcholine, neomycin and F_1 ; 6, phosphatidylcholine, D-609 and F_1 .

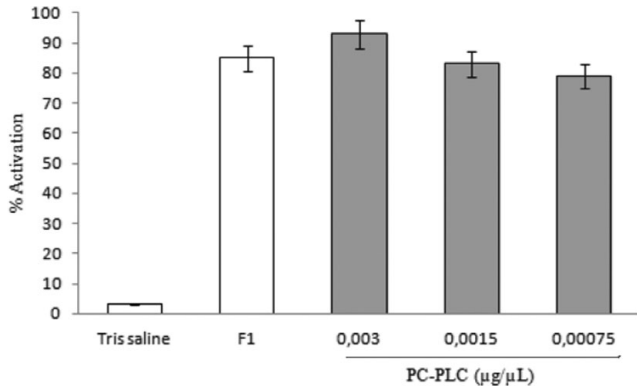


Figure 10 Effect of microinjection of phospholipase C (PLC) that is specific for phosphatidylcholine (PC-PLC) from *Bacillus cereus*. Oocytes matured *in vitro* were injected with 30 nl of PC-PLC from *Bacillus cereus* (0.003, 0.0015, 0.00075 µg/µl). Values are the mean \pm standard error of the mean (SEM) of three experiments. Each experiment was performed on a different animal.

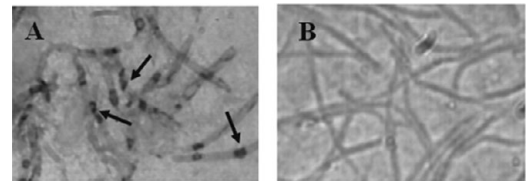


Figure 11 (A) Immunolocalization of phospholipase C (PLC) that is specific for phosphatidylcholine (PC-PLC) in *Rhinella arenarum* spermatozoa (magnification $\times 1600$). (B) Negative control (magnification $\times 1600$). Arrows indicate the presence of PC-PLC in sperm heads.

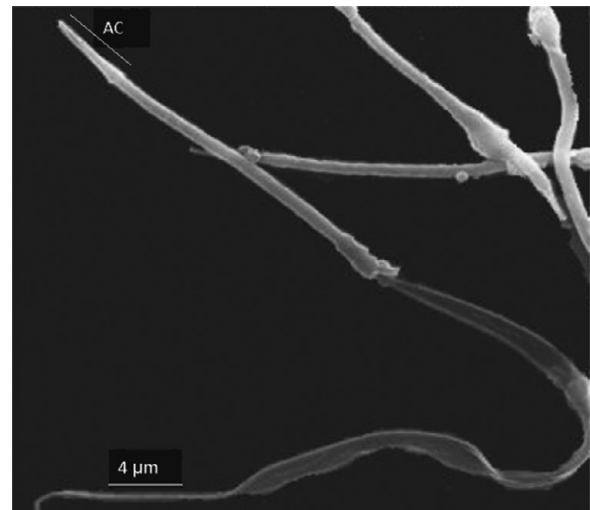


Figure 12 Scanning micrograph of *Rhinella arenarum* sperm. Magnification $\times 3600$. AC, acrosome.

matured *in vitro* were injected with different concentrations of PC-PLC (0.003; 0.0015, 0.00075 µg/µl) and activation was scored 20 min after injection.

The results obtained (Fig. 10) indicated that the injection of this enzyme induced activation at percentages similar to the ones obtained by injection of the F_1 fraction.

Immunolocalization of PC-PLC

The cellular location of the PC-PLC in sperm was studied using antibodies obtained by immunization of rabbits with PC-PLC from *Bacillus cereus*. The assays were performed with sperm obtained by swim-up method and permeabilized according to Materials and methods. The images reveal the presence of PC-PLC distributed throughout the sperm head showing a

discontinuous pattern (Fig. 11). The acrosomal status was confirmed by scanning electron microscopy from aliquots of sperm suspensions (Fig. 12).

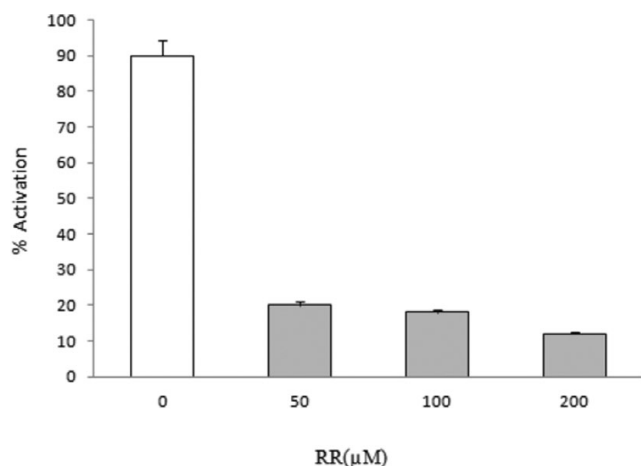


Figure 13 Inhibition of the activation produced by F_1 with ruthenium red (RR). The oocytes were preinhibited with RR and subsequently microinjected with fraction F_1 . Activation was measured at 20 min.

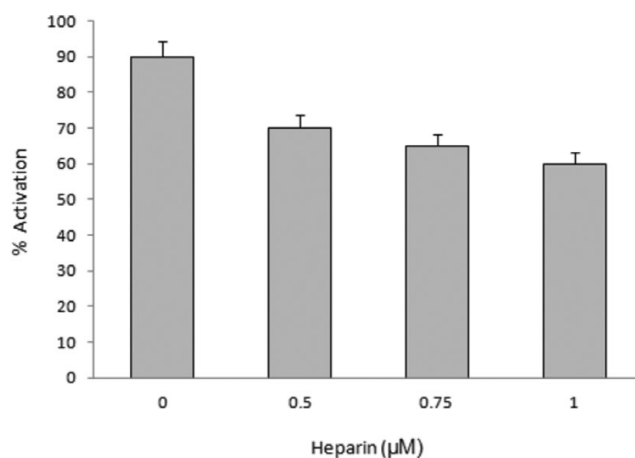


Figure 14 Inhibition of the inositol triphosphate (IP_3) receptor on the activation induced by microinjection of F_1 . The oocytes were preinhibited with heparin and subsequently microinjected with fraction F_1 . Activation was measured at 20 min.

Participation of IP_3R and RyR on oocytes activation induced by F_1

Oocytes matured *in vitro* were microinjected with different heparin doses (0–1 μ M), antagonist of the IP_3 receptor or ruthenium red (50–200 μ M) antagonist of RyR for 15 min. Then they were microinjected with F_1 (0.01 mg/ml). The apparition of signs of activation was assessed after 20 min. The results in Fig. 13 indicate the inhibition of the RyR significantly blocks the activation induced by F_1 in a dose-dependent manner, suggesting the participation of this receptor in the process.

In the case of the inhibition of the IP_3/IP_3 receptor, a decrease was observed in the percentage of activation induced by F_1 (30%) after treatment with heparin, but activation was not blocked, suggesting that the participation of this receptor would not be critical in the process (Fig. 14).

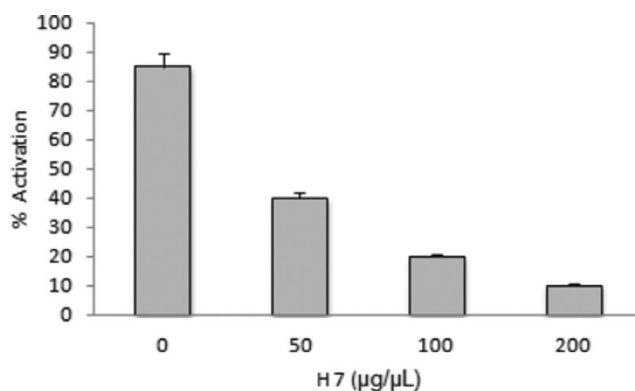


Figure 15 Effect of inhibition of protein kinase C (PKC) on the activation induced by microinjection of F_1 . H7 was microinjected (0, 50, 100 or 200 μ g/ μ L) and 20 min later activation was induced with F_1 .

Effect of inhibition of PKC on oocytes activation induced by F_1

Taking into account that PC-PLC produces DAG, a PKC activator, we studied the effect of the inhibition of this kinase on the activation induced by F_1 injection.

We used 1,5-isoquinolinesulfonyl-2-methylpiperazine (H7) as PKC inhibitor. The oocytes pretreated with H7 for 15 min were microinjected with F_1 (0.01 mg/ml). The signs of activation were assessed 20 min after F_1 injection. The results (Fig. 15) showed that H7 inhibited the activation induced by F_1 in a dose-dependent manner.

Discussion

During fertilization, sperm fusion triggers an increase in egg cytosolic Ca^{2+} that traverses the egg in a wave-like fashion (Nuccitelli *et al.*, 1993; Horner & Wolfner, 2008). These transient increases in $[Ca^{2+}]_i$ are essential for cortical granule exocytosis, meiosis resumption and pronuclear formation (Bement & Capco, 1989; Oterino *et al.*, 1997; Ducibella *et al.*, 2002). The rise in IP_3 concentration is responsible for inducing Ca^{2+} release from the endoplasmic reticulum (ER) by binding to the IP_3 receptor (Jaffe *et al.*, 2001). The increase in IP_3 is caused mainly by the hydrolysis of phosphatidyl 4,5-bisphosphate (PIP_2) by a family of specific phospholipases.

Our results indicated the presence of a 24 kDa protein with PLC activity in the biologically active sperm fraction of *Rhinella arenarum*.

The experiments *in vitro* using phospholipid inhibitors indicated the presence of PLC activity specific for phosphatidylcholine (PC-PLC) in the biologically active fraction of the sperm extract of *Rhinella arenarum*. It is interesting to note that the biological activity is similar in the whole sperm extract and in the reacted sperm extract, which would indicate that this enzyme would not be an acrosomal enzyme.

This type of phospholipase (PC-PLC) has been identified in preparations from bull sperm (Sheikhnejad & Srivastava, 1986; Hinkovska-Galchev & Srivastava, 1992).

The concentration of phospholipids present in the mature oocytes of *Rhinella arenarum* is as follows: phosphatidylcholine 186.23 nmol/mg of protein, phosphatidylethanolamine 41.2 nmol/mg of protein, phosphatidylinositol 11.3 nmol/mg of protein, sphingomyelin 8.5 nmol/mg of protein, phosphatidylserine 4.0 nmol/mg of protein (Sánchez Toranzo *et al.*, 2007). Phosphatidylcholine, the most abundant phospholipid in this species, plays a major role in intracellular signalling methods and is a source of lipidic second messengers such as DAG, phosphatidic acid (PA) and arachidonic acid (AA) (Sánchez Toranzo *et al.*, 2007; Mateos *et al.*, 2008).

Stith *et al.* (1993) showed that DAG and IP₃ levels increase during *Xenopus* egg activation (Berridge, 1984). Their work indicates that *Xenopus* fertilization triggers a large increase in egg DAG (two- to four-fold) and IP₃ (up to seven-fold). DAG increase is rapid, occurring within a minute of sperm addition, as would be expected if PKC activation controlled events such as cortical granule (CG) exocytosis and cortical contraction (Bement & Capco, 1989; Bement, 1992; Douglas & Kline, 1992; Xue-qing *et al.*, 2006). They also found that approximately 99% of the DAG increase was derived from non-PIP₂ sources. DAG may also act as a fusogen during cortical granule-plasma membrane fusion (Whitaker & Zimmerberg, 1987; Petcoff *et al.*, 2008). The DAG mass increases from 48 pmol to 110 pmol/cell post fertilization (Stith *et al.*, 1997).

These previous data support our results, in which we describe the presence in the *Rhinella arenarum* sperm extract of a protein with PC-PLC activity. This enzyme, which presents PLC activity *in vitro*, is capable of forming DAG and is inhibited by D-609 but not by neomycin or n-butanol. D-609 is a potent inhibitor of PC-PLC, while neomycin and n-butanol are specific inhibitors of PI-PLC and of PLD respectively. This result suggests that the biologically active protein in the sperm extract would be a PC-PLC. DAG production by the effect of the PC-PLC

present in the sperm extract would be responsible for the activation of the homologous oocytes.

Our results of the injection of a commercial PC-PLC into mature oocytes, which induced activation at percentages similar to the ones obtained by the injection of the F₁ fraction, confirm the presence of a PC-PLC in the active fraction of sperm extract in this species. However, the molecular weight of the protein isolated in our active fraction is 24 kDa, quite small if we compare it with the 74 kDa of the PLC ζ described in mammals (Kurokawa *et al.*, 2007), or with the 66 kDa of the PC-PLC of mouse fibroblasts (Ramoni *et al.*, 2004, Spadaro *et al.*, 2006) or with the 40 kDa of human PC-PLC (Ramoni *et al.*, 2001; Roldan & Shi, 2007; Szumilo & Rahden-Staron, 2008). It is possible that PC-PLC may have undergone physiological proteolysis due to the extraction methodology. In this sense, Kurokawa *et al.* (2007) demonstrated that, in mammals, PLC fragments (27 kDa) in sperm factor retain activity, suggesting that spontaneous proteolysis of PLCs may play a physiological role in cellular function.

The finding that PLC fragments consistent with enzyme cleavage can be observed in fresh intact sperm and in sperm extracts (Kurokawa *et al.*, 2005, 2007) suggests that the 24 kDa protein with PC-PLC activity identified in the *Rhinella arenarum* sperm extract could also be a product of the proteolysis of the enzyme.

In sea urchin eggs, *in vivo* studies have demonstrated that PKC phosphorylates and solubilizes the sperm nuclear lamina in a Ca²⁺-dependent manner (Stephens *et al.*, 2002), and that PKC is required for the eggs respiratory burst after activation (Heinecke & Shapiro, 1992).

Other reports on mammalian eggs have attributed a role to PKC in the regulation of cortical granule exocytosis, resumption of the cell cycle, and second polar body formation at fertilization (Halet *et al.*, 2004). In sea urchins, DAG may also contribute to Ca²⁺ release from the ER by stimulating a pathway leading to the production of cyclic ADP ribose, which mobilizes Ca²⁺ from RyRs on ER (Horner & Wolfner, 2008; Dale & DeFelice, 2011).

Our results indicated that the inhibition of PKC by treatment with H-7 inhibits the activation produced by the sperm fraction, suggesting that this second messenger is implicated in the signalling route (Fig. 15), which would support the idea that in the biologically active fraction of sperm extract there would be a protein with PC-PLC activity that would be responsible for inducing the activation in the mature oocytes of *Rhinella arenarum*.

In the mature oocytes of *Rhinella arenarum* the presence of RyRs has been described, their participation in the activation process having also been demonstrated (Ajmat *et al.*, 2013). Our results show that if the RyRs is blocked, F₁ is unable to induce activation,

thus confirming the importance of this receptor in the activation of the oocytes of this species. There is data from other species that support this pathway, since the microinjection of cyclic ADP ribose or ryanodine in mouse oocytes is capable of inducing oocyte activation (Ayabe *et al.*, 1995).

The immunomarkation of *Rhinella arenarum* sperm with anti-PC-PLC antibodies shows that the PC-PLC is distributed in the whole sperm head and that it is intracytoplasmic, since in the assays performed with non-permeabilized sperm no signalling was observed (Hinkovska-Galchev & Srivastava, 1992).

The PLC δ was found in the lipid rafts present in the plasma membrane, as reported previously by Yamaga *et al.* (2007).

The heterogeneous distribution of immunostaining in the head of *Rhinella arenarum* sperm (Fig. 11) could be attributed to the presence of PC-PLC related to lipid rafts (Kawano *et al.*, 2011).

In conclusion, in this study we demonstrated that a fraction of the extract of reacted sperm of *Rhinella arenarum* purified by FPLC was able to induce activation when it was microinjected into homologous oocytes matured in vitro and has a 24 kDa protein with PLC activity specific for phosphatidylcholine. Immunolocalization revealed that the enzyme is found throughout the sperm head. The transduction pathway of this enzyme involves de activation of PKC and the mayor participation of RyRc in the release mechanisms of Ca²⁺.

Although the amount of proteins microinjected into the oocytes is greater than the amount present in a single spermatozoon, the present work proposes a mechanism of oocyte activation with a future biotechnological application that directly involves a phospholipase specific for PC.

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