Zygote **22** (November), pp. 483–495. © Cambridge University Press 2013
doi:10.1017/S0967199413000348 First Published Online 9 September 2013

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

Federico Bonilla[1], *Carlos Minahk*[3], *María Teresa Ajmat*[2], *Graciela Sánchez Toranzo*[2] *and Marta Inés Bühler*[2]

Departamento de Biología del Desarrollo-INSIBIO. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán; and Departamento de Química Biológica – Instituto Superior de Investigaciones Biológicas-INSIBIO, Universidad Nacional de Tucumán, Tucumán, Argentina

Date submitted: 17.09.2012. Date revised/accepted: 22.11.2012.

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 (IP_3) 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: Diacylglycerol, Oocyte activation, PC-PLC, *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; Dale *et al.*, 2010). In many non-mammalian species, such as sea urchin and amphibian, the observed Ca^{2+} increase in the egg comprises a single transient rise but in mammals there is a series of repetitive Ca^{2+} oscillations (Swann *et al.*, 2004, Miyazaki & Ito, 2006).

In amphibians, this Ca^{2+} 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 Ca^{2+} increase at fertilization in frog eggs is thought to be mediated by inositol triphosphate (IP_3) receptors in the egg endoplasmic reticulum (Nuccitelli

[1]All correspondence to: Federico Bonilla. Departamento de Biología del Desarrollo, Instituto Superior de Investigaciones Biológicas (INSIBIO), Chacabuco 461–2° piso, T4000ILI San Miguel de Tucumán, República Argentina. Tel: +54381 4247752 int. 7093. Fax: +54 381 4247752 int. 7004. e-mail: bonilla@fbqf.unt.edu.ar
[2]Departamento de Biología del Desarrollo-INSIBIO. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán, Chacabuco 461 (4000), Tucumán, Argentina.
[3]Departamento de Química Biológica - Instituto Superior de Investigaciones Biológicas-INSIBIO. Universidad Nacional de Tucumán, Chacabuco 461 (4000), Tucumán, Argentina.

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-biphosphate (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; Szumiło & 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 PLCγ, which is activated by tyrosine phosphorylation (Runft & Jaffe, 2000) and PLCζ, 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 PLCζ 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, PLCγ activation induced cortical granule exocytosis (Sato *et al.*, 2000, 2006; Carroll *et al.*, 1997).

The species-specific ryanodine receptor (RyRc), 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). RyRc 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

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

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 μg/μl.

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 μg/μl 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 μM) 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 °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 μl) 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 μg. 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-μm 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 μg/μl) on PC (0.30 μM) and phosphatidylinositol (PI) (0.50 μM).

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 μl of the fraction with biological activity obtained with the FPLC (Fraction F1) was incubated in 300 μl Dulbecco's phosphate-buffered saline (PBS; Irvine Scientific®) with PC (0.26 μmol) and PI

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

(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 F_1, 20 μl of different dilutions of F_1 were seeded in 2 mm thick wells on an egg yolk agar plate at 1% (2 mm thick). Serial dilutions were made from the F_1 fraction (protein concentration 0.1 μg/μl). 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 μg/μl) and Tris-saline as a negative control.

Effect of phospholipase inhibitors on F_1 activity

Fraction F_1 was pre-treated with different phospholipase inhibitors for 30 min and then incubated with comercial 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 μg/ml), neomycin (5 mM) and D-609 (50 μg/ml).

Participation of IP_3R, RyR and PKC on F_1 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 (F_1) 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

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

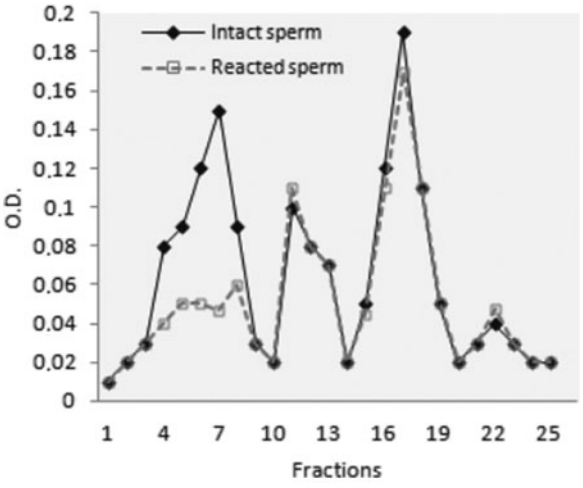

Figure 1 Size exclusion chromatography of intact sperm extract and acrosome-reacted sperm extract. The solid line indicates intact sperm extract (–) and the broken line indicates acrosome-reacted sperm extract (– –). The protein was detected at OD_{280}. Aliquots of all the fractions were injected into 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* (+).

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 F_1

Electrophoretic analysis of the active sperm fractions F_1

F1 from FPLC, which exhibited highest biological activity, were analyzed by SDS-PAGE.

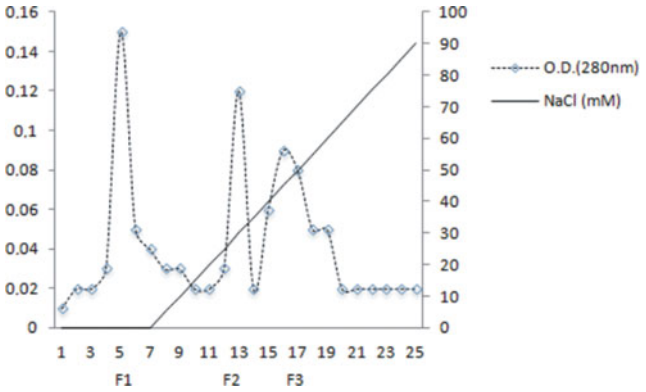

Figure 2 Mono Q column using an fast protein liquid chromatography (FPLC) system of fraction 18 from size exclusion chromatography of sperm extract. Fractions (0.30 ml) from each peak were collected (F_1, F_2, F_3) and concentrated to give a final protein concentration of 0.1 μg/μl.

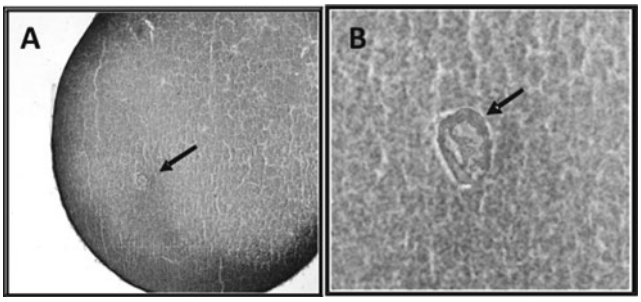

Figure 3 Cytological preparations. (*A*) Formation of female pronucleus 120 min post-microinjection of F_1 (magnificatioin ×400). (*B*) Hematoxylin–eosin (magnification ×1000). Arrows show the pronucleus.

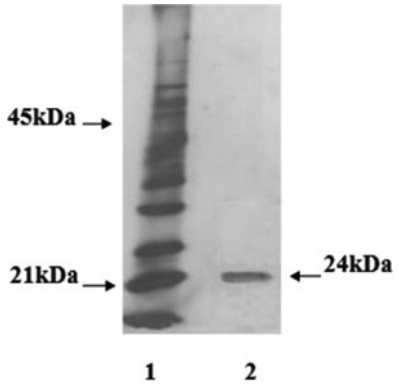

Figure 4 Electrophoresis of F_1. Lane 1, Molecular weight markers. Lane 2, F_1 from the Mono Q column.

Electrophoretic profiles obtained by SDS-PAGE (Fig. 4) indicate that the fraction with biological activity (F_1) is a protein of approximately 24 kDa and isoelectric point (IEP) of 8.8 (Fig. 5).

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

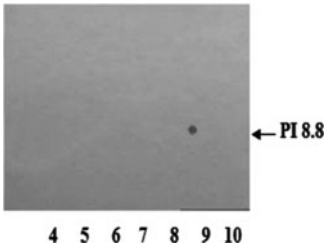

Figure 5 Isoelectric point of F_1. F_1 was subjected to bidimensional electrophoresis. Silver staining.

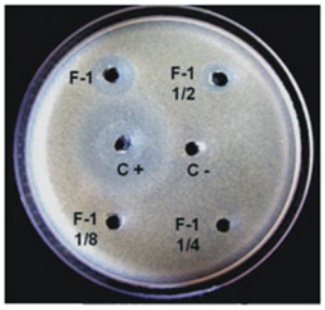

Figure 6 Effect of different F_1 concentrations on egg lipids. F_1 was seeded on egg yolk agar. The halo produced by F_1 was measured at 60 min.

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 PLA_2 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 μg/ml), a specific inhibitor of PC-PLC, and n-butanol (50 μg/ml), a specific inhibitor of phospholipase D (PLD).

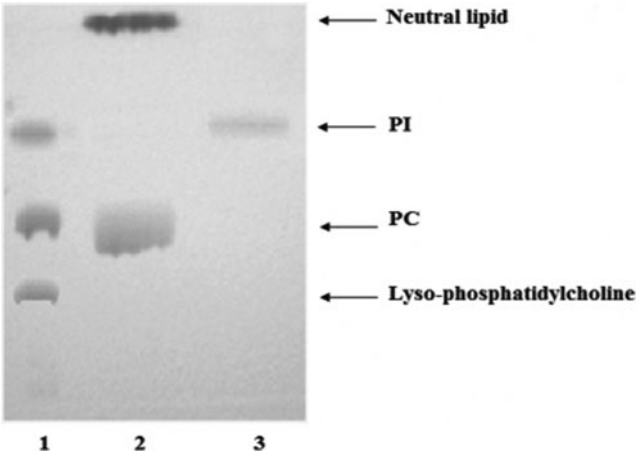

Figure 7 Thin layer chromotography (TLC) of hydrolysis products from phosphatidylcholine (PC) and phosphatidylinositol (PI). PC and PI were exposed to F_1 for 60 min at 37°C. The lipids obtained were recovered in chloroform/methanol and processed with thin layer chromatography. Lane 1, markers (lyso-PC,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

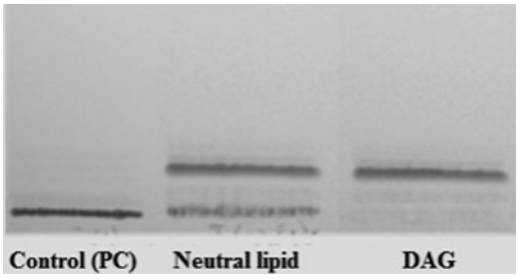

Figure 8 Thin layer chromotography (TLC) of neutral lipids formed in the incubation of phosphatidylcholine (PC) with F_1. The neutral lipids formed in the incubation of PC with F_1 were subjected to thin layer chromatography. TLC revealed the presence of 1,2 diacylglycerol. Commercial diacylglycerol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

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

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

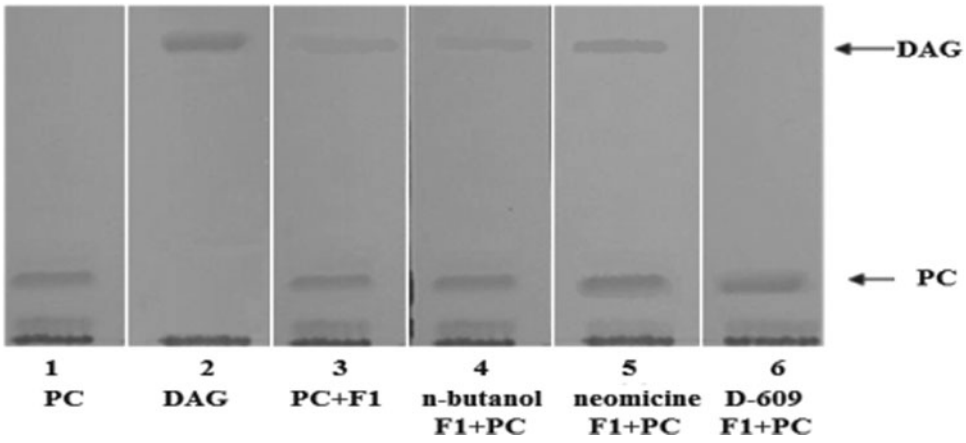

Figure 9 Thin layer chromotography (TLC) of the products resulting from the effect of F_1 on PC in the presence of inhibitors. 1, phosphatidylcholine; 2, diacylglycerol (DAG) as control; 3, hydrolysis products of phosphatidylcholine by F_1; 4, phosphatidylcholine, n-butanol and F1; 5, phosphatidylcholine, neomycin and F_1; 6, phosphatidylcholine, D-609 and F_1.

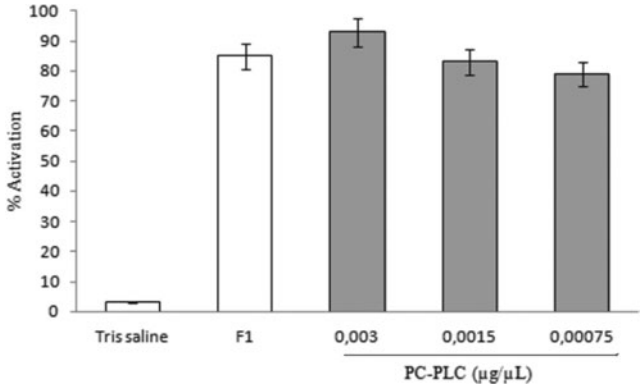

Figure 10 Effect of microinjection of phospholipase C (PLC) that is specific for phosphatidylcholine (PC-PLC) from *Bacillus cereus*. Oocytes matured *in vitro* were injected with 30 nl of PC-PLC from *Bacillus cereus* (0.003, 0.0015, 0.00075 µg/µl). Values are the mean ± standard error of the mean (SEM)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

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.

Immunolocation 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).

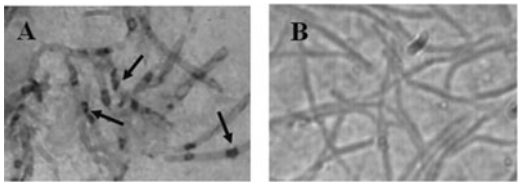

Figure 11 (*A*) Immunolocation of phospholipase C (PLC) that is specific for phosphatidylcholine (PC-PLC) in *Rhinella arenarum* spermatozoa (magnification ×1600). (*B*) Negative control (magnification ×1600). Arrows indicate the presence of PC-PLC in sperm heads.

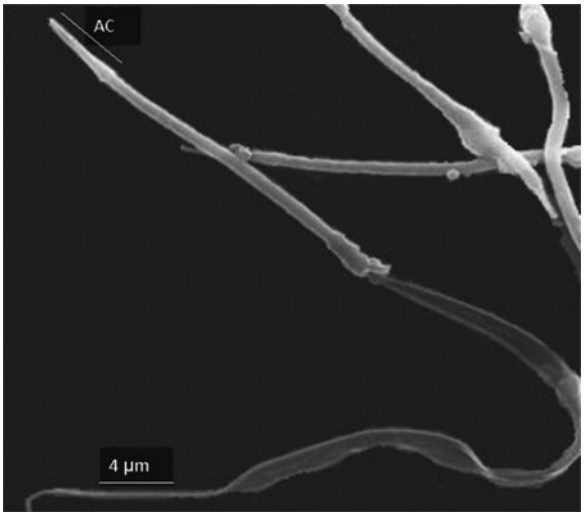

Figure 12 Scanning micrograph of *Rhinella arenarum* sperm. Magnification ×3600. AC, acrosome.

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

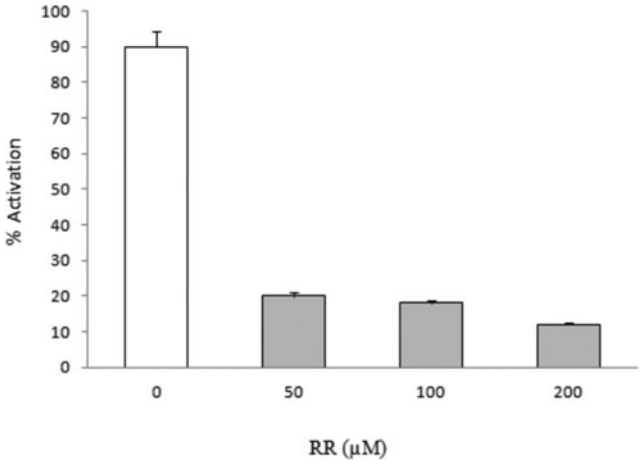

Figure 13 Inhibition of the activation produced by F_1 with ruthenium red (RR). The oocytes were preinhibited with RR and subsequently microinjected with fraction F_1. Activation was measured at 20 min.

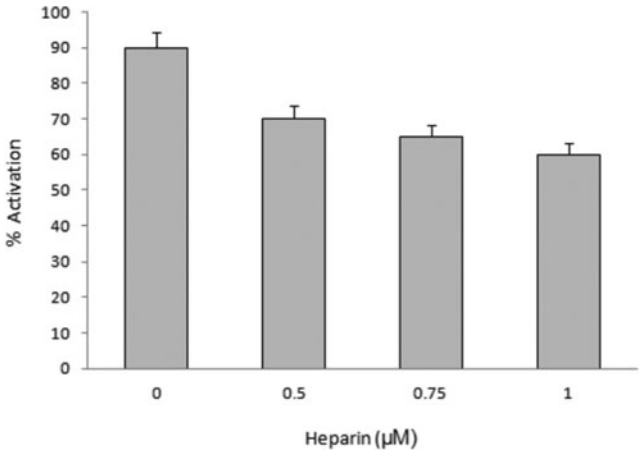

Figure 14 Inhibition of the inositol triphosphate (IP_3) receptor on the activation induced by microinjection of F_1. The oocytes were preinhibited with heparin and subsequently microinjected with fraction F_1. Activation was measured at 20 min.

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 μM), antagonist of the IP3 receptor or ruthenium red (50–200 μM) 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/IP3 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).

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.

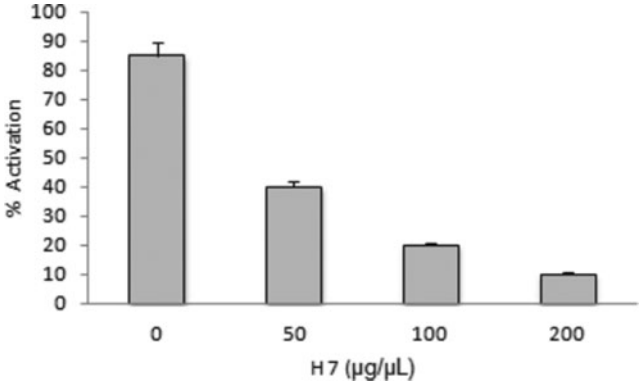

Figure 15 Effect of inhibition of protein kinase C (PKC) on the activation induced by microinjection of F_1. H7 was microinjected (0, 50, 100 or 200 μg/μl) and 20 min later activation was induced with F_1.

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.

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

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 IP_3 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 IP_3 (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, Wu X *et al.*, 2006). They also found that approximately 99% of the DAG increase was derived from non-PIP_2 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 F_1 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 PLCζ 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; Szumiło & Rahden-Staroń, 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 Ca^{2+}-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 Ca^{2+} release from the ER by stimulating a pathway leading to the production of cyclic ADP ribose, which mobilizes Ca^{2+} 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, F_1 is unable to induce activation,

https://doi.org/10.1017/S0967199413000348 Published online by Cambridge University Press

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 PLCδ 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. Immunolocation 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 Ca^{2+}.

Although the amount of proteins microinjected into the oocytes is greater that 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.

Acknowledgements

This work was supported by a grant from the *Science* Council of the National University of Tucumán (CIUNT) and the National Agency for the Promotion of *Science* and Technology (FONCYT). We are grateful to Dr Hugo Borsetti for providing antibodies.

References

Ajmat, M.T., Bonilla, F., Zelarayán, L. & Bühler, M.I. (2011). Participation of inositol trisphosphate and ryanodine receptors in *Bufo arenarum* oocyte activation. *Zygote* **19**, 171–80.

Ajmat, M.T., Bonilla, F, Hermosilla, P.C., Zelarayán, L. & Bühler, M.I. (2013). Role of phospholipase A2 pathway in regulating activation of *Bufo arenarum* oocytes. *Zygote* **21**, 214–20.

Ayabe, T., Kopf, G.S. & Schultz, R.M. (1995). Regulation of mouse egg activation: presence of ryanodine receptors and effects of microinjected ryanodine and cyclic ADP ribose on uninseminated and inseminated eggs. *Development* **7**, 2233–44.

Bembenek, J.N., Richie, C.T., Squirrell, J.M., Campbell, J.M., Eliceiri, K.W., Poteryaev, D., Spang, A., Golden, A. & White, J.G. (2007). Cortical granule exocytosis in *C. elegans* is regulated by cell cycle components including separase. *Development* **134**, 3837–48.

Bement, W. (1992). Signal transduction by calcium and protein linase C during egg activation. *J. Exp. Zool.* **263**, 382–97.

Bement, W. & Capco, D.G. (1989). Activators of protein kinase C trigger cortical granule exocytosis, cortical contraction, and cleavage furrow formation in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **108**, 885–92.

Berridge, M. (1984). Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**, 345–60.

Berridge, M. (1997). Elementary and global aspects of calcium signalling. *J. Physiol.* **499**, 291–306.

Bonilla, F., Ajmat, M.T., Sánchez Toranzo, G., Zelarayán, L., Oterino, J. & Bühler, M.I. (2008). Activation of amphibian oocytes by sperm extracts. *Zygote* **16**, 303–8.

Bühler, M.I., Petrino, T., Zelarayán, L. & Legname, A. (1994). Effects of cytoplasmic components upon sperm aster development in *Bufo arenarum* eggs. *Dev. Biol.* **164**, 398–401.

Busa, W.B., Ferguson, J.E., Joseph, S.K., Williamson, J.R. & Nuccitelli, R. (1985). Activation of frog *(Xenopus laevis*) eggs by inositol trisphosphate. Characterization of Ca^{2+} release from intracellular stores. *J. Cell Biol.* **101**, 677–82.

Carroll, D.J., Ramarao, C.S., Mehlmann, L.M., Roche, S., Terasaki, M. & Jaffe, L.A. (1997). Calcium release at fertilization in starfish eggs is mediated by phospholipase Cγ. *J. Cell Biol.* **138**, 1303–11.

Coward, K., Ponting, C.P., Zhang, N., Young, C., Huang, C.J., Chou, C.M., Kashir, J., Fissore, R.A. & Parrington, J. (2011). Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLCζ) in pufferfish. *Mol. Reprod. Dev.* **78**, 48–56.

Cox, L.J., Larman, M.G., Saunders, C.M., Hashimoto, K., Swann, K. & Lai, F.A. (2002). Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca^{2+} oscillations, activation and development of mouse oocytes. *Reproduction* **124**, 611–23.

Dale, B. & DeFelice, L. (2011). Polyspermy prevention: fact and artifacts? *J. Ass. Reprod. Genet.* **28**, 199–207.

Dale, B., DeFelice, L. & Ehrenstein, G. (1985). Injection of a soluble sperm extract into sea urchin eggs triggers the cortical reaction. *Experientia* **41**, 1068–70.

Dale, B., Wilding, M., Coppola, G. & Tosti, E. (2010). How do spermatozoa activate oocytes? *Reprod. Biomed. Online* **21**, 1–3.

Dong, J.B., Tang, T.S. & Sun, Z.F. (2000). *Xenopus* and chicken sperm contain a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. *Biochem. Biophys. Res. Commun.* **268**, 947–51.

Douglas, K. & Kline, J.T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80–9.

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R.M., Kopf, G.S., Fissore, R., Madoux, S. & Ozil, J.P. (2002). Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev. Biol.* **250**, 280–91.

Evans, J.P. & Kopf, G.S. (1998). Molecular mechanisms of sperm-egg interactions and egg activation. *Andrologia* **30**, 297–307.

Folch, J., Lees, M. & Stanley, G.H.S. (1957). A simple method for the isolation and purification of total lipid from animal tissues. *J. Biochem.* **226**, 497–509.

Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C., Izumi, T. & Perry, A.C.F. (2004). Mammalian phospholipase C zeta induces oocyte activation from the sperm perinuclear matrix. *Dev. Biol.* **274**, 370–83.

Galione, A. & Summerhill, R. (1995). Regulation of ryanodine receptors by cyclic ADP-ribose. In *Ryanodine Receptors* (ed. V. Sorrentino), pp. 51–70. CRC Press, Boca Raton, FL, USA.

Galione, A., McDougall, A., Busa, W. B., Willmott, N., Gillot, I. & Whitaker, M. (1993). Redundant mechanisms of calcium-induced calcium release underlying calcium waves during fertilization of sea urchin eggs. *Science* **261**, 348–52.

Gould, M. & Stephano, J.L. (1991). Peptides from sperm acrosomal protein that initiate development. *Dev. Biol.* **146**, 509–18.

Halet, G., Tunwell, R. & Parkinson, S.J. (2004). Conventional PKCs regulate the temporal pattern of Ca^{2+} oscillations at fertilization in mouse eggs. *J. Cell Biol.* **164**, 1033–44.

Heinecke, J.W. & Shapiro, B.M. (1992). The respiratory burst oxi dase of fertilization. A physiological target for regulation by protein kinase C. *J. Biol. Chem.* **267**, 7959–62.

Hinkovska-Galchev, V. & Srivastava, P.N. (1992). Phosphatidylcholine and phosphatidylinositol-specific phospholipases C of bull and rabbit spermatozoa. *Mol. Reprod. Dev.* **33**, 281–6.

Horner, V. & Wolfner, M. (2008). Transitioning from eg to embryo: triggers and mechanisms of egg activation. *Dev. Dyn.* **237**, 527–44.

Jaffe, L.A., Giusti, A.F., Carroll, D.J. & Foltz, K.R. (2001). Ca^{2+}signalling during fertilization of echinoderm eggs. *Semin. Cell. Dev. Biol.* **12**, 45–51.

Jones, K.T. (1998). Protein Kinase C action at fertilization overstated or undervalued? *Rev. Reprod.* **3**, 7–12.

Jones, K.T., Cruttwell, C., Parrington, J. & Swann, K. (1998). A mammalian sperm cytosolic phospholipase C activity generates inositol triphosphate and causes Ca^{2+} release in sea urchin egg homogenates. *FEBS Lett.* **437**, 297–300.

Jones, K.T., Matsuda, M., Parrington, J., Katan, M. & Swann, K. (2000). Different Ca^{2+} releasing abilities of sperm extracts compared with tissue extracts and phospholipase C isoforms in sea urchin eggs homogenates and mouse eggs. *Biochem. J.* **346**, 743–9.

Kawano, N., Yoshida, K., Miyado, K. & Yoshida, M. (2011). Lipid rafts: keys to sperm maturation, fertilization and early embryogenesis. *J. Lipids* doi:10.1155/2011/264706

Krapf, D., O'Brien, E.D., Cabada, M.O., Visconti, P.E. & Arranz, S.E. (2009). Egg water from the amphibian *Bufo arenarum* modulates the ability of homologous sperm to undergo the acrosome reaction in the presence of the vitelline envelope. *Biol. Reprod.* **2**, 311–9.

Kurokawa, M., Sato, K. & Fissore, R.A. (2004). Mammalian fertilization: from sperm factor to phospholipase Cζ. *Biol. Cell* **96**, 37–45.

Kurokawa, M., Sato, K., Wu, H., He, C., Malcuit, C., Black, S.J., Fukami, K. & Fissore, R.A. (2005). Functional, biochemical, and chromatographic characterization of the complete $[Ca^{2+}]$ oscillation-inducing activity of porcine sperm. *Dev. Biol.* **285**, 376–92.

Kurokawa, M., Yoon, S., Alfandari, D., Fukami, K., Sato, K. & Fissore, R.A. (2007). Proteolytic processing of phospholipase Cζ and $[Ca^{2+}]_i$ oscillations during mammalian fertilization. *Dev. Biol.* **312**, 407–18.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680–5.

Larman, M.G., Saunders, C.M., Carroll, J., Lai, F.A. & Swann, K. (2004). Cell cycle-dependent Ca^{2+} oscillations in mouse embryos are regulated by nuclear targeting of PLCζ. *J. Cell Sci.* **117**, 2513–21.

Lin, Y.W. & Schuetz, A.W. (1985). Intrafollicular action of estrogen in regulation pituitary-induced ovarian progesterone synthesis and oocyte maturation in *Rana pipiens*: temporal relationship and locus of action. *Gen. Comp. Endocrinol.* **58**, 421–35.

Machaty, Z., Bonk, A.J., Kuhholzer, B. & Prather, R.S. (2000). Porcine oocyte activation induced by a cytosolic sperm factor. *Mol. Reprod. Dev.* **57**, 290–5.

Malcuit, C., Knott, J.G., He, C., Wainwright, T., Parys, J.B., Robl, J.M. & Fissore, R.A. (2005). Fertilization and inositol 1,4,5-trisphosphate (IP_3) induced calcium release in type-1 inositol 1,4,5-trisphosphate receptor down regulated bovine eggs. *Biol. Reprod.* **73**, 2–13.

Martinez, M.L. & Cabada, M.O. (1996). Assessment of the acrosome reaction in *Bufo arenarum* spermatozoa by immunostaining: comparison with other methods. *Zygote* **4**, 181–90.

Mateos, M.V., Uranga, R.M., Salvador, G.A. & Giusto, N.M. (2008). Activation of phosphatidylcholine signalling during oxidative stress in synaptic endings. *Neurochem. Int.* **53**, 199–206.

Mehlmann, L., Chattopadhyay, A., Carpenter, G. & Jaffe, L. (2001). Evidence that phospholipase C from sperm is not responsible for initiating Ca^{2+} release at fertilization in mouse eggs. *Dev. Biol.* **236**, 492–501.

Minahk, C., Kim, K.W., Nelson, R., Trigatti, B., Lehner, R. & Vance, D.E. (2008). Conversion of low density lipoprotein-associated phosphatidylcholine to triacylglycerol by primary hepatocytes. *J. Biol. Chem.* **283**, 6449–58.

Miyazaki, S. & Ito, M.(2006). Calcium signals for egg activation in mammals. *J. Pharm. Sci.* **100**, 545–52

Mizote, A., Okamoto, S. & Iwao, Y. (1999). Activation of *Xenopus* eggs by proteases: possible involvement of a sperm protease in fertilization. *Dev. Biol.* **208**, 79–92.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 393–8.

Nuccitelli, R., Yim, D.L. & Smart, T. (1993). The sperm-induced Ca^{2+} wave following fertilization of the *Xenopus* egg requires the production of $Ins(1,4,5)P_3$. *Dev. Biol.* **158**, 200–12.

Oterino, J., Sánchez Toranzo, G., Zelarayán, L. & Bühler, M.I. (1997). Polyspermy in *Bufo arenarum* oocytes matured *in vitro*. *Zygote* **5**, 267–71.

Oterino, J., Sánchez Toranzo, G., Zelarayán, L., Valz-Gianinet, J.N. & Bühler, M.I. (2001). Cortical granule exocytosis in *Bufo arenarum* oocytes matured *in vitro*. *Zygote* **9**, 251–9.

Parrington, J., Swann, K., Shevchenko, V.I., Sesay, A.K. & Lai, F.A. (1996). Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. *Nature* **25**, 364–8.

Parrington, J., Jones, K.T., Lai, F.A. & Swann, K. (1999). The soluble sperm factor that causes Ca^{2+} release from sea-urchin (*Lytechinus pictus*) egg homogenates also triggers Ca^{2+} oscillations after injection into mouse eggs. *Biochem. J.* **341**, 1–4.

Parrington, J., Davis, L.C., Galione, A. & Wessel, G. (2007). Flipping the switch: how a sperm activates the egg at fertilization. *Dev. Dyn.* **236**, 2027–38.

Petcoff, D.W., Holland, W.L. & Stith, B.J. (2008). Lipid levels in sperm, eggs, and during fertilization in *Xenopus laevis*. *J. Lipid Res.* **49**, 2365–78.

Raisman, J.S., de Cunio, R.W., Cabada, M.O., del Pino, E.J. & Mariano, M.I. (1980). Acrosome breakdown in *Leptodactylus chaquensis* (Amphibia, Anura) spermatozoa. *Dev. Growth Differ.* **22**, 289–97.

Ramoni, C., Spadaro, F., Menegon, M. & Podo, F. (2001). Cellular localization and functional role of phosphatidylcholine-specific phospholipase C in NK cells. *J. Immunol.* **167**, 2642–50.

Ramoni, C., Spadaro, F., Barletta, B., Dupuis, M.L. & Podo, F. (2004). Phosphatidylcholine-specific phospholipase C in mitogen-stimulated fibroblasts. *Exp. Cell Res.* **299**, 370–82.

Rice, A., Parrington, J., Jones, K.T. & Swann, K. (2000). Mammalian sperm contain a Ca^{2+} sensitive phospholipase C activity that can generate $InsP_3$ from PIP_2 associated with intracellular organelles. *Dev. Biol.* **227**, 125–35.

Richert, S., Luche, S., Chevallet, M., Van Dorsselaer, A., Leize-Wagner, E. & Rabilloud, T. (2004). About the mechanism of interference of silver staining with peptide mass spectrometry. *Proteomics* **4**, 909–16.

Rogers, N.T., Hobson, E., Pickering, S., Lai, F.A., Braude, P. & Swann, K. (2004). Phospholipase Cζ causes Ca^{2+} oscillations and parthenogenetic activation of human oocytes.*Reproduction* **128**, 697–702.

Roldan, E.R. & Shi, Q.X. (2007). Sperm phospholipases and acrosomal exocytosis. *Front Biosci.* **1**, 89–104.

Runft, L.L. & Jaffe, L.A. (2000). Sperm extract injection into ascidian eggs signals Ca^{2+} release by the same pathway as fertilization. *Development* **127**, 3227–36.

Runft, L.L., Jaffe, L.A. & Mehlmann, L.M. (2002). Egg activation at fertilization: where it all begins. *Dev. Biol.* **245**, 237–54.

Runft, L.L., Carroll, D.J., Gillett, J., Giusti, A. F., O'Neill, F.J. & Foltz, K.R. (2004). Identification of a starfish egg PLCγ that regulates Ca^{2+} release at fertilization. *Dev. Biol.* **269**, 220–36.

Sánchez Toranzo, G., Oterino, J., Zelarayán, L., Bonilla, F. & Bühler, M.I. (2007). Spontaneous and LH-induced maturation in *Bufo arenarum* oocytes: importance of gap junctions. *Zygote* **15**, 65–80.

Sato, K., Tokmakov, A.A., Iwasaki, T. & Fukami, Y. (2000). Tyrosine kinase-dependent activation of phospholipase Cγ is required for calcium transient in *Xenopus* egg fertilization. *Dev. Biol.* **224**, 453–69.

Sato, K., Fukami, Y. & Stith, B.J. (2006). Signal transduction pathways leading to Ca^{2+} release in a vertebrate model system: lessons from *Xenopus* eggs. *Semin. Cell. Dev. Biol.* **17**, 285–92.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K. & Lai, F.A. (2002). PLCζ: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* **129**, 3533–44.

Sheikhnejad, R.G. & Srivastava, P.N. (1986). Isolation and properties of a phosphatidylcholine-specific phospholipase C from bull seminal plasma. *J. Biol. Chem.* **261**, 7544–9.

Singer, W.D., Brown, H.A. & Sternweis, P.C. (1997). Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu. Rev. Biochem.* **66**, 475–509.

Snow, P., Yim, D.L., Leibow, J.D., Saini, S. & Nuccitelli, R. (1996). Fertilization stimulates an increase in inositol trisphosphate and inositol lipid levels in *Xenopus* eggs. *Dev. Biol.* **180**, 108–18.

Spadaro, F., Cecchetti, S., Sanchez, M., Ausiello, C.M., Podo, F. & Ramoni, C. (2006). Expression and role of phosphatidylcholine-specific phospholipase C in human NK and T lymphocyte subsets. *Eur. J. Immunol.* **36**, 3277–87.

Stephens, S., Beyer, B., Balthazar-Stablein, U., Duncan, R., Kostacos, M., Lukoma, M., Green, G.R. & Poccia, D. (2002). Two kinase activities are sufficient for sea urchin sperm chromatin decondensation *in vitro*. *Mol. Reprod.* **62**, 496–503.

Stith, B.J., Goalstone, M., Silva, S. & Jaynes, C. (1993). Inositol 1,4,5-trisphosphate mass changes from fertilization through first cleavage in *Xenopus laevis*. *Mol. Biol. Cell* **4**, 435–43.

Stith, B.J., Woronoff, K., Espinoza, R. & Smart, T. (1997). sn-1,2-diacylglycerol and choline increase after fertilization in *Xenopus laevis*. *Mol. Biol. Cell* **4**, 755–65.

Stricker, S.A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev. Biol.* **211**, 157–76.

Swann, K., Larman, M.G., Saunders, C.M. & Lai, F. (2004). The cytosolic sperm factor that triggers Ca^{2+} oscillations and egg activation in mammals is a novel phospholipase C: PLCζ. *Reproduction* **127**, 431–9.

Szumiło, M. & Rahden-Staroń, I. (2008). Biol. role of phosphatidylcholine-specific phospholipase C in mammalian cells. *Postepy. Hig. Med. Dosw.* (*Online)* **62**, 593–8.

Talevi, R., Dale, B. & Campanella, C. (1985). Fertilization and activation potentials in *Discoglossus pictus* (Anura) eggs: A delayed response to activation by pricking. *Dev. Biol.* **111**, 316–23.

Whitaker, M.J. & Swann, K. (1993). Lighting the fuse at fertilization. *Development* **117**, 1–12.

Whitaker, M. & Zimmerberg, J. (1987). Inhibition of secretory granule discharge during exocytosis in sea urchin eggs by polymer solutions. *J. Physiol.* (*London)* **389**, 527–39.

Wilding, M. & Dale, B. (1998). Soluble extracts from ascidian spermatozoa trigger intracellular calcium release independently of the activation of the ADP ribose channel. *Zygote* **6**, 149–55.

Wilding, M., Kyozuka, K., Ruso, G.L., Tosti, E. & Dale, B. (1997). A soluble extract from human spermatozoa activates ascidian oocytes. *Dev. Growth Differ.* **39**, 329–36.

Wolny, Y.M., Fissore, R.A., Wu, H., Reis, M.M., Colombero, L.T., Ergün, B., Rosenwaks, Z. & Palermo, G.D. (1999). Human glucosamine-6-phosphate isomerase, a homologue of hamster oscillin, does not appear to be involved in Ca^{2+} release in mammalian oocytes. *Gamete Biol.* **53**, 277–87.

Wu, H., Smyth, J., Luzzi, V., Fukami, K., Takenawa, T., Black, S.L., Allbritton, N.L. & Fissore, R. (2001). Sperm factor induces intracellular free calcium oscillations by stimulating the phosphoinositide pathway. *Biol. Reprod.* **64**, 1338–49.

Wu, X., Zhang, X., Li, W., Cheng, H., Kuai, Y., Wang, S. & Guo, Y.-L. (2006). Translocation of classical PKC and cortical granule exocytosis of human oocyte in germinal vesicle and metaphase II stage. *Acta Pharmacologica Sinica* **27**, 1353–8.

Yamaga, M., Kawai, K., Kiyota, M., Homma, Y. & Yagisawa, H. (2007). Recruitment and activation of phospholipase C (PLC)-d1 in lipid rafts by muscarinic stimulation of PC12 cells: contribution of p122RhoGAP/DLC1, a tumor-suppressing PLCd1 binding protein. *Adv. Enzyme Regul.* **48**, 41–54.

Yue, C., White, K.L., Reed, W.A. & Bunch, T.D. (1995). The existence of inositol 1,4,5-trisphosphate and ryanodine receptors in mature bovine oocytes. *Development* **8**, 2645–54.