Zygote **16** (November), pp. 303–308. © 2008 Cambridge University Press
doi:10.1017/S0967199408004814 First Published Online 11 July 2008 Printed in the United Kingdom

Activation of amphibian oocytes by sperm extracts

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Date submitted: 24.10.07. Date accepted: 20.02.08

Summary

In the fertilization of most animals, egg activation is accompanied by an increase in cytoplasmatic Ca^{2+}; however, the mechanism through which the fertilizing sperm induce this phenomenon is still controversial. An increase in intracellular free Ca^{2+} is required to trigger egg activation events, a process that includes cortical granule exocytosis, resumption and completion of meiosis and DNA replication, and culminates in the first mitotic cleavage. In this work, we investigated the effect of microinjection and incubation of different fractions of homologous sperm extract on the activation of *Bufo arenarum* oocytes matured *in vitro*. Two heat treatment-sensitive fractions obtained by chromatography were able to induce oocyte activation. The sperm fraction, which contained a 24 kDa protein, induced 90% activation when it was microinjected into the oocytes. Whilst the sperm fraction, which contained a 36 kDa protein, was able to induce about 70% activation only when it was applied on the oocyte surface.

Keywords: *Bufo arenarum*, Oocyte activation, Sperm extracts

Introduction

In almost all animal species studied, spermatozoa activate eggs by causing transient increases in the intracellular free Ca^{2+} concentration (Dale, 1990; Miyasaki *et al*., 1993; Nuccitelli *et al*., 1993). In most non-mammals, such as *Xenopus* and echinoderm, only one transient Ca^{2+} rise occurs in the fertilized eggs. The Ca^{2+} rise starts from the site of sperm attachment and travels across the entire egg in the form of a wave (Miyasaki *et al*., 1993; Swann & Lai, 1997). This Ca^{2+} signal is sufficient to trigger the events associated with egg activation and embryonic development (Whitaker & Swann, 1993; Stricker, 1999).

Several models have been proposed to explain how the spermatozoon achieves Ca^{2+} release. Some authors hypothesized that, after gamete fusion, the sperm may introduce a soluble factor that triggers the activation process in the oocyte (Dale, 1985; Wilding & Dale, 1998; Machaty *et al*., 2000; 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). In intact eggs and egg homogenates, mammalian sperm extract triggers Ca^{2+} release by stimulating IP_3 production (Stith *et al*., 1993; Jones *et al*., 1998b, 2000; Rice *et al*., 2000; Wu *et al*., 2001, Saunders *et al*., 2002), which indicates the involvement of a IP-specific phospholipase C (PLCζ) in the signal transduction mechanism.

Another hypothesis proposes the interaction between sperm and oocyte surface. In the frog *Xenopus laevis*, unfertilized eggs can be activated by a sperm extract from the newt *Cynops pyrrhogaster*, this activation is accompanied by propagative Ca^{2+} release across the entire egg surface (Iwao *et al*., 1995). In vertebrates, the surface application of a peptide derived from a sperm surface supports the hypothesis of egg activation that involves a signal transducing receptor for sperm in the oocyte's plasma membrane (Shilling *et al*., 1998). An extract obtained from *Xenopus* sperm showed hydrolytic activity, which suggests that a tryptic protease from sperm is involved in fertilization, most probably participating in egg activation (Mizote, 1999). In sea urchin and starfish eggs, these early plasma membrane interactions somehow cause the activation of a Src family kinase, which leads to the activation of phospholipase Cγ and the production of IP_3, which releases Ca^{2+} from the endoplasmic reticulum (Jaffe *et al*., 2001). The Ca^{2+} release during

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the fertilization of *Xenopus* eggs requires type I IP_3-gated Ca^{2+} channels (Snow *et al*., 1996), but not SH_2 domain-mediated activation of PLCγ or Gq-mediated activation of PLCβ (Runft *et al*., 1999).

In anuran eggs, after sperm contact, cortical granules exocytose their content, which modifies the egg surface and the vitelline envelope (Gómez *et al*., 1984; Oterino *et al*., 2001, 2006).

In *Bufo arenarum* oocytes, the mechanism by which spermatoza induce oocyte activation has not yet been elucidated. The purpose of the present work was to investigate the ability of the different fractions, obtained by chromatography from *Bufo arenarum* sperm extract, to induce oocyte activation either by microinjection or by external application.

Materials and methods

Animals

Sexually mature *Bufo arenarum* males and females were collected in the northwestern area of Argentina from May to August (winter animals) and from September to December (summer animals) 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 amphibian Ringer solution (AR) (NaCl 6.6 g/l, $CaCl_2$ 0.15 g/l, KCl 0.15 g/l) and centrifuging at 1085 *g* for 10 min; then, the pellet was resuspended in AR. To select motile sperms, we used a swim-up procedure. The high quality sperm suspension was centrifuged at 1085 *g* for 10 min. Sperm were resuspended in calcium-free Tris-buffered saline (NaCl 7.59 g/l, Tris–HCl 2.40 g, pH 7.4) and lysed by three cycles of freezing (−70 °C) and thawing (25 °C). Lysate was observed under a microscope to verify that intact sperm or sperm heads were not present. The lysate was centrifuged for 30 min at 16 000 *g* and the supernatant was collected as sperm extract.

The sperm extract with a final protein concentration of 2.5 μg/μl was run through size exclusion chromatography using a Bio-Gel P-60 chromatography column. The column was washed with calcium-free Tris-buffered saline, and the fractions were stored at −20 °C until use.

Electrophoresis

SDS-PAGE of sperm fractions was performed according to Laemmli (1970) with 7.5% running gels. Proteins were stained with Coomassie brilliant blue R-250.

Oocyte maturation

Denuded oocytes were obtained according to Lin & Schuetz (1985). Follicle cells were removed by incubation of defolliculated oocytes in AR that contained 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.

The experiments were performed with *in vitro* matured *Bufo arenarum* oocytes. Meiosis reinitiation was induced by progesterone treatment (2.5 μM) and scored by the presence of a white spot in the animal pole.

Microinjection and incubation

The biological activity of the different fractions was assayed in *Bufo arenarum* oocytes matured *in vitro* by microinjection or external exposure.

Different fractions from the sperm extract were microinjected using ICSI (intracytoplasmic sperm injection) micropipettes Humagen^{tm} Fertility Diagnostics. The injection volume was 30 nl. The microinjections were carried out in calcium-free Tris-buffered saline at 20 °C. Injection of the buffer alone did not have any measurable effect on oocytes.

In vitro cultures were carried out at room temperature (22–25 °C) using plastic multiwell culture dishes (Costar 3524). Randomized samples of 20 freshly denuded oocytes were distributed into separate wells containing 2 ml AR; the reagents were added (5 μl) directly to the culture medium. Two-well duplicates were routinely run in each experimental group.

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

Results

Size exclusion chromatography of sperm extract

Sperm extract obtained according to the Materials and methods was run using a Bio-Gel P-60 chromatography column and the resulting fractions were screened. Results showed four protein peaks and 25 fractions (Fig. 1).

Effect of the microinjection of different sperm extract fractions on the activation of *Bufo arenarum* oocytes

The ability of the different sperm extract fractions to induce activation was determined by microinjecting each of them into *Bufo arenarum* oocytes that had been

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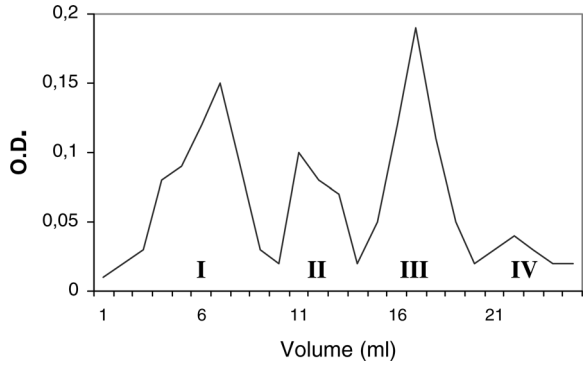

Figure 1 Size exclusion chromatography of sperm extract. The sperm extract with a final protein concentration of 2.5 µg/µl was run through size exclusion chromatography using a Bio-Gel P-60 chromatography column. The column was washed with calcium-free Tris-buffered saline and the 25 fractions obtained were stored at −20 °C until use.

matured *in vitro*. Groups of 20 oocytes were injected with 30 nl of sperm extract fractions and cultured in AR at 25 °C. Activation signs were checked 30 min after injection. As the control, another group of oocytes was injected with 30 nl calcium-free Tris-buffered saline.

Results showed that the injection of the fractions 17, 18 and 19, which correspond to protein peak III (Fig. 1), induced about 50, 80 and 30% oocyte activation, respectively. The activated oocytes exhibited all the signs of activation (Fig. 2*b*, *d*; Fig. 3).

To investigate if the active factor was a protein, the fractions were incubated at 90 °C for 30 min before injection into the oocytes. Heat treatment totally abolished the ability of all sperm fractions assayed to induce oocyte activation.

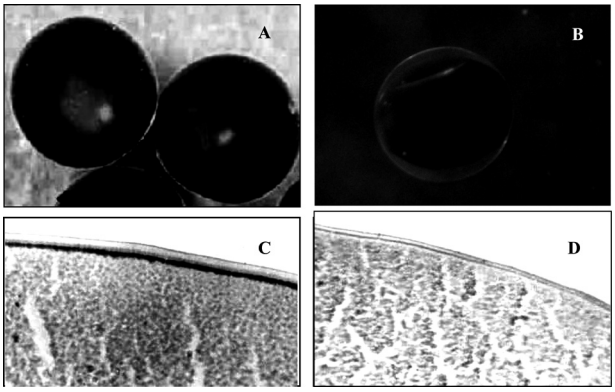

Figure 2 (*a*) Oocyte matured *in vitro* exhibiting a white spot on the animal hemisphere. (*b*) Oocyte exhibiting the characteristic external signs of activation: elevation of the vitelline envelope and disappearance of the white spot. (*c*) Mature oocyte exhibiting a line of cortical granules beneath the plasma membrane. Alcian-Blue (×400). (*d*) In-vitro matured oocyte fixed 30 min after activation; the cortical granules have disappeared. Alcian-Blue (×400).

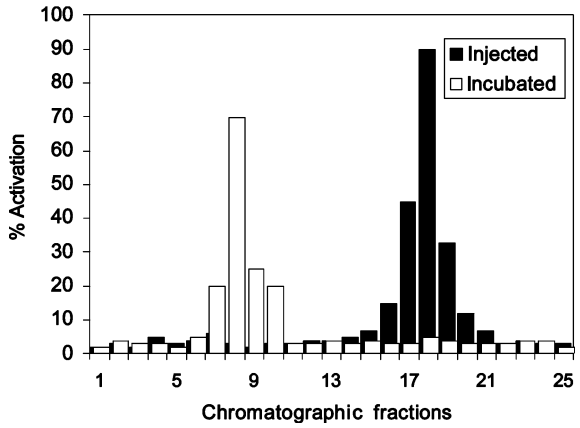

Figure 3 Biological activity of the chromatographic fractions. Oocytes matured *in vitro* were incubated or microinjected with the different fractions. Mature oocytes were injected with 30 nl or incubated in the different sperm fractions. Activation parameters were scored after 20 min. Values are the mean ± SEM of three experiments (60–80 oocytes per experiment). Each experiment was performed on a different animal.

Effect of the incubation with different sperm extract fractions on the activation of matured *Bufo arenarum* oocytes

To study whether the application of the sperm fractions on the outer surface of the oocytes was able to induce activation, incubation experiments were carried out.

Groups of 20 oocytes were cultured in the different sperm fractions and the activation signs were scored after 30 min of incubation at 25 °C. As the control, another group of oocytes was cultured in calcium-free Tris-buffered saline.

Results (Fig. 3) indicated that the ability to induce oocytes activation was restricted to the 6 to 9 fractions, which correspond to protein peak I (Fig. 1). Fraction 8 induced activation in 70% of oocytes, which exhibited all signs of activation. Incubation in heat-treated sperm fractions did not induce activation.

Electrophoretic analysis of the active sperm fractions

Sperm fractions 8 and 18, which showed highest biological activity, were analysed by SDS-PAGE to determine their macromolecular composition. In fraction 8, electrophoretic analysis revealed the presence of a protein of approximately 36 kDa and in fraction 18 another protein of approximately 24 kDa was present (Fig. 4).

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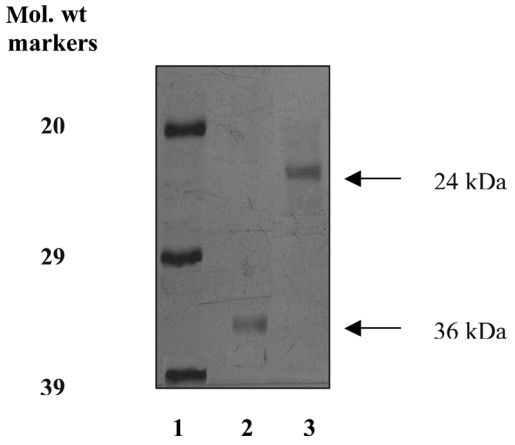

Figure 4 Electrophoresis of the biologically active fractions. Fraction 8, which showed activity when it was microinjected and fraction 18, which was able to induce activation when applied on the oocyte surface were analysed by SDS gel electrophoresis. Lane 1: molecular weight markers. Lane 2: fraction 8. Lane 3: fraction 18.

Discussion

Effect of the microinjection of different fractions of sperm extract on the activation of *Bufo arenarum* oocytes

Although it is a well accepted fact that oocytes are activated by an increase in free Ca^{2+} level at fertilization, the details of how spermatozoa induce the release of Ca^{2+} from intracellular stores is still controversial.

The hypothesis that the Ca^{2+} increase during fertilization is initiated by the introduction of factors into the egg because of sperm–egg fusion is supported by current evidence. Several candidates have been suggested as the egg-activation protein from sperm. The first was a 33 kDa protein purified from hamster sperm that had high sequence homology with the enzyme glucosamine-6-phosphate isomerase (Parrington *et al*., 1996; Swann & Lai, 1997). This protein is present in several species and tissues (Wolny *et al*., 1999). However, recent studies indicated that immunodepletion of this protein in sperm extract failed to abolish the Ca^{2+}-releasing ability of the extract, so that it is not likely to be the egg-activation protein.

Another protein proposed as the activating factor in sperm extract is a sperm-specific isoform of PLC, PLCζ, of 74 kDa, which showed a higher sensitivity to Ca^{2+} than other PLC isoforms (Saunders *et al*., 2002; Kouchi *et al*, 2004). The sperm-specific PLCζ would be responsible for Ca^{2+} oscillations in mammals (Kurokawa *et al*., 2004). An extract of *Xenopus* sperm was able to stimulate Ca^{2+} release when injected into mouse eggs, which indicated that the sperm factor appears not to be species specific in vertebrates (Dong *et al*., 2000).

Our results indicate that a protein of 24 kDa, present in the sperm extract of *Bufo arenarum*, was able to induce activation when it was microinjected in homologous oocytes matured *in vitro*. The molecular weight of the active protein obtained in *Bufo arenarum* sperm extract is different to that of the 33 kDa protein described in mammal sperm extract. In addition, the 74 kDa PLCζ protein proposed as sperm factor is very different from the 24 kDa protein, which is able to induce activation in *Bufo arenarum* eggs.

Other experiments have shown that injection of demembranated sperm heads activated mouse eggs, which suggests that the sperm-derived egg-activating protein may be associated with the sperm perinuclear material (Kuretake *et al*., 1996; Kimura *et al*., 1998).

Effect of the incubation with different sperm extract fractions on the activation of *Bufo arenarum* oocytes

A possibility for signal transduction at fertilization is that spermatozoa might use transmembrane proteins to trigger Ca^{2+} release (Sato *et al*., 2000). This hypothesis involves the Ca^{2+}-releasing second messenger IP_3. In support of this hypothesis, the protease cathepsin B or a similar protease of 380 kDa present in *Xenopus* sperm extract caused an increase in intracellular Ca^{2+} when applied to *Cynops* eggs (Mizote *et al*., 1999; Iwao, 2000), but it has not been applied to *Xenopus* eggs.

Other results suggest that Ca^{2+} release is initiated in frog eggs when disintegrin molecules on the sperm surface contact an integrin in the egg surface (Iwao & Fujimura, 1996; Shilling *et al*., 1998).

All the molecules proposed as agonists to trigger Ca^{2+} released at fertilization have molecular weights higher than the protein present in *Bufo arenarum* sperm extract capable of activating the eggs when it was added to the culture medium. Interestingly, it had no effect when microinjected into the egg cytoplasm.

In this study, we demonstrated the presence in sperm extract from *Bufo arenarum* of two proteins that were able to induce oocyte activation. The sperm fraction containing a 24 kDa protein induced 90% activation when it was microinjected into the oocytes, while the sperm fraction containing a 36 kDa protein was able to induce about 70% activation only when it was applied on the oocyte surface.

It is possible that mechanisms for oocyte activation involve signals from soluble sperm factors together with signals generated by the interaction of the sperm with receptors on the oocyte plasma membrane.

https://doi.org/10.1017/S0967199408004814 Published online by Cambridge University Press

Acknowledgements

This work was supported by a grant from the Science Council of the National University of Tucumán (CIUNT).

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