

Egg water from the amphibian *Bufo arenarum* induces capacitation-like changes in homologous spermatozoa

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Abstract

Mammalian sperm acquire fertilizing capacity after residing in the female tract, where physiological changes named capacitation take place. In animals with external fertilization as amphibians, gamete interactions are first established between sperm and molecules of the egg jelly coat released into the medium. Since dejellied oocytes are not normally fertilized, the aim of this study was to determine if the jelly coat of the toad *Bufo arenarum* promotes a “capacitating” activity on homologous sperm. We found that sperm incubation in diffusible substances of the jelly coat (egg water) for 90–180 s is sufficient to render sperm transiently capable of fertilizing dejellied oocytes. The fertilizing state was correlated with an increase of protein tyrosine phosphorylation and a decrease of sperm cholesterol content. Inhibition of either the increase in tyrosine phosphorylation or cholesterol efflux affected the acquisition of fertilizing capacity. Phosphorylation and fertilization could be promoted with NaHCO_3 and also by addition of beta cyclodextrin. Moreover, sperm could gain the ability to fertilize dejellied oocytes in the presence of these compounds. These data indicate that sperm should undergo a series of molecular changes to gain fertilizing capacity; these changes are reminiscent of mammalian sperm capacitation and take place before the acrosome reaction.

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Introduction

Mammalian sperm do not fertilize an egg immediately upon ejaculation. Over 50 years ago, independent reports suggested that ejaculated mammalian spermatozoa require a period of residence in the female reproductive tract before being capable of fertilization (Austin, 1952; Chang, 1951). The changes that take place during this period confer to the spermatozoa the ability to adhere to the zona pellucida to undergo the acrosome reaction and initiate oocyte fusion. This process is known as “capacitation” and includes changes in intracellular pH, alterations in membrane lipid architecture and protein distribution and initiation of complex signal transduction pathways (Baldi et al., 2002; Visconti and Kopf, 1998; Visconti et al.,

2002). In animals with external fertilization as amphibians, gamete interactions are first established between the spermatozoa and molecules released into the medium by the egg jelly coat (JC) (Miceli and Cabada, 1998). This JC surrounds the vitelline envelope (analogous to the zona pellucida of mammals) and is formed by the oviduct secretion during ovulation. Some molecules of the JC are released from the jelly matrix during spawning. The solution containing the substances that diffuse from the JC is called egg water (EW).

The requirement of the jelly coats for amphibian fertilization was established many years ago (Kambara, 1953; Newport, 1851). The passage of sperm through the jelly layers has been regarded as an important step in fertilization and was sometimes proposed to be a sperm “capacitating” requisite, by analogy with the concept developed in mammals (Shivers and James, 1970). EW of the toad *Bufo arenarum* was reported to “activate” homologous free spermatozoa before they penetrate into the jelly coats (Barbieri and Cabada, 1969; Barbieri and del Pino, 1975; Barbieri and

Abbreviations: EW, egg water; M β CD, methyl- β -cyclodextrin.

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Raisman, 1969). Dejellied oocytes of different amphibian species can still be fertilized after reintroduction of the diffusible jelly components (EW) in the insemination media (Barbieri, 1976; Barbieri and Oterino, 1972; Barbieri and Villecco, 1966; Elinson, 1971; Katagiri, 1973). Unlike sea urchin, a necessary condition for *Bufo* spermatozoa to fertilize the oocytes is to reach the vitelline envelope with its acrosome-intact or at least not completely reacted (Omata and Katagiri, 1996; Raisman et al., 1980; Yoshizaki and Katagiri, 1982). Acrosomal integrity is rapidly lost when spermatozoa are incubated in hypotonic solutions (resembling the osmolarity of the jelly at the time of fertilization). It was shown that L-HGP, a component of the EW, was able to avoid spontaneous acrosome breakdown caused by hypoosmotic stress (Arranz and Cabada, 2000; Krapf et al., 2006). As a consequence, acrosome integrity might be maintained until spermatozoa contact and bind to the vitelline envelope (Barisone et al., 2007).

The first demonstration of a physiological role for egg jelly macromolecules in amphibian fertilization was reported by al Anzi and Chandler (1998). Allurin, a 21 kDa protein from *Xenopus* EW, exhibits sperm chemoattractant activity (Olson et al., 2001; Xiang et al., 2004, 2005).

In this paper we report that short exposure to EW rendered sperm transiently capable of fertilizing dejellied oocytes. The fertilizing state was correlated with an increase of protein phosphorylation in tyrosine residues, and with sperm cholesterol loss. Moreover, these physiological modifications could be mimicked *in vitro* with incubations in media containing combinations of NaHCO_3 and the synthetic cholesterol acceptor methyl- β -cyclodextrin. As far as we are aware, this is the first report of sperm physiological changes induced by the jelly coat that take place at early stages of fertilization in amphibians.

Materials and methods

Reagents

Tyrphostin A1, A25 and genistein were purchased from Calbiochem (San Diego, CA) (kindly provided by Dr Patricia Miranda). H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), chelerythrine, dibutyryl cAMP and IBMX were obtained from Sigma. Methyl- β -cyclodextrin (MBCD) was from Aquaplex, Cyclodextrin Technologies Development (Gainesville, FL) (kindly provided by Dr Luis S. Mayorga). Anti-phosphotyrosine antibody (clone 4G10) was obtained from Upstate (Lake Placid, NY). All other reagents were of the highest analytical grade.

Animals

B. arenarum sexually mature specimens (150 g) were collected in the neighborhood of Rosario city and maintained in the dark in a moist chamber between 15 and 17 °C until used. Experiments were performed in accordance with the guide for the care and use of laboratory animals of Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR.

Preparation of gametes

Sperm suspensions were obtained as described by elsewhere (Valz-Gianinet et al., 1991). After washing, spermatozoa were suspended in ice cold Ringer ST medium to a final concentration of $1\text{--}1.4 \times 10^8$ cells/ml and used within 3 h.

Uterine oocytes (referred to as oocytes) were obtained according to Barisone et al. (2007). Oocytes were dejellied as described (Barisone et al., 2007).

Egg water

EW was obtained as described (Diaz Fontdevila et al., 1991). Final protein concentration was 0.3 mg/ml (Arranz and Cabada, 2000). EW solutions were referred to as percentages of EW (30–80% EW), instead of stating the EW protein concentration since its chemical composition includes glycoproteins, inorganic ions and lipids (Diaz Fontdevila et al., 1991; Ishihara et al., 1984; Katagiri, 1973).

Assessment of tyrosine phosphorylation of sperm proteins

Sperm suspensions were diluted to 1.4×10^7 cells/ml in the appropriate media depending on the experiment performed. Osmotic pressure of media was in all cases similar to Ringer ST. When the effect of NaHCO_3 was analyzed, a modified Ringer media was used (80 mM NaCl instead of 110 mM, 25 mM HEPES, pH 7.6) adding NaCl to maintain constant osmolarity. It is important to notice that addition of NaHCO_3 did not significantly change the pH. After the incubation period, sperm were concentrated by centrifugation at $650 \times g$ for 5 min (4 °C), the sperm pellet resuspended in 10 μl of ice cold lysis buffer (1% Triton X-100, 1 mM NaVO_3 , 1 mM PMSE, 5 mM EDTA, 150 mM NaCl, 10 mM Tris pH 7.6) and stored 10 min on ice with 3×5 s vortexing. Samples were then centrifuged at $18,000 \times g$ for 5 min (4 °C), the supernatant mixed with sample buffer containing 50 mM DTT, incubated 10 min at 70 °C and then subjected to SDS–PAGE (Laemmli, 1970) in 8% gels. Each lane was loaded with 7×10^6 cells. Proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, UK) at 250 mA (constant) for 2 h at 4 °C. Immunodetection was performed using a dilution 1/2000 (0.5 $\mu\text{g}/\text{ml}$ final concentration) of a monoclonal antibody against anti-phosphotyrosine (clone 4G10) following manufacturer's directions and a dilution 1/5000 of a secondary HRP labeled antibody provided with the enhanced chemiluminescence detection kit (ECL, Amersham Biosciences, UK). For specificity controls, 10 mM phosphotyrosine was added to the antibodies solution and incubated with constant rotation for 30 min at 20 °C prior to use in the immunoblots.

Cholesterol measurements

Aliquots (8×10^6 cells) of *B. arenarum* spermatozoa were incubated for 15 min at 22 °C with the specified MBCD or EW concentration in Ringer ST (500 μl final volume) and concentrated by centrifugation at $650 \times g$ for 5 min at 4 °C. Pellets and supernatants were individually lyophilized for 6 h. Free cholesterol was determined basically as described by Gamble et al. (1978). In brief, to each tube was added 20 μl of a solution of 20 mM sodium desoxycholate and 1% Triton X-100 followed by 25 μl of 95% ethanol and stored at 4 °C for 30 min. Cholesterol was measured by the method of cholesterol oxidase/peroxidase (Wiener, Rosario, Argentina) in a final volume of 1 ml and analyzed in a Gilford Response spectrophotometer at 505 nm (Allain et al., 1974).

In vitro fertilization

In vitro fertilization was carried out at 20 °C, inseminating jelly-intact oocyte strings (110–170 oocytes) with 1×10^4 sperm/ml or jelly free oocytes with 1×10^6 sperm/ml in 10% Ringer medium (0.1 \times Ringer/10 mM Tris pH 7.6). When the effect of NaHCO_3 was analyzed, NaCl was added to maintain constant osmolarity, using modified Ringer medium as stated above for the assessment of tyrosine phosphorylation. After 15 min, oocytes and embryos were transferred to fresh 10% Ringer. Fertilization was evaluated with a stereomicroscope by recording two- or four-cell embryos.

Statistical analysis

Statistical analyses were performed with the ANOVA test. Models were further tested according to Nagarsenker (1984), and Shapiro and Wilk (1965). Significance (*p*) and sample size (*n*) were indicated in each figure legend.

Results

Effect of EW on the fertilizing capacity of sperm

Studies in a number of amphibian species suggest that diffusible factors that are released from the jelly coat play a role in fertilization (Cabada, 1975; Katagiri, 1973, 1986; Olson and Chandler, 1999; Shivers and James, 1971). EW has a direct effect on sperm and may increase their ability to penetrate the oocyte's surface by an increase in sperm motility or other mechanisms. If these mechanisms correspond to a "capacitating" activity of EW, its presence would no longer be necessary once sperm have undergone capacitation. Thus, it should be possible to expose spermatozoa to EW and fertilize dejellied oocytes in EW free media. To test this hypothesis, sperm suspensions were incubated in 80% EW (since it is the maximum concentration achievable after addition of Ringer St medium) before inseminating jelly free oocytes. When sperm were not previously exposed to EW, only 4.3% of oocytes were fertilized (Fig. 1). However, the effect on fertilization was especially pronounced when sperm were incubated in EW for 1.5 or 3 min (48 and 43% of fertilization respectively). Longer incubation periods promoted a lowering in the fertilization rates, down to zero. Due to the technical procedure, EW concentration in the insemination media was 10% in all the conditions assayed. The fact that fertilization could proceed without the further participation of EW suggests that the jelly components promote a change in sperm physiology.

Effect of EW on sperm cholesterol content

Capacitation of mouse sperm requires the presence of BSA in the incubation medium (Visconti et al., 1995a). The role of BSA in capacitation of mammalian sperm has been postulated to involve the removal of cholesterol from the plasma membrane (Suzuki and Yanagimachi, 1989; Visconti et al.,

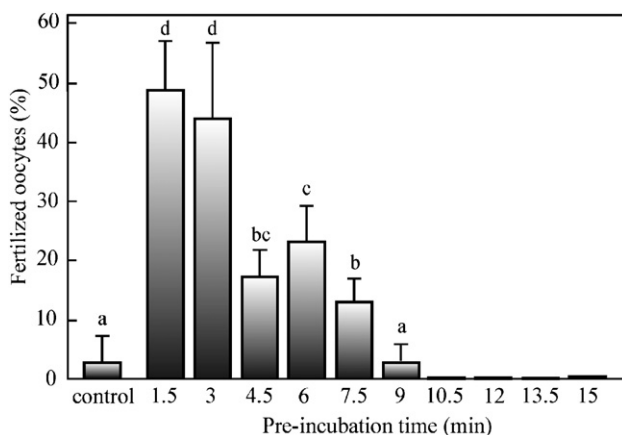


Fig. 1. Effect of sperm incubation in EW on fertilization rates of dejellied eggs. Sperm were incubated in Ringer ST media containing 80% EW for the indicated periods before inseminating dejellied oocytes. Fertilized oocytes were observed at 2- or 4-cell stage embryos. Data represent the mean \pm SEM ($n=3$). The means of groups that have different letters differ significantly ($p<0.05$).

1999a,b). As EW has a number of proteins potentially capable of binding cholesterol, cholesterol loss could play a role in the acquisition of fertilizing capacity of *B. arenarum* sperm upon contact with the jelly coat. To test this hypothesis, we incubated *Bufo* sperm in Ringer ST media (110 mM NaCl, 2 mM KCl, 1.4 mM CaCl₂, 10 mM Tris pH 7.6) with or without EW and measured sperm cholesterol content. The use of Ringer ST medium would prevent an acrosome breakdown and a consequent membrane loss induced by hypotonic stress (Arranz and Cabada, 2000). Cholesterol content of untreated sperm (freshly prepared and stored on ice for no longer than 1 h) was 0.80 ± 0.025 pg/sperm (mean \pm SEM, $n=6$). As observed in Fig. 2A, an EW concentration-dependent efflux of cholesterol was observed. Maximal cholesterol removal was achieved with the maximum EW concentration tested.

Cholesterol efflux may represent a sperm regulatory step for the acquisition of fertilizing capacity. If sperm cholesterol loss was a key element for acquisition of sperm fertilizing capacity, this process could be impaired by preincubation with the cholesterol analogue, cholesterol-SO₄⁻. Preincubation with cholesterol-SO₄⁻ would block cholesterol-binding sites in the egg jelly, lowering their ability to bind cholesterol from sperm. As shown in Fig. 2B, preincubation of gametes with increasing concentrations of cholesterol-SO₄⁻ lowered fertilization rates of intact oocytes, suggesting that the cholesterol efflux from *B. arenarum* sperm is necessary for acquisition of fertilizing capacity.

Time and concentration dependence of protein tyrosine phosphorylation on EW incubation

While the molecular bases underlying the capacitating events in mammalian sperm are not fully understood, they are consistent with the activation of signal transduction cascades. Among them, capacitation is associated with an increase in tyrosine phosphorylation of a subset of proteins in sperm from several mammalian species, including the mouse (Visconti et al., 1995a), cow (Galantino-Homer et al., 1997), human (Leclerc et al., 1996; Osheroff et al., 1999), pig (Kalab et al., 1998) and hamster (Devi et al., 1999; Kulanand and Shivaji, 2001; Visconti et al., 1999c). To examine if there is correlation between the acquisition of fertilizing capacity and protein tyrosine phosphorylation in *B. arenarum*, sperm were incubated under conditions that support fertilization. After incubations, phosphorylation on tyrosine residues was analyzed. When sperm suspensions were incubated in EW, a concentration-dependent increase in the phosphorylation of tyrosine residues was observed in proteins from Mr 50,000 to 200,000 (Fig. 3A). Phosphorylation was observed 30 s after the addition of 80% EW (Fig. 3B). Ponceau S staining was carried out to show equal protein loads in each lane (Fig. 3B, right panel). These proteins were specifically phosphorylated on tyrosine residues since the immunoreactivity was completely abolished when the antibody solution was preincubated with 10 mM *O*-phosphotyrosine for 30 min (not shown). Fig. 3C shows the immunoblotting EW proteins, indicating that the phosphoproteins detected do not belong to EW.

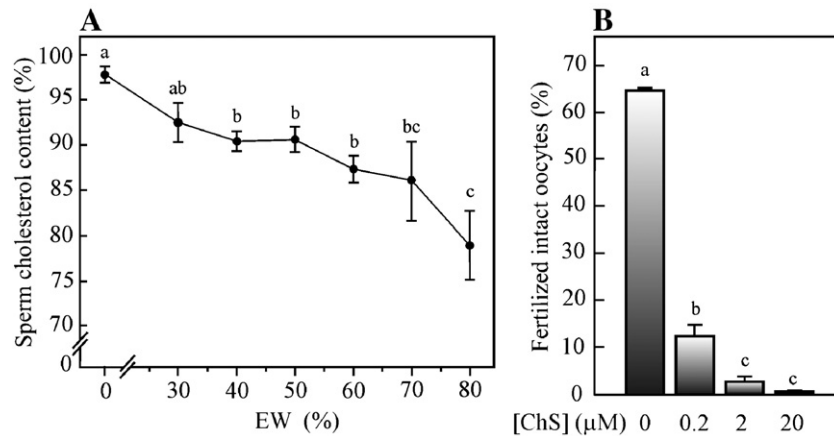


Fig. 2. Sperm cholesterol content and its role in fertilization. (A) Sperm suspensions (8×10^6 cells) were incubated 15 min in the specified EW concentration. Cholesterol sperm content was normalized within each experiment with respect to the cholesterol content of the untreated control sample (100% equivalent to 0.80 ± 0.025 pg/sperm). Data represent the mean \pm SEM ($n=4$). The means of groups that have different letters differ significantly ($p < 0.01$). (B) Intact oocyte strings were incubated for 90 min in 10% Ringer at 22 °C containing the specified cholesterol- SO_4 concentration, before insemination with 1×10^4 sperm/ml. After 15 min, the strings were transferred to fresh 10% Ringer between 110 and 170 oocytes were used in each experiment. The means of groups that have different letters differ significantly (mean \pm SEM; $n=3$; $p < 0.002$).

Effects of tyrosine kinase inhibitors on sperm protein phosphorylation and fertilization

To find out if phosphorylation inhibition had any effect on fertilization, sperm were incubated in media containing the tyrosine kinase inhibitor tyrphostin A25. After 30 min, sperm were exposed to 80% EW. Phosphorylation was inhibited in a concentration-dependent manner, with an IC_{50} (50% phosphorylation inhibition) of about 2 μM (Fig. 4A), based on densitometric analysis (Gel-Pro Analyzer, Version 3.0, Media Cybernetics). No inhibition was observed when the inactive analogue tyrphostin A1 was assayed at 200 μM . Genistein also displayed an inhibitory effect on phosphorylation with an IC_{50} between 2 and 20 μM (data not shown).

Since we observed that sperm incubated in EW were capable of fertilizing dejellied oocytes in a condition that tyrosine

phosphorylation is detected, we examined whether this phosphorylation has functional consequences. Sperm suspensions were incubated in different concentrations of tyrphostin A25 for 30 min. These suspensions were then used to inseminate intact oocytes, a condition where fertilizing capacity is naturally acquired. As shown in Fig. 4B, inhibition of tyrosine phosphorylation was accompanied by a significant reduction of fertilization rates. However, no effect was observed when sperm were preincubated in the presence of the inactive analogue tyrphostin A1, correlated with no inhibition of tyrosine phosphorylation. The inhibitor concentration was held during all the experiments by keeping the inhibitor in both the incubation and the insemination media. The effect of tyrphostin A25 on fertilization did not appear to be due to any effect on the oocyte itself since insemination of jellied oocytes in tyrphostin A25 is achieved with untreated sperm (data not shown).

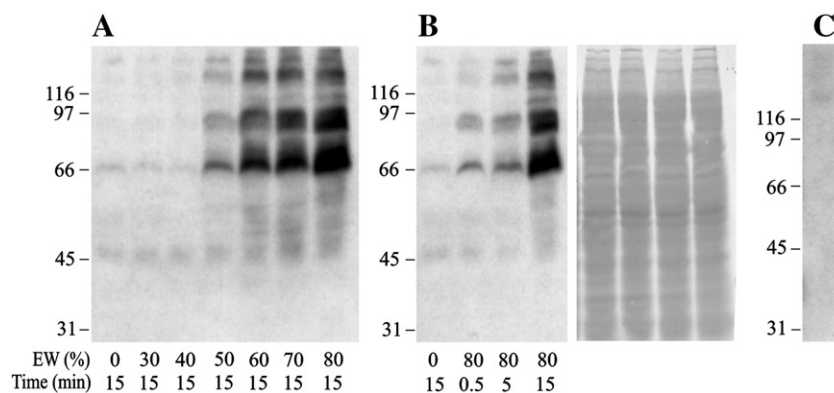


Fig. 3. Time course and concentration dependence of EW-induced protein phosphorylation in *B. arenarum* sperm. (A and B) Sperm suspensions were incubated in EW (0–80%) for 0.5 to 15 min at 22 °C. Each lane contains 1% Triton X-100 soluble proteins of 7×10^6 spermatozoa subjected to Western immunoblotting analysis by using the anti-PY antibody. The right blot of panel B was stained with Ponceau S as a loading control. (C) EW (12 μg) assayed with anti-PY antibody. Molecular weight standards are indicated at the left of each blot ($\text{Mr} \times 10^3$).

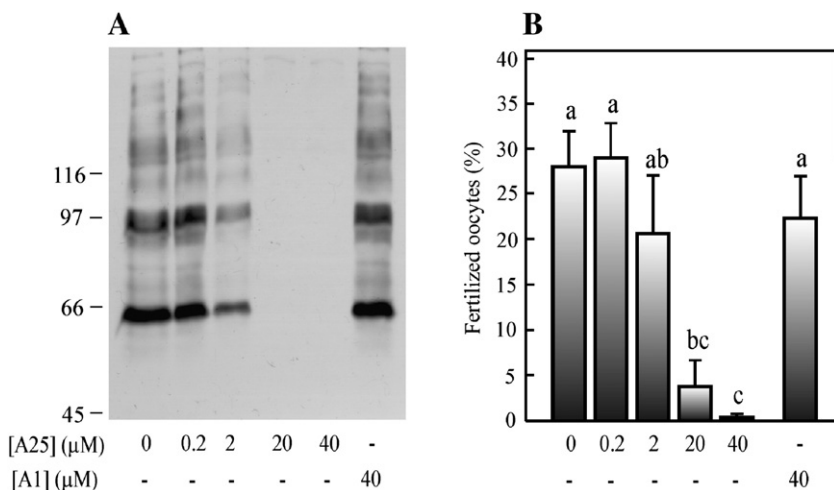


Fig. 4. Effect of protein kinase inhibitors on protein tyrosine phosphorylation and fertilization. (A) Sperm were incubated for 30 min at 19 °C in Ringer ST containing tyrphostin A25 or A1 before exposure to 80% EW for 15 min at 22 °C. Phosphoproteins were detected as in Fig. 3. While tyrphostin A25 inhibited protein tyrosine phosphorylation, the inactive analogue tyrphostin A1 had no effect at 200 μM. (B) Sperm were incubated for 30 min at 19 °C in Ringer ST containing tyrphostin A25 or A1 before inseminating jelly-intact oocytes, keeping the inhibitor concentration in the insemination media. Two- or four-cell embryos were counted. Data represent the mean±SEM, $n=3$. The means of groups that have different letter differ significantly ($p<0.0002$).

Relationship between cholesterol efflux and sperm tyrosine phosphorylation

EW components were able to sequester cholesterol from *B. arenarum* spermatozoa. However, in addition to cholesterol removal, EW molecules could induce on sperm other physiological modifications. We examined whether cholesterol removal by cyclodextrin in medium devoid of EW was sufficient to activate phosphorylation of *B. arenarum* sperm proteins. β -Cyclodextrins are water-soluble cyclic heptasac-

charides able to effectively solubilize non-polar substances (Pitha et al., 1988). They have been widely used to promote cholesterol efflux from a wide variety of cells (Kilsdonk et al., 1995; Yancey et al., 1996). Sperm cholesterol content was measured after incubations in Ringer ST media with different concentrations of methyl- β -cyclodextrin (M β CD). As shown in Fig. 5A, a concentration-dependent cholesterol efflux was observed. At 1 and 2 mM M β CD, the loss of cholesterol was around one half of the total loss. Maximum cholesterol removal was observed with 10 mM M β CD, the maximum concentration

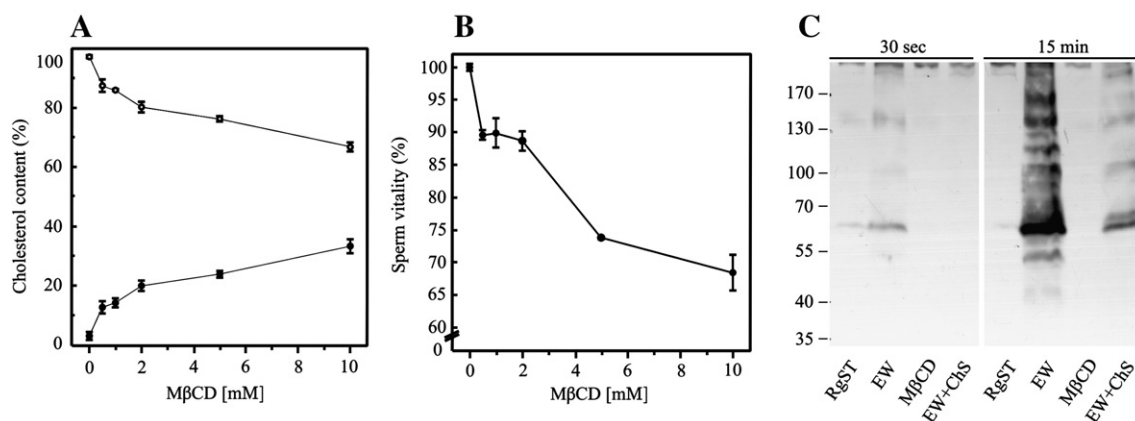


Fig. 5. Sperm cholesterol efflux in M β CD and its relation with tyrosine phosphorylation. (A) Sperm were incubated for 15 min at 22 °C in Ringer ST media containing the indicated concentrations of M β CD. Remaining cholesterol (open circles) and released cholesterol (filled circles) were normalized within each experiment with respect to the cholesterol content of the untreated control sample (100%). Cholesterol remaining at 0.5 mM M β CD or higher concentrations was significantly less than in control sample ($p<0.001$). There were no significant differences between the cholesterol remaining at 0.5 mM and 1 mM, and between the cholesterol remaining at 2 mM and 5 mM M β CD. Data represent mean±SEM ($n=4$). (B) Assessment of *B. arenarum* sperm vitality was performed with trypan blue staining after incubations in M β CD. The results express percentages of unstained spermatozoa normalized within each experiment with respect to the unstained spermatozoa of the 0 mM M β CD treatment (100%). Sperm vitality was in all cases significantly less than in control ($p<0.001$). At least 200 spermatozoa were counted in each case ($p<0.001$) (mean±SEM, $n=3$). (C) Sperm were incubated for the indicated periods of time in Ringer ST, 80% EW, 2 mM M β CD or 80% EW preincubated 30 min with 20 μM cholesterol sulfate. Protein tyrosine phosphorylation was detected as in Fig. 3.

tested. To analyze a possible vitality loss caused by cholesterol removal or toxic drug effects, sperm vitality was assessed by trypan blue staining after the M β CD treatment. Samples showed high vitality percentages ranging between 88% and 90% for M β CD concentrations between 0.5 and 2 mM (Fig. 5B). Lower vitality (74 and 68%) was observed at 5 and 10 mM M β CD. Therefore, *Bufo* sperm lose approximately 20% of cholesterol maintaining membrane integrity. Interestingly, the amount of cholesterol removed after incubation for 15 min in 80% EW (Fig. 2A) was analogous to the amount removed with 2 mM M β CD during the same period (Fig. 5A). To study a possible relationship between cholesterol efflux and tyrosine phosphorylation, sperm suspensions were incubated either for 30 s or 15 min in media containing 80% EW, 2 mM M β CD or 80% EW preincubated with 20 μ M cholesterol-sulfate. As shown in Fig. 5C, 2 mM M β CD failed in promoting tyrosine phosphorylation. However, when 80% EW was preincubated with 20 μ M cholesterol-sulfate for 30 min prior to sperm exposure, a concentration sufficient to block fertilization (Fig. 2B), the increase in protein tyrosine phosphorylation was inhibited. Altogether, these experiments suggest that, similar to what it is observed for mammalian fertilization, cholesterol efflux is necessary but not sufficient for the promotion of sperm protein tyrosine phosphorylation.

Role of M β CD and HCO $_3^-$ in mediating the fertilizing capacity acquisition of *B. arenarum* sperm

The presence of NaHCO $_3$ is required for *in vitro* capacitation of mouse sperm. The associated changes of tyrosine phosphorylation occur upon activation of sperm adenylyl cyclase induced by HCO $_3^-$ (Visconti et al., 1995a). Membrane permeable cAMP analogues can substitute for NaHCO $_3$ in promoting phosphorylation, through direct activation of PKA (Visconti et al., 1995b). We examined whether tyrosine phosphorylation of *Bufo* sperm proteins could be, as in the case of mouse sperm, stimulated by NaHCO $_3$. Sperm suspensions were incubated in modified Ringer ST media (see Materials and methods)

containing different NaHCO $_3$ concentrations. The ionic strength was kept constant in all samples. As shown in Fig. 6A, 30 mM NaHCO $_3$ did induce protein tyrosine phosphorylation. Interestingly, when sperm were preincubated with 2 mM M β CD for 15 min prior to exposure to different concentrations of NaHCO $_3$, tyrosine phosphorylation was induced at lower concentrations. A striking biphasic effect was displayed by NaHCO $_3$ when sperm were pre-treated with M β CD: tyrosine phosphorylation was induced at 1 and 5 mM NaHCO $_3$ but no phosphorylation was observed at higher concentrations. To test whether PKA could also play a role in *B. arenarum* sperm tyrosine phosphorylation, we incubated sperm with the membrane permeable cAMP analogue dibutyryl-cAMP. However, db-cAMP did not induce protein phosphorylation in incubations up to 2 h and concentrations up to 1 mM in the absence or presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Fig. 6B).

Since NaHCO $_3$ supported tyrosine phosphorylation, we examined if NaHCO $_3$ could substitute for EW in supporting acquisition of sperm fertilizing capacity. As previously demonstrated, dejellied oocytes inseminated in the absence of EW or with sperm that were not previously exposed to EW were not fertilized (Fig. 1). We performed two sets of experiments where different NaHCO $_3$ concentrations were tested on sperm with respect to their ability to fertilize dejellied oocytes. Only in one set of experiments sperm were previously incubated in the presence of 2 mM M β CD. Under no preincubation with M β CD, sperm were able to fertilize when insemination was carried out in media containing 30 mM NaHCO $_3$ (Fig. 7). Fertilization rates were four times higher in 30 mM NaHCO $_3$ than in 1 to 15 mM. However, when sperm were previously incubated in media containing 2 mM M β CD, fertilization was observed at lower NaHCO $_3$ concentrations. Both results demonstrate that acquisition of fertilizing sperm capacity could be supported in the absence of EW. *In vitro* fertilization performed at higher concentrations of NaHCO $_3$ with sperm previously incubated in media containing M β CD 2 mM displayed reduced fertilization rates (Fig. 7); interestingly,

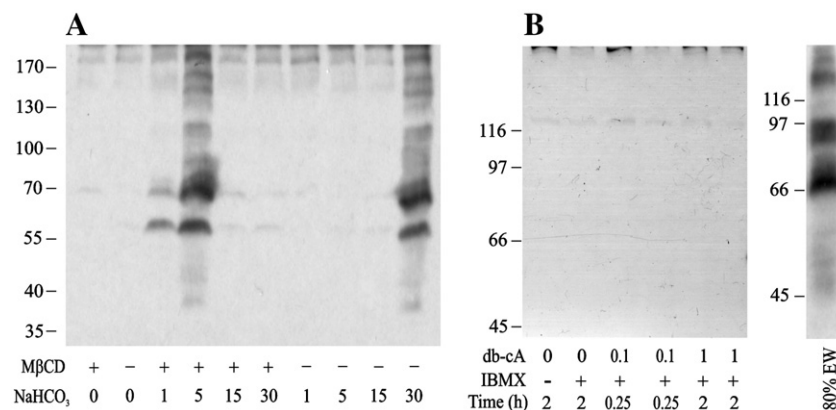


Fig. 6. Dependence of tyrosine phosphorylation induced by NaHCO $_3$ on M β CD pre-treatment and stimulation of PKA by a cAMP analogue. (A) Sperm suspensions were incubated in the absence or presence of 2 mM M β CD for 15 min prior to exposure to the specified NaHCO $_3$ concentrations (in mM) for 15 min. (B) Sperm were incubated in the presence of db-cAMP (db-cA, in mM) and 0.1 mM IBMX as specified. Right panel shows a sample incubated with 80% EW for 15 min at 22 °C. Protein tyrosine phosphorylation was detected as in Fig. 3A.

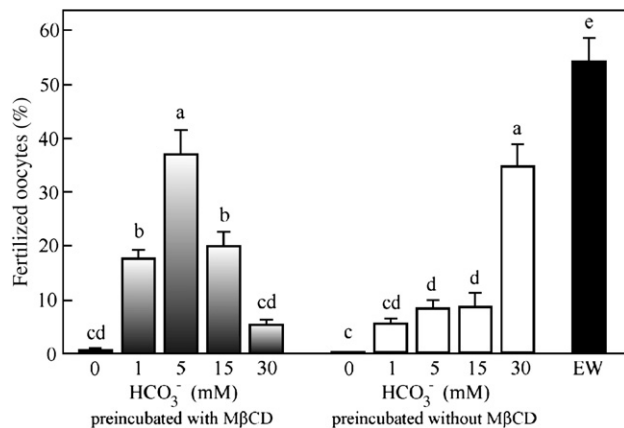


Fig. 7. Effects of MβCD incubation on the ability of sperm to fertilize dejellied oocytes in media containing NaHCO₃. Sperm suspensions were incubated for 15 min in the absence or presence of 2 mM MβCD and diluted ten times before inseminating dejellied oocytes in media containing the specified NaHCO₃ concentrations. Embryos and oocytes (at least 110) were recorded after 2–4 h. Data represent the mean ± SEM ($n=4$). The means of groups that have different letters differ significantly ($p < 0.05$).

these conditions are correlated with the levels of protein tyrosine phosphorylation shown in Fig. 6A.

Discussion

Successful fertilization by free-spawning organisms such as amphibians can occur only if a series of constraints are overcome before the sperm makes contact with the egg. *Bufo* sperm reach the egg envelope with an intact acrosome (Raisman et al., 1980; Yoshizaki and Katagiri, 1982). In *B. arenarum*, spermatozoa can bind to dejellied oocytes but no fertilization takes place (Krapf et al., unpublished results). Accordingly, previous reports showed that the acrosome of *B. japonicus* sperm bound to the vitelline envelope of dejellied oocytes is not reacted (Omata and Katagiri, 1996). These data indicate that spermatozoa depend on egg jelly components to fertilize, suggesting a key role of the jelly coat in the sperm acquisition of fertilizing capacity.

One of the characteristics of mammalian sperm capacitation is the ability of the acrosome-intact spermatozoa to undergo the acrosome reaction in response to ZP stimulation (Ward and Storey, 1984). Thus, the similarity between the unresponsiveness of acrosome reaction to egg envelope components in non-capacitated mammalian sperm with the lack of fertilizing capacity of *Bufo* sperm when no EW is present was of interest. We were able to set up conditions where fertilization of dejellied *Bufo* oocytes was restored by short preincubations of sperm in EW media before insemination. Olson and Chandler (1999) have shown different results in *X. laevis*, where incubation of sperm in EW did not restore fertilization of dejellied eggs. In those experiments, the preincubation media contained approximately 10 times less diffusible proteins and the osmolarity was about 10 times higher than our preincubation medium. Moreover, the authors restricted the preincubation periods to 5 min. Considering the short period of fertilizing capacity that *Bufo* sperm displayed in our conditions, it could be possible that they

failed to find a “fertility window” under their conditions. We showed that this presumably “capacitated state” of *B. arenarum* sperm, as first hypothesized in amphibians by Shivers and James over 35 years ago (1970), correlates to physiological changes caused by the jelly coat components to the sperm. These changes are not related to the occurrence of an acrosome reaction since EW of *B. arenarum* does not promote acrosome exocytosis of homologous spermatozoa (Arranz and Cabada, 2000; Krapf et al., 2006).

In mammals, these changes are associated with sperm cholesterol efflux and protein tyrosine phosphorylation. We measured the amount of cholesterol in *B. arenarum* sperm and found it to be similar to that of *X. laevis* (Bernardin et al., 1992). After incubations of 15 min in Ringer ST supplemented with 80% EW, approximately 20% of cholesterol was released from sperm. This efflux was dependent on EW concentration. Since a fast efflux was noticed, it is most likely that the pool of cholesterol released was located in the sperm plasma membrane (Yancey et al., 1996). This cholesterol loss could not be ascribed to an acrosome breakdown since the acrosome reaction is not induced under the assay conditions (Arranz and Cabada, 2000). We found that the cholesterol efflux is a prerequisite for fertilization since the incubation of oocytes with cholesterol-SO₄⁻ impairs fertilization.

In addition, the fertilizing capacity acquired during incubation in EW was found to be correlated with the tyrosine phosphorylation of a subset of proteins of Mr 50,000 to 200,000. The induced phosphorylation could be detected within 30 s and found it to be maximal at 15 min, with no further increase thereafter (not shown). Inhibition of tyrosine phosphorylation could be accomplished with the tyrosine kinase inhibitor tyrphostin A25. The role of tyrosine kinases during fertilizing capacity acquisition was further supported by the inhibitory effects of tyrphostin A25 on fertilization of jelly-intact oocytes.

In mouse sperm, increasing of cytoplasmic cAMP levels follows direct activation of a sperm adenylyl cyclase by HCO₃⁻ (Okamura et al., 1985; Visconti et al., 1990). Unlike the modulation of mouse sperm phosphorylation by cAMP (Visconti et al., 1995b), a cAMP analogue could not substitute for EW in promoting protein tyrosine phosphorylation. However, tyrosine phosphorylation of *B. arenarum* sperm proteins was induced by NaHCO₃. The regulation of the transmembrane signal transduction pathway leading to the promotion of tyrosine phosphorylation by EW or HCO₃⁻ could be modulated by cholesterol removal. The removal of cholesterol was necessary for activation of phosphorylation, although it could be circumvented with high NaHCO₃ concentration (Fig. 7). This fact became evident when both tyrosine phosphorylation promoted by EW and fertilization of intact oocytes were impaired when cholesterol release from sperm was abrogated. How cholesterol removal regulates this pathway is still not known. Previous studies suggested that cholesterol modifies biophysical properties of biological membranes, including the ability of membrane proteins to undergo conformational changes that may control their functions (Cross and Overstreet, 1987; Rochwerger and Cuasnicu, 1992). Thus, high concentrations of cholesterol in the membrane might inhibit membrane

protein function. This indirect effect of cholesterol on membrane protein function might stabilize those membrane and transmembrane events that are part of the intrinsic regulatory nature of fertilizing capacity acquisition. The dependence of the EW-induced increase in tyrosine phosphorylation on cholesterol membrane content could possibly be ascribed to an increase of the permeability of the sperm to certain ions, such as HCO_3^- , upon removal of cholesterol from the sperm plasma membrane. In fact, this could be similar to the regulation of some membrane-associated ion transporters by sterols (Shouffani and Kanner, 1990; Vemuri and Philipson, 1989). Cholesterol might concentrate in specialized plasma membrane microdomains known as lipid rafts. In this regard, the removal of cholesterol upon contact with components of the egg jelly or EW could mediate the disruption of lipid raft domains causing a shift in the overall membrane fluidity of the sperm plasma membrane, playing a role in the transmembrane movement of HCO_3^- (Sleight et al., 2005).

The effect of NaHCO_3 on phosphorylation could be related to intracellular pH changes rather than direct adenyl cyclase activation since a rise of intracellular cAMP concentration did not promote phosphorylation. In this regard, a $\text{Na}^+/\text{HCO}_3^-$ cotransporter was identified and localized in the flagellar plasma membrane of sea urchin (Gunaratne et al., 2006) and mouse sperm (Demarco et al., 2003). Alkalinization of intracellular pH was found to alter the phosphorylation pattern of epididymal bovine sperm proteins, but this effect could also occur via kinases other than PKA or potentially on protein phosphatases (Carr and Acott, 1989). A possible role of PKA in *Bufo* sperm capacitation cannot be discarded yet.

In summary, we identified physiological modifications that take place in *B. arenarum* sperm before the acrosome reaction. These changes are guided by female factors secreted by the oviduct that are found in the jelly coat. We could correlate these modifications (rise in tyrosine phosphorylation and cholesterol efflux) with the acquisition of fertilizing capacity. Moreover, HCO_3^- was shown to be a key element of both tyrosine phosphorylation induction and acquisition of fertilizing capacity. Using this ion in combination with M β CD, we defined an EW-free medium for fertilization. We can conclude that capacitation-like changes of spermatozoa take place in an animal with external fertilization as *B. arenarum*, suggesting that sperm capacitation is not exclusive of mammalian species.

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References

al Anzi, B., Chandler, D.E., 1998. A sperm chemoattractant is released from *Xenopus* egg jelly during spawning. *Dev. Biol.* 198, 366–375.

- Allain, C.C., Poon, L.S., Chan, C.S., Richmond, W., Fu, P.C., 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* 20, 470–475.
- Arranz, S.E., Cabada, M.O., 2000. Diffusible highly glycosylated protein from *Bufo arenarum* egg-jelly coat: biological activity. *Mol. Reprod. Dev.* 56, 392–400.
- Austin, C.R., 1952. The capacitation of the mammalian sperm. *Nature* 170, 326.
- Baldi, E., Luconi, M., Bonaccorsi, L., Forti, G., 2002. Signal transduction pathways in human spermatozoa. *J. Reprod. Immunol.* 53, 121–131.
- Barbieri, F.D., 1976. Diffusible factors in anuran fertilization. *Acta Physiol. Lat. Am., Supl.* 26, 1–9.
- Barbieri, F.D., Cabada, M., 1969. The role of the diffusible factor released by the egg jelly in fertilization of the toad egg. *Experientia* 25, 1312–1313.
- Barbieri, F.D., del Pino, E.J., 1975. Jelly coats and diffusible factor in anuran fertilization. *Arch. Biol. (Liege)* 86, 311–321.
- Barbieri, F.D., Oterino, J.M., 1972. A study of the diffusible factor released by the jelly of the eggs of the toad, *Bufo arenarum*. *Dev. Growth Differ.* 14, 107–117.
- Barbieri, F.D., Raisman, J.S., 1969. Non-gametic factors involved in the fertilization of *Bufo arenarum* oocytes. *Embryologia (Nagoya)* 10, 363–372.
- Barbieri, F.D., Vilecco, E.I., 1966. A fertilizin like substance in the jelly coat of the toad *Bufo arenarum*. *Arch. Zool. Ital.* 51, 227.
- Barisone, G.A., Krapf, D., Correa-Fiz, F., Arranz, S.E., Cabada, M.O., 2007. Glycoproteins of the vitelline envelope of amphibian oocyte: biological and molecular characterization of ZPC component (gp41) in *Bufo arenarum*. *Mol. Reprod. Dev.* 74, 629–640.
- Bernardin, G., Gornati, R., Rapelli, S., Rossi, F., Berra, B., 1992. Lipids of *Xenopus laevis* spermatozoa. *Dev. Growth Differ.* 34, 329–335.
- Cabada, M.O., 1975. Some female coordinating factors in amphibian fertilization. *Dev. Growth Differ.* 17, 187–195.
- Carr, D.W., Acott, T.S., 1989. Intracellular pH regulates bovine sperm motility and protein phosphorylation. *Biol. Reprod.* 41, 907–920.
- Chang, M.C., 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* 168, 697–698.
- Cross, N.L., Overstreet, J.W., 1987. Glycoconjugates of the human sperm surface: distribution and alterations that accompany capacitation in vitro. *Gamete Res.* 16, 23–35.
- Demarco, I.A., Espinosa, F., Edwards, J., Sosnik, J., De La Vega-Beltran, J.L., Hockensmith, J.W., Kopf, G.S., Darszon, A., Visconti, P.E., 2003. Involvement of a $\text{Na}^+/\text{HCO}_3^-$ cotransporter in mouse sperm capacitation. *J. Biol. Chem.* 278, 7001–7009.
- Devi, K.U., Jha, K., Shivaji, S., 1999. Plasma membrane-associated protein tyrosine phosphatase activity in hamster spermatozoa. *Mol. Reprod. Dev.* 53, 42–50.
- Diaz Fontdevila, M.F., Bloj, B., Cabada, M.O., 1991. Effect of egg water from *Bufo arenarum* on the fertilizing capacity of homologous spermatozoa. *J. Exp. Zool.* 257, 408–414.
- Elinson, R.P., 1971. Fertilization of partially jellied and jellyless oocytes of the frog *Rana pipiens*. *J. Exp. Zool.* 176, 415–428.
- Galantino-Homer, H.L., Visconti, P.E., Kopf, G.S., 1997. Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3′/5′-monophosphate-dependent pathway. *Biol. Reprod.* 56, 707–719.
- Gamble, W., Vaughan, M., Kruth, H.S., Avigan, J., 1978. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J. Lipid Res.* 19, 1068–1070.
- Gunaratne, H.J., Nomura, M., Moy, G.W., Vacquier, V.D., 2006. A sodium bicarbonate transporter from sea urchin spermatozoa. *Gene* 375, 37–43.
- Ishihara, K., Hosono, J., Kanatani, H., Katagiri, C., 1984. Toad egg-jelly as a source of divalent cations essential for fertilization. *Dev. Biol.* 105, 435–442.
- Kalab, P., Peknicova, J., Geussova, G., Moos, J., 1998. Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Mol. Reprod. Dev.* 51, 304–314.
- Kambara, S., 1953. Role of jelly envelopes of toad eggs in fertilization. *Annot. Zool. Jpn.* 26, 78–84.
- Katagiri, C., 1973. Chemical analysis of toad egg-jelly in relation to its ‘sperm capacitating’ activity. *Dev. Growth Differ.* 15, 81–92.

- Katagiri, C., 1986. The role of oviducal secretions in mediating gamete fusion in the toad, *Bufo bufo japonicus*. Adv. Exp. Med. Biol. 207, 151–166.
- Kilsdonk, E.P., Yancey, P.G., Stoudt, G.W., Bangerter, F.W., Johnson, W.J., Phillips, M.C., Rothblat, G.H., 1995. Cellular cholesterol efflux mediated by cyclodextrins. J. Biol. Chem. 270, 17250–17256.
- Krapf, D., Vidal, M., Arranz, S.E., Cabada, M.O., 2006. Characterization and biological properties of L-HGP, a glycoprotein from the amphibian oviduct with acrosome-stabilizing effects. Biol. Cell 98, 403–413.
- Kulanand, J., Shivaji, S., 2001. Capacitation-associated changes in protein tyrosine phosphorylation, hyperactivation and acrosome reaction in hamster spermatozoa. Andrologia 33, 95–104.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
- Leclerc, P., de, L.E., Gagnon, C., 1996. Cyclic adenosine 3',5'-monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. Biol. Reprod. 55, 684–692.
- Miceli, D.C., Cabada, M.O., 1998. Amphibian fertilization. Trends Comp. Biochem. Physiol. 5, 249–265.
- Nagarsenker, P.B., 1984. On Bartlett's test for homogeneity of variances. Biometrika 71, 405–407.
- Newport, G., 1851. On the impregnation of the ovum in the amphibian. Philos. Trans. R. Soc. Lond. (First Series Part 1) 141, 169–242.
- Okamura, N., Tajima, Y., Soejima, A., Masuda, H., Sugita, Y., 1985. Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. J. Biol. Chem. 260, 9699–9705.
- Olson, J.H., Chandler, D.E., 1999. *Xenopus laevis* egg jelly contains small proteins that are essential to fertilization. Dev. Biol. 210, 401–410.
- Olson, J.H., Xiang, X., Ziegert, T., Kittelson, A., Rawls, A., Bieber, A.L., Chandler, D.E., 2001. Allurin, a 21-kDa sperm chemoattractant from *Xenopus* egg jelly, is related to mammalian sperm-binding proteins. Proc. Natl. Acad. Sci. U. S. A. 98, 11205–11210.
- Omata, S., Katagiri, C., 1996. Involvement of carbohydrate moieties of the toad egg vitelline coat in binding with fertilizing sperm. Dev. Growth Differ. 38, 663–672.
- Osheroff, J.E., Visconti, P.E., Valenzuela, J.P., Travis, A.J., Alvarez, J., Kopf, G.S., 1999. Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. Mol. Hum. Reprod. 5, 1017–1026.
- Pitha, J., Irie, T., Sklar, P.B., Nye, J.S., 1988. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. Life Sci. 43, 493–502.
- Raisman, J.S., 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, 289–297.
- Rochwerger, L., Cuasnicu, P.S., 1992. Redistribution of a rat sperm epididymal glycoprotein after in vitro and in vivo capacitation. Mol. Reprod. Dev. 31, 34–41.
- Shapiro, S.S., Wilk, M.B., 1965. Distributional fitting, assumption testing. Biometrika 52, 591–611.
- Shivers, C.A., James, J.M., 1970. Capacitation of frog sperm. Nature 227, 183–184.
- Shivers, C.A., James, J.M., 1971. Fertilization of antiserum-inhibited frog eggs with “capacitated” sperm. Biol. Reprod. 5, 229–235.
- Shouffani, A., Kanner, B.I., 1990. Cholesterol is required for the reconstruction of the sodium- and chloride-coupled, gamma-aminobutyric acid transporter from rat brain. J. Biol. Chem. 265, 6002–6008.
- Sleight, S.B., Miranda, P.V., Plaskett, N.W., Maier, B., Lysiak, J., Scrable, H., Herr, J.C., Visconti, P.E., 2005. Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation. Biol. Reprod. 73, 721–729.
- Suzuki, F., Yanagimachi, R., 1989. Changes in the distribution of intramembranous particles and filipin-reactive membrane sterols during in vitro capacitation of golden hamster spermatozoa. Gamete Res. 23, 335–347.
- Valz-Gianinet, J.N., del Pino, E.J., Cabada, M.O., 1991. Glycoproteins from *Bufo arenarum* vitelline envelope with fertility-impairing effect on homologous spermatozoa. Dev. Biol. 146, 416–422.
- Vemuri, R., Philipson, K.D., 1989. Influence of sterols and phospholipids on sarcolemmal and sarcoplasmic reticular cation transporters. J. Biol. Chem. 264, 8680–8685.
- Visconti, P.E., Kopf, G.S., 1998. Regulation of protein phosphorylation during sperm capacitation. Biol. Reprod. 59, 1–6.
- Visconti, P.E., Muschietti, J.P., Flawia, M.M., Tezon, J.G., 1990. Bicarbonate dependence of cAMP accumulation induced by phorbol esters in hamster spermatozoa. Biochim. Biophys. Acta 1054, 231–236.
- Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995a. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121, 1129–1137.
- Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995b. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121, 1139–1150.
- Visconti, P.E., Galantino-Homer, H., Ning, X., Moore, G.D., Valenzuela, J.P., Jorgez, C.J., Alvarez, J.G., Kopf, G.S., 1999a. Cholesterol efflux-mediated signal transduction in mammalian sperm. Beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. J. Biol. Chem. 274, 3235–3242.
- Visconti, P.E., Ning, X., Fornes, M.W., Alvarez, J.G., Stein, P., Connors, S.A., Kopf, G.S., 1999b. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Dev. Biol. 214, 429–443.
- Visconti, P.E., Stewart-Savage, J., Blasco, A., Battaglia, L., Miranda, P., Kopf, G.S., Tezon, J.G., 1999c. Roles of bicarbonate, cAMP, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. Biol. Reprod. 61, 76–84.
- Visconti, P.E., Westbrook, V.A., Chertihin, O., Demarco, I., Sleight, S., Diekmann, A.B., 2002. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J. Reprod. Immunol. 53, 133–150.
- Ward, C.R., Storey, B.T., 1984. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev. Biol. 104, 287–296.
- Xiang, X., Burnett, L., Rawls, A., Bieber, A., Chandler, D., 2004. The sperm chemoattractant “allurin” is expressed and secreted from the *Xenopus* oviduct in a hormone-regulated manner. Dev. Biol. 275, 343–355.
- Xiang, X., Kittelson, A., Olson, J., Bieber, A., Chandler, D., 2005. Allurin, a 21 kD sperm chemoattractant, is rapidly released from the outermost jelly layer of the *Xenopus* egg by diffusion and medium convection. Mol. Reprod. Dev. 70, 344–360.
- Yancey, P.G., Rodriguez, W.V., Kilsdonk, E.P., Stoudt, G.W., Johnson, W.J., Phillips, M.C., Rothblat, G.H., 1996. Cellular cholesterol efflux mediated by cyclodextrins. Demonstration Of kinetic pools and mechanism of efflux. J. Biol. Chem. 271, 16026–16034.
- Yoshizaki, N., Katagiri, C., 1982. Acrosome reaction in sperm of *Bufo bufo japonicus*. Gamete Res. 6, 342–352.