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Vitelline Envelope of *Bufo arenarum*: Biochemical and Biological Characterization[1]

Gustavo A. Barisone,[3] Jerry L. Hedrick,[4] and Marcelo O. Cabada[2,3]

Division Biología del Desarrollo,[3] Instituto de Biología Molecular y Celular de Rosario (IBR) (CONICET-UNR); Facultad de Ciencias Bioquímicas y Farmacéuticas (UNR), Rosario S2002LRK, Argentina
Section of Molecular and Cellular Biology,[4] University of California, Davis, California 95616

ABSTRACT

Vitelline envelopes (VEs) of *Bufo arenarum* were isolated in order to study their composition and their role in fertilization. VEs are composed of four glycoproteins, with molecular masses of 120, 75, 41, and 38 kDa. To characterize its biological properties, we quantitatively determined sperm-VE binding and the induction of the acrosome reaction. Heterologous binding of *B. arenarum* sperm to *Xenopus laevis* VE components was observed with about one-third the efficiency of homologous binding. Equivalent binding of *X. laevis* sperm to the *B. arenarum* VE was observed. When *B. arenarum* sperm were incubated with fluorescein isothiocyanate-labeled VE, the labeled glycoproteins bound to the anterior end of the sperm head, showing a lateral distribution. Induction of the acrosome reaction was evaluated by incubating sperm in hypotonic saline media with VE glycoproteins. VEs induced the acrosome reaction in a time- and concentration-dependent manner. The acrosome reaction was maximal after 10 min. The half-maximal effect was obtained at a glycoprotein concentration of 1 μg/ml. Specificity was determined using fertilization envelope glycoproteins, which failed to induce the acrosome reaction. The *B. arenarum* VE is biochemically similar to other egg envelopes. It also seems that its biological properties are similar to other species in regard to sperm binding and induction of the acrosome reaction. However, as far as we are aware, this is the first observation of the VE inducing the sperm acrosome reaction in amphibians. The relatively small differences observed in heterologous sperm-VE binding in *X. laevis* and *B. arenarum* are inconsistent with the current paradigm that species specificity in fertilization is regulated at the sperm-VE binding step.

acrosome reaction, fertilization, gamete biology, sperm

INTRODUCTION

Animal eggs are surrounded by specialized extracellular matrices that serve different functions during fertilization. The extracellular matrix of amphibian oocytes consists of a definite structure, the vitelline envelope (VE), and a number of jelly coat layers. The VE is composed of glycoproteins that are synthesized and secreted during oocyte maturation in the ovary; the jelly layers are produced by oviductal secretions that overlay the oocytes as they flow along the oviduct after being released from the ovary [1, 2].

Gamete interactions resulting in successful fertilization involve many complex processes. The relevance of the egg extracellular matrix in the early events of fertilization has been clearly and extensively documented in many animal species; indeed, it mediates sperm-oocyte binding, induction of the acrosome reaction, sperm penetration, and prevention of polyspermy. The egg extracellular matrix is also proposed to mediate species-specific gamete recognition.

The molecular mechanisms that explain many of these functions have been frequently studied in mammals [3–8]. In other species, however, many of the roles of the egg extracellular matrix have not been clearly demonstrated or are still controversial. In sea urchin, the acrosome reaction is known to be induced in a species-specific way by at least two components of the jelly coat [9]. Furthermore, a sperm receptor on the egg surface has been reported [10–14]. In starfish, the acrosome reaction is also triggered by the jelly coat [15, 16]. In amphibians, conflicting results have been published on the role of the egg extracellular matrix. Sperm binding was attributed to the VE glycoprotein of M_r 41, known as ZPC, in *B. japonicus* and in *X. laevis* [17]. The egg jelly in amphibians is essential for fertilization, but its specific function is not yet clear.

In this report we study the biochemical composition of the VE of *B. arenarum*. We analyzed its biological properties and, in particular, sperm binding and induction of the acrosome reaction, in order to provide data to better understand the role of this specialized extracellular matrix in amphibian fertilization. We present results that demonstrate a functional involvement of the VE in sperm binding and in the induction of the acrosome reaction in *B. arenarum*.

MATERIALS AND METHODS

Animals and Gametes

B. arenarum specimens were collected in the neighborhoods of Rosario, Argentina, and were kept in a moist chamber at 15°C. Testes were dissected from male toads, and spermatozoa were obtained by mincing the organs in Ringer-Tris solution (0.11 M NaCl, 2 mM KCl, 1.4 mM $CaCl_2$, 10 mM Tris, pH 7.2) at 4°C. The homogenate was filtered through gauze and the suspension was centrifuged for 10 min at 130 × *g* at 4°C to remove blood cells and tissue debris. The sperm suspension was centrifuged for 10 min at 650 × *g* at 4°C. Pelleted spermatozoa were resuspended in Ringer-Tris solution, and the concentration of cells was estimated by measuring absorbance at 410 nm.

Sperm suspensions to be used in quantitative sperm binding assays were prepared by mincing testes in Ringer-Tris and filtering them through gauze. Sperm suspensions were kept in isotonic medium no longer than 10 min, and were diluted to the desired concentration of cells immediately before use. Dilutions were made so that the final medium for the binding assay was hypotonic (10% Ringer-Tris).

Female specimens were kept in a moist chamber at 20–22°C for 1 day before stimulation with one homologous hypophysis homogenate injected intracoelomically. After 10–12 h, oocyte strings were collected from ovisacs [18].

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[2]Correspondence: Marcelo O. Cabada, Área Biología, Fac. Cs. Bioquímicas y Farmacéuticas, U.N.R., Suipacha 531, Rosario S2002LRK, Argentina. FAX: 54 341 4804601; e-mail: cabada@elsitio.net

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Envelope Isolation and Solubilization

To isolate VEs, oocyte strings were hydrated in 10% Ringer-Tris (10% Ringer solution, 10 mM Tris, pH 7.6) for 30 min, and dejellied with 1% thyoglycolic acid solution pH 8.0. Oocytes were thoroughly washed with ice-cold 10% Ringer-Tris containing 10 mM EDTA, and homogenized with a Potter-Elvehjem homogenizer (Thomas Scientific, Swedesboro, NJ). Vitelline envelopes were obtained by filtering the homogenate through a double sheet of 30-mesh screen, and were extensively washed with the same buffer [19]. All procedures were carried out at 4°C. VEs were solubilized in distilled water by heating at 100°C for 5–10 min.

To isolate fertilization envelopes (FEs), oocyte strings were fertilized and dejellied when embryos were at the two-cell stage. Washing, homogenization, and filtration were performed essentially as described for VEs. FEs were solubilized in distilled water by heating at 100°C for 10–20 min.

Solubilized VEs and FEs were centrifuged at 10 000 × *g* for 20 min at 4°C, and the supernatant solutions were stored at −20°C for up to 2 mo. Envelope glycoprotein concentration was measured by the Folin phenol (Lowry) method [20].

Chemical Deglycosylation

Deglycosylation of VE components was carried out with trifluoromethanesulfonic (TFMS) acid, using a Glycofree Deglycosylation kit (Oxford GlycoSciences, U.K.), according to the manufacturer's instructions.

Electrophoresis

Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed essentially according to the method of Laemmli [21]. Dissolved VE samples were diluted with an appropriate volume of 5× sample buffer with or without β-mercaptoethanol (β-ME), boiled for 5 min, and loaded onto 10% acrylamide mini gels with 5% stacking gel, and electrophoresed for about 1 h at 20 mA/gel (MiniProtean II gel system; BioRad, Hercules, CA). To determine apparent molecular masses, we coelectrophoresed samples with molecular mass standards (BioRad). After electrophoresis, gels were processed for Coomassie Brilliant Blue staining [22] or silver staining (silver stain kit, Sigma Chemical Company, St. Louis, MO). Glycoprotein bands were visualized with periodic acid/Schiff staining (PAS) [23] or a combination of PAS and silver staining [24].

Labeling of VE Glycoproteins

Solubilized VEs were labeled with fluorescein isothiocyanate (FITC) [22]. Briefly, 4 mg of glycoprotein in 1 ml of 0.1 M sodium carbonate (pH 9) were incubated with 50 μl of FITC (1 mg/ml in dimethyl sulfoxide) for 8 h at 4°C. NH_4Cl was added to 50 mM and the solution was incubated for 2 h. The unbound dye was separated by dialysis in 0.1× PBS overnight at 4°C. The dialyzed glycoproteins were further washed and concentrated using Centricon 10 (Millipore Corp., Bedford, MA); washings were carried out with 10% Ringer, 20 mM Tris, pH 7.6. The ratio of coupling was estimated by measuring the absorbance at 495 nm and 280 nm [22]. Glycoprotein concentration (mg/ml) was estimated as $(Abs_{280} - 0.35 \times Abs_{495})/1.4$ [25]. The purity and integrity of the labeled VE components were analyzed by SDS-PAGE.

Fertilization Inhibition Assays

Batches of 50–60 dejellied oocytes were incubated in 10% Ringer-Tris containing different dilutions of anti-VE polyclonal antibodies [26] (treated) or preimmune rabbit serum (controls). After 30 min at 18–20°C, eggs were washed with 10% Ringer-Tris, and 500 μl of fertilization media (50% egg water in 10% Ringer-Tris) was added. Twenty-five microliters of a sperm suspension ($OD_{410nm} = 0.9$; approximately 7×10^6 cells/ml) was then added to each batch of oocytes. Fertilization medium was washed out 25 min later, eggs were kept in 10% Ringer-Tris, and fertilization levels were determined 4–10 h later by counting the number of embryos at the 4- or 8-cell stage.

Acrosome Reaction Assays

Sperm were incubated with solubilized VEs in a hypotonic medium (10% Ringer-Tris). In the acrosome reaction assay as a function of incubation time, 40 μl of stock sperm suspension in Ringer-Tris ($OD_{410nm} = 1.5$, approximately 16×10^6 sperm/ml) was diluted 10-fold in distilled water or VE solution containing no salt (see *Solubilization Procedure*, above) in order to obtain hypotonic incubation media without (controls) or with (treated) VE glycoproteins. Glycoprotein concentration was 1 μg/μl in the final incubation mixture. Suspensions were kept at 18°C, and 100-μl aliquots were taken at 1, 10, and 30 min. Ringer-Tris (1.4 ml) was immediately added to each aliquot to turn the medium back to isotonic. Sperm were pelleted at 550 × *g* for 5 min at room temperature, and finally resuspended in 20 μl of Ringer-Tris.

In the acrosome reaction assay as a function of VE or FE protein concentration, 10 μl of stock sperm suspension in Ringer-Tris was diluted 10-fold in VE or FE solutions of different glycoprotein concentrations, ranging from 0.01 to 1000 μg/μl in the final incubation mixture. Sperm were incubated for 10 min at 18°C, and 1.4 ml Ringer-Tris was added. Sperm were pelleted as described above, and resuspended in 20 μl of Ringer-Tris.

Assessment of Acrosome Status

Acrosomal status was assessed using an indirect immunofluorescence technique [27]. Briefly, sperm were immobilized on polylysine-coated coverslips, fixed with 3% formaldehyde in PBS, and permeabilized with 0.2% Triton X-100. Acrosomal content was detected using an antigen-specific primary antibody and an FITC-coupled secondary antibody. In this way, fluorescence in the anterior end of the sperm head indicated presence of the acrosomal content and, therefore, nonreacted sperm. About 100–150 cells were evaluated in each of 4 repetitions for every condition assayed. Preparations were counted under an Olympus BH-2 epifluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Sperm Binding: Quantitative Assays

Sperm binding to homologous and heterologous VEs was assayed using a direct binding method developed by Vo and Hedrick [17]. Briefly, ligands of interest were coupled to silanized glass slides using a macromolecule concentration of 0.2 mg/ml protein. Glass slides were washed and incubated with sperm suspensions. Slides were extensively washed in PBS (3 times for 20 min). Samples were fixed with 3% formaldehyde/PBS, and bound sperm were counted with a light microscope.

Sperm Binding: Fluorescence Microscopy

Sperm suspensions (about 30×10^6 cells/ml) in Ringer-Tris were fixed in 3% formaldehyde (final concentration) for 20 min at 20–22°C. To avoid sperm agglutination, the suspensions were homogenized by gently flicking the tubes every 2–3 min. Fixed sperm were washed twice with PBS (2-fold volume), and finally resuspended in the same buffer (starting volume). One hundred-microliter samples were pelleted at 1200 × *g* for 5 min, and resuspended in 100 μl of either labeled VE (FITC-VE, 1 mg/ml) or Ringer-Tris (controls). After incubating for 45 min at 20–22°C, sperm were washed twice with PBS, pelleted as above, and mounted in 0.1% phenylendiamine/50% glycerol and PBS for fluorescence microscopy.

Statistical Analysis

In the fertilization inhibition experiments data were analyzed with nonparametric ANOVA techniques and multiple comparisons based on the Kruskal Wallis method (range addition) [28].

In quantitative binding assays, data were analyzed by analysis of covariance (ANCOVA) to study the relationship between variables (number of sperm bound versus sperm concentration) for the different ligands assayed. Each data set was further analyzed by ANOVA to determine significant differences for each sperm concentration tested. For heterologous binding, data were analyzed by ANOVA using the Scheffé method for multiple comparisons [29].

In the acrosome reaction assays, data were analyzed by ANOVA to establish the significance of the percentage of acrosome-reacted sperm in VE, FE, or control media.

In all cases, comparisons were considered to be significantly different when P values were < 0.05 [29].

RESULTS

Electrophoresis Analysis

Solubilized VE components were analyzed by SDS-PAGE to determine the VE macromolecular composition. Samples were reduced with β-mercaptoethanol, and gradient slab gels of 7%–15% acrylamide concentration were

run. Alternatively, samples were analyzed in 10% gels. Coomasie Blue staining of the gels revealed four major bands, with apparent molecular masses of 120, 75, 41, and 38 kDa, respectively (hereafter termed gp120, gp75, gp41, and gp38; Fig. 1, lane 1). Similar gels were also stained with silver, with the PAS method for detecting glycoproteins or with the PAS-silver method. The staining pattern was similar in all cases (Fig. 1, lane 2), indicating that all of the macromolecular components described are glycoproteins.

Electrophoresis of nonreduced samples (β-mercaptoethanol omitted from the loading buffer) also resulted in four major bands, three of which had different electrophoretic mobilities when compared with their reduced counterparts. The estimated apparent molecular masses of the nonreduced bands were 120, 73.5, 34, and 32 kDa (Fig. 1, lane 3).

Solubilized VEs were deglycosylated with TFMS, resulting in nonselective cleavage of both *O*- and *N*-linked oligosaccharide chains [30], leaving the primary structure of the protein intact. Deglycosylation yielded five major bands upon SDS-PAGE analysis (reducing conditions), with apparent molecular masses of 99, 86, 64, 37, and 31 kDa (Fig. 1, lane 4).

The FE composition was also analyzed by SDS-PAGE. Under reducing conditions four major bands were detected. When compared to VE, only one band was observed to have a different electrophoretic mobility; the VE component gp75 changed to a diffuse, ill-defined band with an apparent molecular mass of 69–66 kDa in the fertilization envelope (Fig. 1, lane 5). When deglycosylated FE was compared to deglycosylated VE, the same mass difference described above was observed (i.e., the deglycosylated polypeptide derived from gp69/66 had a lower molecular weight; data not shown). This observation suggests the gp75 to gp69/66 conversion at fertilization may involve proteolysis.

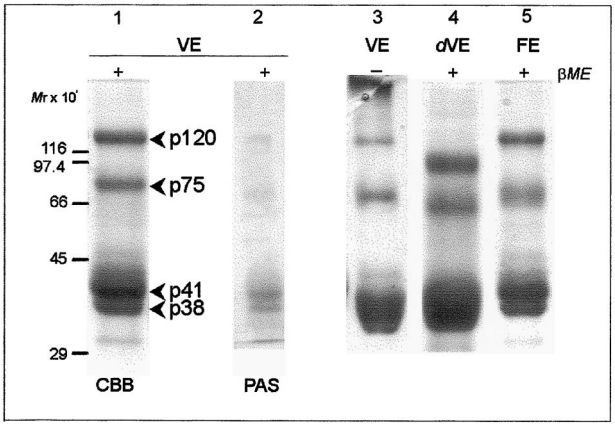

FIG. 1. Electrophoretic analysis of *Bufo arenarum* VE. Solubilized VE glycoproteins were separated on a 10% gel under reducing and denaturing conditions. The gels were stained with Coomasie Brilliant Blue (lane 1) or periodic acid/Schiff (PAS, lane 2). Molecular mass markers are displayed on the left. Four major bands were observed. Thirty micrograms of protein were loaded onto gels. Nonreduced VE (lane 3) and deglycosylated VE (dVE; lane 4) and fertilization envelopes (FE; lane 5) were analyzed in similar gels.

Inhibition of Fertilization with Anti-VE Antibodies

We studied the effect of anti-VE antibodies on in vitro fertilization levels. Assays involving the incubation of oocytes with different dilutions of polyclonal anti-VE serum showed an inhibitory effect of antibodies on fertilization. In three independent experiments, a significant decrease in fertilization (evaluated as the number of cleaving eggs) was consistently observed (data not shown). Almost 100% inhibition of fertilization was achieved with 10-fold dilutions of the serum, whereas fertilization levels did not differ from controls when 1000-fold dilutions were used ($P < 0.05$). No significant decrease in fertilization ($P < 0.05$) was observed in controls with normal rabbit serum, even at high concentrations.

Sperm Binding to the VE

An important aspect in the fertilization process is the recognition and binding of spermatozoa to the VE. We carried out experiments to quantify sperm binding to dissolved VEs. We used a quantitative sperm binding assay that involved the covalent coupling of glycoproteins to glass slides. The assay was designed so that only live, motile sperm could interact with the immobilized ligands [17]. To test the validity of the assay for our system of *B. arenarum* sperm, we initially compared sperm binding to homologous VE and to BSA as a function of sperm concentration. Negative controls were run using silanized glass with no coupled macromolecules. As shown in Figure 2A, sperm bound in a concentration-dependent manner to the VE. Sperm binding became concentration-independent at levels above 3×10^6 sperm/ml. The binding dependence on sperm concentration to the VE and the lower sperm binding to BSA and control glass validated the sperm binding assay. Statistical analysis (ANCOVA) indicated that the variable ''# sperm bound'' behaved differently for the conditions tested; it was constant for glass controls but not for VE or BSA binding. However, the number of sperm bound to VE-treated glass was significantly higher than for BSA controls ($P < 0.05$) when sperm suspensions of a concentration of 3×10^6 cells/ml or higher were used. *B. arenarum* sperm bind specific ligands in the VE, as described for other species [17].

Species specificity of the binding was checked using *X. laevis* VE as a ligand. As indicated in Figure 2B, we observed that *B. arenarum* sperm bind *X. laevis* VEs. Binding to both *Xenopus* and *Bufo* VEs was significantly higher than controls ($P \approx 0$). However, sperm bound to *X. laevis* VEs about one-third less efficiently than they did to the homologous envelope components. The reverse experiment was also carried out; *X. laevis* sperm bound *B. arenarum* VEs at about the same rates (data not shown).

We also evaluated sperm binding to VEs using fluorescence microscopy. VE glycoproteins were labeled with FITC. After extensive dialysis and filtration, the labeling reaction was checked by SDS-PAGE; all four components of the envelope proved to be labeled, and no free dye was observed (data not shown). Sperm suspensions were fixed with formaldehyde before incubation with FITC-VE, in order to avoid an acrosome reaction during the long incubation periods. As shown in Figure 3, incubation of nonreacted sperm with fluorescent VEs resulted in staining of the sperm head. In different repetitions of the experiment using different sperm preparations, a distinct pattern was always observed; the fluorescent label was localized at the anterior end of the sperm head, showing a rather lateral distribution (Fig. 3B). This pattern was not due to different planes of the sperm being focused, because it was repetitive and consistent in hundreds of cells observed in different viewing fields in independent experiments. Some fluorescence was

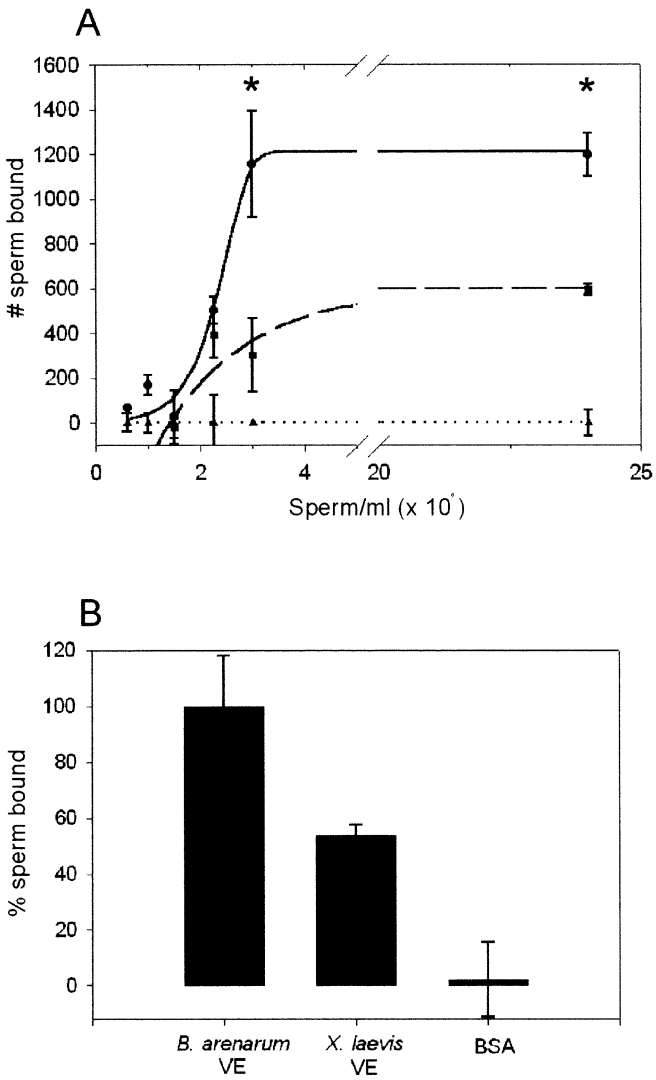

FIG. 2. Sperm binding to the VE. **A**) Sperm-envelope binding as a function of sperm concentration. Sperm suspensions were prepared in 10% Ringer-Tris. A protein concentration of 0.2 mg/ml was used for coupling. Data points for VE, BSA, and controls (no macromolecules bound to glass) are indicated by circles, squares, and triangles, respectively. Error bars represent the SD of three measurements. Trend lines for VE, BSA, and controls are indicated by the solid line, dashed line, and dotted line, respectively. Asterisks indicate date points with a significant difference ($P < 0.05$) between VE and BSA binding. **B**) *Bufo arenarum* sperm binding to homologous and heterologous VE. The sperm binding assay was performed on *Bufo arenarum* and *Xenopus laevis* VE glycoproteins. Sperm suspensions were used at a concentration of 4×10^6 cells/ml in 10% Ringer-Tris; 0.2 mg/ml protein was used for coupling. All data represent the mean number of sperm bound subtracted from background binding (control slides with no macromolecules; number of sperm bound 124 ± 31), relative to the number of sperm bound to the homologous VE components (% sperm bound = [# sperm bound − control]/[# sperm bound to *Bufo arenarum* VE − control] × 100). Error bars represent the SD of three measurements. Multiple comparison techniques indicated that all three data sets are significantly different from each other ($P \approx 0$).

also observed at the sperm tail, but it was much less intense, nonlocalized, and attributed to nonspecific binding.

Induction of the Acrosome Reaction

We tested whether the solubilized macromolecular components of the VE induced acrosome breakdown in homologous sperm. Acrosomal status was evaluated using a technique developed in our laboratory [27]. Sperm suspensions were incubated with solubilized VEs for different periods of time. For each experiment, controls were run in parallel (i.e., sperm were incubated in saline medium without VE components for the same times). In a series of four experiments, the number of acrosome-reacted spermatozoa was consistently higher ($P < 0.005$) in treated than in control suspensions, the difference being maximal after a 10-min incubation time (Fig. 4A). Different sperm preparations were used for each experiment, and at least 100 spermatozoa were counted in each case. Considerable variation was found among the single experiments; this was probably due to differences in the basal values (percentage of sperm spontaneously reacted in saline medium) of different sperm preparations. However, statistical analysis confirmed that both data sets (control versus VE) behaved differently ($P \approx 0$); the percentage of acrosome-reacted sperm in control experiments is statistically not different for the 1- or 10-min incubation time, but a significant increase is observed for 30-min incubation; on the other hand, in VE media a significant increase is observed between 1 and 10 min, but not between 10 and 30 min of incubation.

The VE induced the acrosome reaction in a concentration-dependent manner (Fig. 4B). At low protein concentrations (<0.1 μg/ml) no differences were observed between treated and control suspensions (data not shown); however, the number of reacted spermatozoa significantly increased ($P < 0.005$) when VE components were present in the incubation media at a concentration of 1 μg/ml, and reached a maximum (about 85% reacted sperm) at 10 μg/ml.

To check if the effect observed was specifically induced by the VE components, we carried out the same experiment using solubilized FEs. Controls were run by incubating sperm in saline solution (basal) and with solubilized VEs (induced). In all cases, no significant difference was observed between FE-treated and control suspensions, although incubation with VEs in the same experiment resulted in a high rate of acrosome-reacted sperm (Fig. 4B); that is, no significant induction of acrosome reaction was observed with fertilization envelopes, even at high glycoprotein concentrations of up to 1000 μg/ml.

DISCUSSION

We used *B. arenarum* as an amphibian model for the study of the early events of fertilization. In particular, we were interested in improving our understanding of sperm binding and induction of the acrosome reaction in amphibians. *B. arenarum* provides a suitable model for such studies because spermatozoa can easily be obtained; they are stable and remain motile long enough to allow for functional studies in which cell integrity is essential.

The VE of *B. arenarum* consists of four major components as analyzed by SDS-PAGE under reducing conditions. All components are glycoproteins because they are stained by the PAS method; furthermore, deglycosylation results in all of the bands shifting down in PAGE. This indicates that oligosaccharides are present in all four components. Removal of *O*- and *N*-linked oligosaccharides resulted in five bands instead of four. This seems to indicate that one of the glycoprotein bands (the 120-kDa component) consists of at least two different proteins. Partial proteolytic cleavage, however, is also possible. Nevertheless, microheterogeneity of the glycosidic moiety of the VE components has been well documented in other species. Analysis of nonreduced samples indicates the presence of disulfide bonds in at least three of the components. Non-

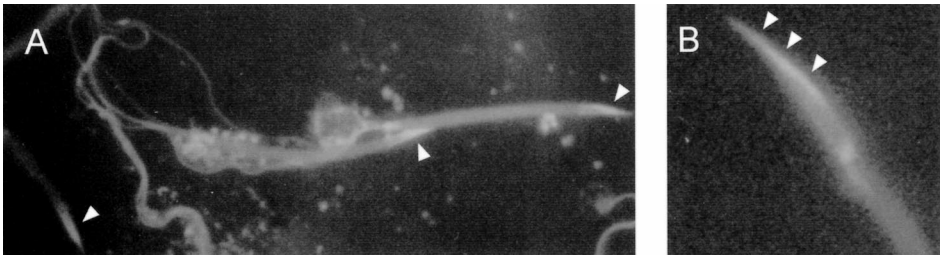

FIG. 3. Sperm binding to the VE. Sperm suspensions were fixed with formaldehyde in isotonic medium to avoid acrosome breakdown, washed, and incubated with FITC-labeled VE glycoproteins. Sperm were washed and prepared for fluorescence microscopy. Fluorescence was observed to be associated with the sperm head, as indicated by arrowheads in **A**. Details of the anterior end of the sperm head are shown in **B** at higher magnification. Magnification ×1500 in **A**; ×2500 in **B**.

reduced components show higher electrophoretic mobilities than β-mercaptoethanol-treated components. This can be explained by the presence of internal disulfide bonds. If this is the case, breaking these bonds would result in a change in the folding of the macromolecule and probably in the interaction of some domains with SDS, which in turn, would result in a different electrophoretic mobility.

The macromolecular composition of the VE of *B. arenarum* very much resembles that of *X. laevis*. In this species, four major components have been determined: gp120 (ZPAX), gp69 (ZPA), gp41 (ZPC), and gp37 (ZPB) [17]. The cDNAs corresponding to these proteins have been cloned and shown to be homologous to mammalian zona pellucida glycoproteins [17, 31]. In *B. arenarum*, the four major components detected have similar molecular masses to those reported in *X. laevis*. Further experimental evidence is needed to evaluate homology between components in both species.

The importance of the VE in the fertilization process has been well documented in different species [3, 7, 32–35]. This is confirmed in our study by the effect of anti-VE antiserum on fertilization. Anti-VE antibodies in the media inhibit fertilization in a specific, concentration-dependent manner. Antibodies could prevent sperm from interacting with the VE, possibly by masking binding sites on the egg extracellular matrix. Steric effects should also be considered, because immunoglobulins could hinder access of ligands to binding sites. Antibodies could also interfere with other steps of fertilization, such as the acrosome reaction or penetration of the sperm through the VE to reach the plasma membrane. Further experiments are needed to understand the molecular basis of this inhibition. However, it is clear that blocking some sites on the VE renders eggs unfertilizable.

Localization of sperm receptors for the VE ligands was studied using fluorescence microscopy techniques. Formaldehyde-fixed sperm were used for this assay in order to avoid acrosome breakdown during the incubation for long times. Therefore, our results apply for nonreacted sperm; plasma membrane receptors seem to be present in a discrete area at the anterior end of the sperm head. Binding of labeled VE components to the inner acrosomal membrane using similar techniques remains to be studied.

Heterologous binding of *B. arenarum* sperm to *X. laevis* VE was also demonstrated. This would indicate the presence of somewhat conserved binding sites. Only a 3-fold difference in binding of heterologous sperm and VE was observed. This observation seems to be inconsistent with the current paradigm of sperm-egg envelope binding being a species-specific step in fertilization. However, these findings do not necessarily reflect any biological significance. *B. arenarum* and *X. laevis* do not share a common habitat nor do they exhibit the same reproductive behavior. If behavioral and geographical considerations are taken into account, then cross-species sperm-VE interaction may be regarded as not biologically relevant. On the other hand, this finding may be quite important if evolutionary relationships are considered. In this regard, cross-species binding may be the result of some protein or glycosylation motifs being conserved in the two species through evolution from common VE glycoprotein ancestors. This of course is just speculative.

The triggering of the acrosome reaction has long been controversial. This exocytosis must take place at the right moment and place during the fertilization process [36]. A nonreacted sperm does not penetrate the oocyte envelope. On the other hand, if the acrosome reaction occurs far from the VE, the reacted sperm can reach the VE, but fertilization is not achieved, probably due to the loss of acrosomal enzymes [37]. Acrosome breakdown can be induced by several means, including osmotic shock [27], incubation with calcium ionophore A23187 [27], progesterone [38, 39], as well as neoglycoproteins [40]. It is reasonable to assume that in natural conditions the acrosome reaction must be induced by molecules encountered by the spermatozoa on its way toward the egg. In mammals, ZP3 has been demonstrated to be the physiological inducer of the acrosome reaction [4, 41, 42]. On the other hand, acrosome reaction induction has been attributed to egg jelly components in sea urchin [9] and starfish [15, 16]. We provide evidence here that in *B. arenarum*, the acrosome reaction is induced by VE components. The technique we used allowed us to quantitatively assess the effect of different incubation conditions on sperm acrosome breakdown. Although sperm morphology is simple and the sperm head can be observed easily under light microscopy [27], the acrosomal vesicle is not visible unless electron microscopy is used [43]. However, the technique developed by Martinez and Cabada [27] allows a differentiation of acrosome-reacted sperm from nonreacted ones using antibodies. Fluorescent, acrosome-intact sperm are easily differentiated from nonfluorescent, reacted sperm. The pattern is clear-cut and allowed us to observe a sufficient number of sperm to obtain statistically significant data. In vitro, this induction seems to be accomplished within 10 min of sperm-egg contact. A significant number of sperm undergo acrosome breakdown after being in contact with VE components for 1 min; a maximum was reached at 10 min of incubation time in our experimental conditions. We also demonstrated that the induction of the acrosome reaction by the VE is highly specific. The FE, a closely related structure, failed to significantly induce acrosome breakdown. This is partic-

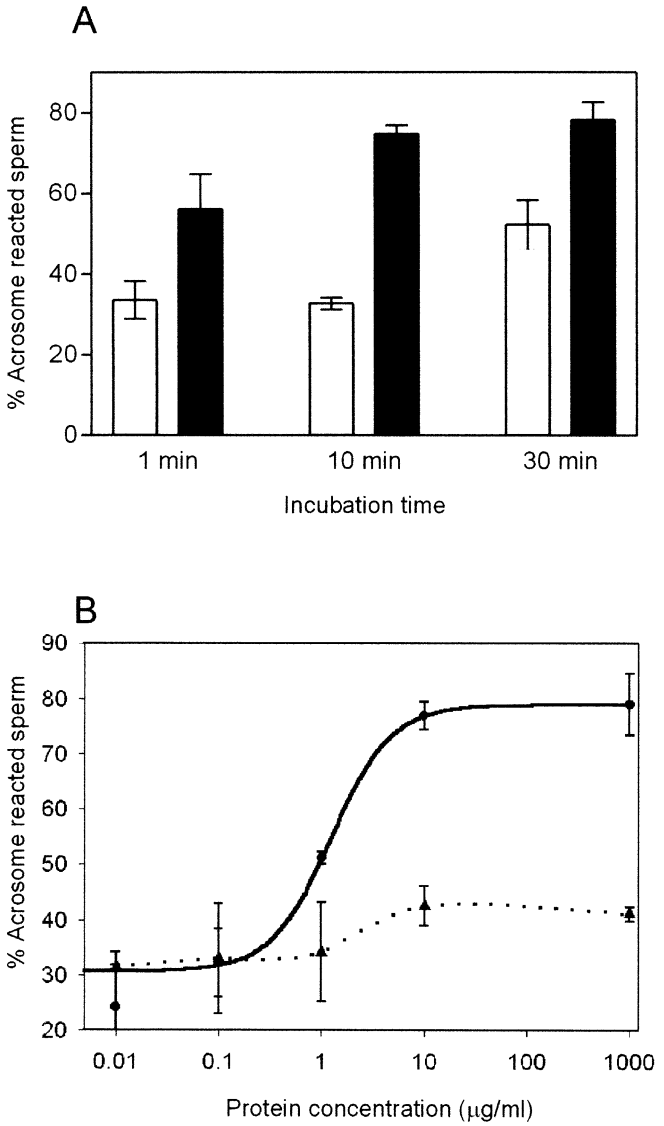

FIG. 4. The VE induced the sperm acrosome reaction. **A**) Effect of incubation time. Sperm suspensions were incubated in saline media with solubilized VE (dark bars) or without any macromolecules (white bars) for 1, 10, or 30 min. A concentration of 1 mg/ml of VE was used, and sperm concentration was 1.6×10^6 cells/ml. Acrosomal status was checked using an immunofluorescence technique. At least 100 spermatozoa were evaluated for each data point in each of 4 independent experiments. Error bars represent the SD of the four measurements. **B**) Effect of protein concentration and specificity. Sperm suspensions (1.6×10^6 cells/ml) were incubated with increasing concentrations (0.01 μg/ml to 1 mg/ml) of VE or FE. Incubations were carried out for 10 min. Circles and triangles illustrate the mean data points for VE and FE, respectively. Error bars indicate the SD of three measurements. Trend lines for VE and FE are represented by the solid line and dotted line, respectively.

ularly interesting because it may be functionally significant regarding the mechanisms involved in the prevention of polyspermy. Currently, we are working on identifying the VE component that is responsible for this biological activity and in the involvement of the glycosidic moieties in the process.

These results are coherent with the findings of Arranz and Cabada [44], who reported a protective effect of egg jelly molecules on acrosome integrity. It is possible to assume that once sperm are released through the male cloaca during amplexus into the surrounding medium, acrosomal breakdown would be avoided as they move through the jelly coat; this would allow sperm to reach the VE with an intact acrosome. Sperm would then interact with the VE; binding would occur to some of the VE components and the acrosome reaction would be triggered. We still do not know the relationship between VE binding and the induction of the acrosome reaction. The same ligand/receptor may or may not be involved. Binding may be a necessary condition for a second interaction (with different VE components) that would lead to acrosome breakdown. It is possible to speculate that capping phenomena could be involved as a previous step to the acrosome reaction. This, however, is speculative; the mechanisms remain to be elucidated.

This study in part confirms what is known in other species, while it provides some interesting new insights into gamete biology; namely, the induction of the acrosome reaction in amphibians and cross-species sperm-egg interaction. These data will need to be correlated with sequence similarity when cDNA and oligosaccharide information are available. We are currently working on this issue through cloning and sequencing strategies. Such studies will provide important information for future analysis of biological roles. Furthermore, a universal terminology for VE components in different species is needed; naming VE glycoproteins in a uniform and consistent way would be useful for reflecting their gene families and evolutionary relationships, and would facilitate the comparison of the macromolecules themselves and of their biological roles in different species.

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