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Rat Caltrin Protein Modulates the Acrosomal Exocytosis During Sperm Capacitation[1]

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ABSTRACT

Caltrin is a small and basic protein of the seminal vesicle secretion that inhibits sperm calcium uptake. The influence of rat caltrin on sperm physiological processes related to fertilizing competence was studied by examining its effect on 1) spontaneous acrosomal exocytosis, 2) protein tyrosine phosphorylation, and 3) sperm-egg interaction. Results show that the presence of caltrin during in vitro capacitation both reduced the rate of spontaneous acrosomal exocytosis without altering the pattern of protein tyrosine phosphorylation, and enhanced the sperm ability to bind to the zona pellucida (ZP). The significantly higher proportion of sperm with intact acrosome observed in the presence of caltrin was accompanied by a strong inhibition in the acrosomal hyaluronidase release. Enhancement of sperm-ZP binding was evident by the increase in the percentage of eggs with bound spermatozoa as well as in the number of bound sperm per egg. Similar results were obtained when the assays were performed using spermatozoa preincubated with caltrin and then washed to remove the unbound protein, indicating that the sperm-bound caltrin was the one involved in both acrosomal exocytosis inhibition and sperm-ZP binding enhancement. Caltrin bound to the sperm head was partially released during the acrosomal exocytosis induced by Ca-ionophore A23187. Indirect immunofluorescence and immunoelectron microscopy studies revealed that caltrin molecules distributed on the dorsal sperm surface disappeared after ionophore exposure, whereas those on the ventral region remained in this localization after the treatment. The present data suggest that rat caltrin molecules bound to the sperm head during ejaculation prevent the occurrence of the spontaneous acrosomal exocytosis along the female reproductive tract. Consequently, more competent spermatozoa with intact and functional acrosome would be available in the oviduct to participate in fertilization.

acrosome reaction, calcium, male reproductive tract, seminal vesicles, sperm capacitation

INTRODUCTION

Fresh mammalian spermatozoa are not able to fertilize oocytes at the time of ejaculation; they become fertilization competent in the female reproductive tract, where they leave behind the seminal plasma and undergo a series of biochemical and physiological changes collectively designated *capacitation* [1]. As a result of membrane modifications produced during capacitation, the spermatozoa can undergo two Ca^{2+}-dependent physiological processes required for fertilization: the acrosome reaction (AR) and hyperactivation.

It is well known that spermatozoa obtained from cauda epididymides and from complete semen differ in their ability to incorporate extracellular Ca^{2+}. Babcock et al. [2] found that the rapid uptake of calcium by bovine epididymal sperm did not occur in sperm separated from ejaculates. On ejaculation, epididymal sperm are suspended in the seminal plasma and are consequently exposed to proteins and other molecules mainly secreted by the seminal vesicles. Among them, caltrin (calcium transport inhibitor), a small and basic secretory protein, binds to the spermatozoa and inhibits the extracellular Ca^{2+} uptake [3, 4]. Caltrin is secreted by the seminal vesicles of bulls (also known as seminalplasmin; UniProtKB/Swiss-Prot entry P06833), guinea pigs (caltrin I and II, gene name *caltrinII*; UniProtKB/Swiss-Prot entry Q91VF8), mice (caltrin I or P12, gene name *Spink3*, MGI: 106202; caltrin II or seminal vesicle secretory protein 7, gene name *Svs7*, MGI: 1930790), and rats (gene name *Spink3*, RGD: 708468). Bovine caltrin or seminalplasmin [5, 6] was detected on the anterior portion of the head and on the principal section of the tail of bovine ejaculated sperm; the same distribution was observed incubating epididymal spermatozoa with purified caltrin [7]. The specific distribution on the surface of ejaculated spermatozoa suggested the existence of caltrin receptors in the plasma membrane distributed over the regions where calcium influx may take place. It was also shown that bovine seminal plasma constituents modulate caltrin activity; the inhibitor effect, which is promoted by interaction with phosphatidylserine, changes to enhancer of sperm Ca^{2+} uptake when caltrin is separated from the anionic phospholipid [8]. Considering these properties, it was postulated that caltrin bound to the spermatozoa prevents Ca^{2+} influx into the acrosomal region and the flagellum, and thus avoids premature acrosomal exocytosis and hyperactivation during sperm ascent through the female reproductive tract [9]. Consequently, the hydrolytic acrosomal enzymes are retained in the organelle until needed, and the spermatozoa can keep moving forward to reach the oviduct, where they are trapped in the distal portion of the isthmus and reside under protective conditions until ovulation.

Two different molecular forms of caltrin have been purified and sequenced from seminal vesicle secretion of guinea pigs [10] and mice [11, 12]. The smaller molecules designated

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caltrin I (also called P12 in mouse [12], which is a Kazal-type serine protease inhibitor), bind to the acrosomal cap of epididymal guinea pig and mouse spermatozoa, respectively, and inhibit extracellular Ca^{2+} uptake, apparently by blockage of calcium channels localized in the head plasma membrane [12, 13]. Guinea pig caltrin I also inhibits Ca^{2+}-induced hyaluronidase release, whereas guinea pig caltrin II [14], which blocks sperm Ca^{2+} uptake as efficiently as caltrin I [10], binds to the principal segment of the tail and retards the onset of sperm hyperactivated motility [13].

We purified and sequenced a small and basic protein from rat seminal vesicle secretion that inhibits extracellular $^{45}Ca^{2+}$ uptake in rat epididymal spermatozoa [11]. It presents 100% sequence identity with pancreatic secretory trypsin inhibitor type II, shares sequence identity with guinea pig and mouse caltrin I proteins (approximately 60%), and specifically binds to the acrosomal region of rat epididymal sperm [15]. Therefore, it was designated rat caltrin, the name that will be used in this paper. Rat caltrin and caltrin I molecules of guinea pig and mouse inhibit the activity of trypsin and acrosomal serine proteases [15]. These three proteins, which are synthesized by the secretory epithelium of the seminal vesicles under strict androgenic control [12, 16, 17], appear to be engaged in the fertilization process regulating not only the sperm extracellular Ca^{2+} uptake and, consequently, Ca^{2+}-dependent processes, but also the activity of sperm enzymes that might have a role in fertilization [18–20].

In order to investigate the role of rat caltrin in sperm function, in the present study we have examined its effect on different physiological events relevant for fertilization, such as spontaneous acrosomal exocytosis, protein tyrosine phosphorylation [21] during capacitation, and sperm ability to interact with zona pellucida (ZP).

MATERIALS AND METHODS

Animals

Immature (26–28 days old) female and adult (90–120 days old) male Wistar or Sprague-Dawley male rats were used. The animals were maintained at 23°C with a 12L:12D cycle. Food and water were available ad libitum, and the animals were killed with carbon dioxide gas. All experimental protocols were approved by the Universidad Nacional de Córdoba and the Instituto de Biología y Medicina Experimental and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Caltrin Purification

Rat caltrin protein was purified from seminal vesicle secretion using a procedure that involves ammonium sulfate fractionation, exclusion chromatography on Sephadex G-50, and cation exchange chromatography on carboxymethylcellulose, as we previously reported [11]. The purity of the protein was assessed by SDS-PAGE according to the method of Schagger and von Jagow [22] or by high-performance liquid chromatography using a Beckman (Fullerton, CA) gradient system with a dual pump, a 165 dual channel variable wavelength detector, and an IBM PC-based data system/controller (Software Beckman System Gold). A Beckman Ultrapore C8 (4.6 × 250 mm) column was used. Eluant A was trifluoracetic acid (TFA), and eluant B was 0.01% TFA in acetonitrile. The column was equilibrated with a mixture of 80% A:20% B for 1 h at a flow rate of 1 ml/min. After sample injection, a gradient from 20% B to 100% B for 1 h was applied. Proteins were detected by recording the absorbance at 280 nm.

Antibody Production and Purification

Monospecific polyclonal antibodies were prepared by injecting purified rat caltrin protein into adult male rabbits as described previously [13], and they were purified by affinity chromatography in a protein A-Sepharose column following the procedure described by Ey et al. [23], as we previously reported [15]. Purified antibodies were stored at −20°C until used.

Culture Medium

A Krebs-Ringer solution modified by Toyoda and Chang [24] that demonstrated to support in vitro fertilization was used as standard medium. It contained 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 21.58 mM Na lactate, 0.5 mM Na pyruvate, 5.56 mM glucose, 4.0 mg/ml BSA, 50 μg/ml streptomycin sulphate, and 75 μg/ml potassium penicillin G. Phenol red (2 mg/l) was added to the medium as a pH indicator.

Preparation of Spermatozoa

Rat sperm were collected from epididymis of adult males. A small puncture was made in the distal portion of the cauda, and a drop of the dense mass of sperm was placed in a conical tube, covered with incubation medium, and allowed to swim up at 37°C. After 10 min, aliquots of the upper sperm layer were transferred to tissue culture wells (16 mm; Costar, Cambridge, MA) containing medium with or without caltrin (0.1 mg/ml) to give a final concentration of 1×10^6 cells/ml. Sperm suspensions were then incubated under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air for different periods of time. Sperm viability was assessed with 0.5% (v/v) eosin (yellowish; Sigma, St. Louis, MO) in saline solution, and the incorporation of the dye was evaluated by light microscopy. The percentage of viability was calculated as the number of sperm that did not incorporate the dye over the total number of sperm cells. For evaluation of motility, 10 μl sperm suspension was placed on prewarmed slides, and the percentage of motile sperm was determined under the light microscope. Only sperm suspensions presenting vigorous motility were used.

Assessment of Acrosomal Status

The status of the acrosome was assessed as described by Larson and Miller [25]. Rat epididymal sperm exposed to different treatments were fixed with 4% p-formaldehyde solution at pH 7.4 for 10 min and then washed with 10 mM ammonium acetate at pH 9.0. Sperm cells were resuspended in a small volume of ammonium acetate, smeared on glass microscope slides, and air dried. Cells were stained for 2 min at room temperature with 0.22% Coomassie Brilliant Blue (CBB) solution prepared in 50% methanol and 10% acetic acid. Stained cells were washed with distilled water, air dried, and covered with a cover slip over a drop of glycerol, and observed under a bright field microscope at 1000× magnification. Spermatozoa with intact acrosome exhibited blue stain over both dorsal (convex) and ventral (concave) surfaces of the sperm head; staining only over the ventral surface was present on the head of acrosome-reacted spermatozoa [26].

Hyaluronidase Release

Release of acrosomal hyaluronidase during the incubation of epididymal spermatozoa in the presence of Ca^{2+} was determined by measuring the enzymatic activity in the supernatant as reported previously [13]. Washed epididymal spermatozoa (2×10^7cells/ml) were incubated at 37°C in the absence or presence of 0.1 mg/ml rat caltrin, concentration that promotes maximal inhibition on sperm Ca^{2+} uptake [11]. At intervals, aliquots were centrifuged to separate the cells, and hyaluronidase activity was assessed in the supernatant following the procedure described by Triana et al. [27], using a colorimetric estimation of liberated *N*-acetylglucosamine end groups [28]. Control experiments without Ca^{2+} were done in the presence and in the absence of rat caltrin, and these activities were subtracted from the experimental values to correct for the spontaneous hyaluronidase release.

Determination of Protein Tyrosine Phosphorylation

Sperm samples were treated as previously reported [29]. Sperm were washed twice with PBS and resuspended in Laemmli sample buffer [30]. After 5 min of incubation, samples were boiled for 5 min and centrifuged at $5900 \times g$ for 5 min. Supernatants were recovered, boiled in the presence of 70 mM 2-β-mercaptoethanol for 5 min, and then centrifuged again at $4000 \times g$ for 3 min. Supernatants were stored at −20°C until use. Solubilized proteins corresponding to approximately 0.75×10^6 spermatozoa/lane were separated on 10% (w/v) polyacrylamide gels with 0.1% (w/v) SDS, and then transferred onto nitrocellulose membranes following the method described by Towbing et al. [31]. Nonspecific protein-binding sites on the membrane were blocked with 2% (w/v) dry skimmed milk in PBS (blocking solution). The membrane was then probed with the monoclonal anti-phosphotyrosine antibody (1:10 000; clone 4G10; Upstate, Lake Placid, NY) in blocking solution for 1 h. After washing with PBS and 0.1% (w/v) Tween 20, the membrane was incubated with anti-

mouse peroxidase-conjugated IgG (1:4000; Vector Laboratories Inc., Burlingame, CA) in blocking solution for 1 h. The membrane was extensively washed, and reactive bands were detected by enhanced chemiluminescence (ECL kit; Amersham Life Science Inc., Oackville, ON, Canada) according to the manufacturer's instructions. Negative controls involved preabsorption of the anti-phosphotyrosine antibody with 40 mM *O*-phosphotyrosine (Sigma) for 1 h. All incubations were performed at room temperature.

Egg Preparation

Immature female rats were superovulated by an injection of 10 IU (i.p.) of equine chronic gonadotropin (eCG; Sigma), followed by an injection of 25 IU (s.c.) of human chorionic gonadotropin (hCG; Sigma) 54 h later. Ovulated oocytes were obtained from the oviducts 13–14 h after hCG administration. Cumulus cells were removed by incubating the oocyte-cumulus complex for 3 min in a medium containing 0.1% hyaluronidase (type IV; Sigma). Zona-intact oocytes were thoroughly washed in culture medium and distributed among treatment groups.

Sperm-Egg Binding Assay

Epididymal sperm (0.5×10^6 cells/ml to 2×10^6 cells/ml) were incubated with ZP-intact eggs in drops of 100 μl fertilization medium in tissue culture dishes under mineral oil at 37°C in an atmosphere of 5% CO_2 in air. The incubations were carried out in the absence or presence of 0.1 mg/ml rat caltrin. At various intervals of time, eggs were removed and transferred serially through several drops of fresh fertilization medium to remove sperm loosely associated with the egg surface. After washing, oocytes were mounted on slides. Coverslips with a mixture of vaseline/paraffin on the corners were applied, and the number of sperm bound per egg was scored under phase-contrast microscope at a 400× magnification.

Indirect Immunofluorescence

Immunofluorescent staining of fresh and acrosome-reacted epididymal spermatozoa was carried out using monospecific polyclonal antibodies against rat caltrin as primary antibody and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Sigma) as secondary antibody as previously reported [15]. Briefly, fresh epididymal spermatozoa were fixed in 0.5% p-formaldehyde in PBS for 30 min at room temperature. On the other hand, epididymal sperm incubated in the presence of rat caltrin (0.1 mg/ml) for 5 h under capacitating conditions and then treated with Ca^{2+} ionophore A23187 to induce AR were also fixed as described above. Fresh sperm fixed immediately after collection were washed with PBS several times and then incubated with rat caltrin (0.1 mg/ml) for 1 h to allow its binding to the cells. After washing, both fixed fresh sperm treated with rat caltrin and fixed reacted sperm cells were spread on acetone-cleaned glass slides to dry and then fixed in absolute ethanol for 20 min. After extensive washing with PBS, the cells were treated with 3% normal goat serum in PBS for 1 h and then exposed to a 1:1000 dilution in 1% BSA-PBS of rabbit anti-rat caltrin antiserum for 1 h. After washing with PBS the slides were treated for 30 min with goat anti-rabbit IgG labeled with FITC, washed with PBS for 1 h, and then covered with a coverslip over a drop of FluorSave Reagent (Calbiochem, La Jolla, CA). The cells were examined for localization of rat caltrin in a fluorescence microscope (MicroStar IV; Leica, Buffalo, NY). Photographs were taken using AGFA PAN 400 film.

Ultrastructural Immunocytochemistry

Fresh and acrosome-reacted epididymal spermatozoa were treated as described for indirect immunofluorescence (IIF). Additionally, the spermatozoa were incubated with 1% goat normal serum in PBS and then treated with rabbit anti-rat caltrin antiserum diluted 1:1000 in 1% BSA-PBS. The cells were washed and treated again with goat normal serum and then incubated with protein A-colloidal gold complex diluted 1:10 in PBS for 45 min. The sperm cells were washed and fixed with 1% glutaraldehyde in 0.01% cacodylate buffer for 1 h and later with 1% osmium tetroxide in the same buffer for 1 h. The cells were embedded in Araldite for 24 h at 60°C, and thin sections cut in a Porter-Blum MT-2 ultramicrotome (Sorvall, Newtown, CT) were mounted on nickel grids, contrasted with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 101 (Siemens) electron microscope.

Protein A-Gold Complex Preparation

Colloidal gold particles around 16 nm average diameter were prepared according to Frens [32] reducing a 0.01% tetrachloriauric acid solution ($HAuCl_4$; Merck) with 1% sodium citrate. About 4 μg protein A (Sigma) was used to stabilize 1 ml colloidal gold adjusted to pH 7.0 with 0.2 M potassium carbonate [33]. The unbound protein A was discarded by centrifugation at $100\,000 \times g$ for 60 min, and the pellet was resuspended in PBS containing 0.01% polyethyleneglycol (molecular weight 20 000 Da; Sigma).

Statistical Analysis

Data in Figures 1 and 2 were analyzed by the Student paired *t*-test, whereas those in Figure 3 were analyzed by chi-square test. The average number and standard deviation of bound sperm per egg in Table 1 and in text were calculated by Student *t*-test with base 2 logarithmic corrections, and differences at $P < 0.05$ were considered to be statistically significant. In all cases, the number of independent experiments in duplicate or triplicate is indicated for each experiment.

RESULTS

Effect of Rat Caltrin on the Acrosomal Integrity of Rat Epididymal Spermatozoa

To investigate the biological role of rat caltrin in sperm function, we analyzed its effect on the acrosomal integrity by evaluating the spontaneous acrosomal exocytosis of epididymal spermatozoa. The incubations were carried out under capacitating conditions in the absence or presence of caltrin at a concentration of 0.1 mg/ml, which demonstrated to support maximal inhibition in rat sperm calcium uptake [11]. At 30, 60, and 90 min of incubation, the sperm acrosomal integrity was examined by monitoring both the activity of hyaluronidase and the occurrence of the spontaneous AR. As shown in Figure 1A, hyaluronidase activity of sperm incubated in the absence of caltrin increased during the first 30 min, remained at almost the same level up to 60 min, and then abruptly increased, reaching the highest values at 90 min. When sperm cells were incubated with caltrin, the enzyme activity increased in a time-dependent manner, but the mean values were statistically reduced to approximately 50% of those registered in the absence of the protein at 30 and 60 min, and to no more than 28% of those recorded at 90 min (Fig. 1A). The percentage of sperm with reacted acrosomes, evaluated in parallel, was significantly lower in spermatozoa incubated in the presence of caltrin and, coincidently, higher differences with the controls were observed at 90 min (Fig. 1B). Exposure to caltrin did not cause detrimental effects on spermatozoa, as both viability and motility of sperm incubated in the presence of caltrin were not significantly different from the controls (65% ± 1% vs. 69% ± 1% and 67% ± 1% vs. 68% ± 2% in the absence and in the presence of caltrin, respectively).

Effect of Rat Caltrin on Protein Tyrosine Phosphorylation

The lower rate of spontaneous AR observed in spermatozoa incubated with caltrin could be a result of a decapacitating action of rat caltrin by its inhibitory effect on sperm calcium uptake. Considering that protein tyrosine phosphorylation is a key event that occurs during sperm capacitation [21] and that rat epididymal sperm incubated under capacitating conditions undergo this process in a time-dependent manner [29], we examined the influence of caltrin on this signal transduction pathway. Sperm were incubated in the absence or presence of rat caltrin (0.1 mg/ml) for 5 h to allow them to get the full state of capacitation [29], and sperm protein extracts were analyzed for tyrosine phosphorylation by Western blot using an anti-phosphotyrosine antibody. In parallel, the sperm acrosomal status was evaluated by staining with CBB. Results shown in Figure 2A revealed a pattern of tyrosine phosphorylation of sperm proteins in a range of 55–148 kDa, which was not altered by the presence of caltrin in the incubation medium. Negative reactions were observed for noncapacitated sperm or

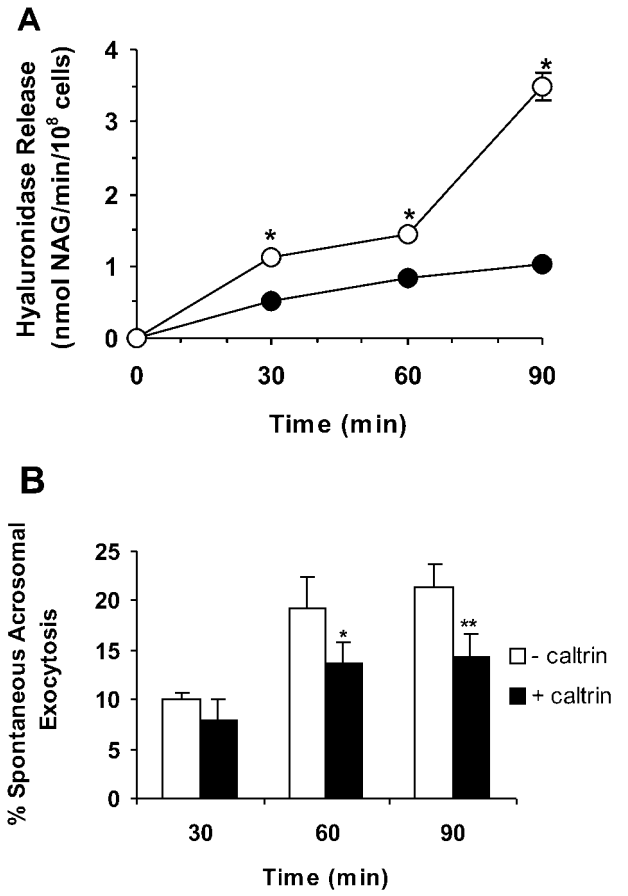

FIG. 1. **A**) Influence of rat caltrin on Ca^{2+}-induced hyaluronidase release during sperm capacitation. Rat epididymal spermatozoa were incubated under capacitation conditions in the absence (open circles) or presence (filled circles) of rat caltrin (0.1 mg/ml). Aliquots were removed and centrifuged at 30, 60, and 90 min, and the enzyme activity was assessed in the supernatant. Data are mean ± SEM of triplicate determinations from three independent experiments. *Significantly different from corresponding control ($P < 0.001$). **B**) Spontaneous acrosomal exocytosis evaluated by staining with CBB. Two hundred spermatozoa per treatment were scored in duplicate, and the percentage of spermatozoa with reacted acrosome was calculated. Values are expressed as mean ± SEM of three independent experiments. Bars labeled with asterisks are significantly different from controls. *$P < 0.02$; **$P < 0.009$.

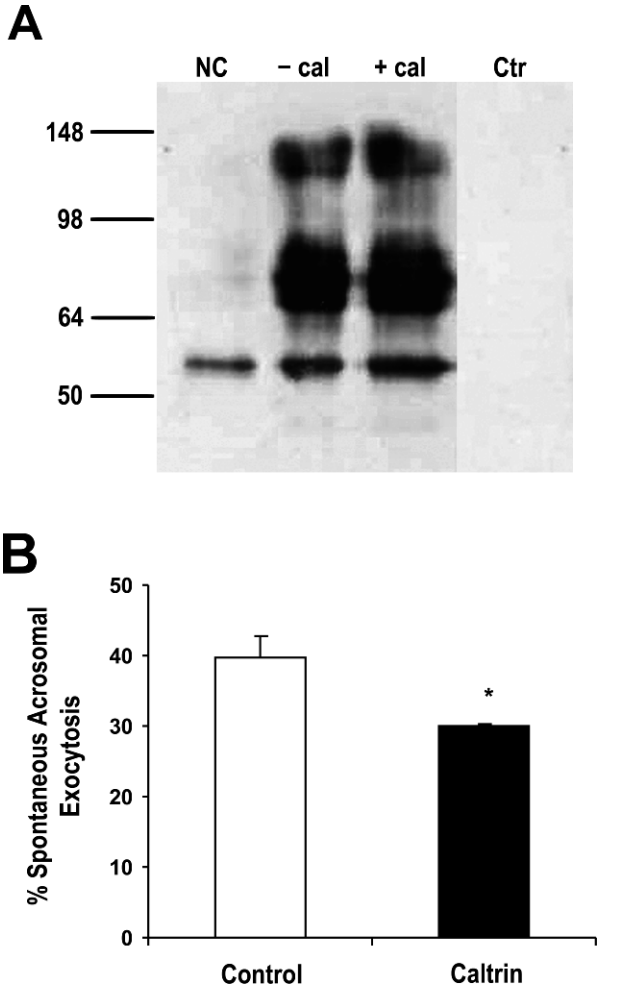

FIG. 2. **A**) Influence of rat caltrin on protein tyrosine phosphorylation during sperm capacitation. Spermatozoa were incubated under capacitation conditions for 5 h in the absence (– cal) or presence of 0.1 mg/ml of rat caltrin (+ cal). Aliquots were then removed, and sperm proteins were analyzed by Western blot using a monoclonal anti-phosphotyrosine antibody. Molecular mass standards (kDa) are shown on the left. Ctr, Protein extract from 5-h incubated sperm revealed without primary antibody; NC, noncapacitated spermatozoa. **B**) Spontaneous acrosomal exocytosis evaluated by staining with CBB. Two hundred spermatozoa per treatment were scored in duplicate, and the percentage of spermatozoa with reacted acrosome was calculated. Values are expressed as mean ± SEM of three independent experiments. *Significantly different from control ($P < 0.001$).

for capacitated sperm not exposed to the primary antibody. In contrast, the presence of caltrin during capacitation significantly reduced the rate of spontaneous acrosomal exocytosis in sperm compared with controls (Fig. 2B). No effects on sperm viability (62% ± 2% vs. 62% ± 3%, n = 4) or motility (61% ± 3% vs. 61% ± 1%, n = 4) were detected at the end of the incubations either in the absence or presence of caltrin.

Effect of Caltrin on Sperm-Egg Interaction

We previously demonstrated that guinea pig caltrin I and rat caltrin inhibit the activity of trypsin and acrosomal serine proteases [15]. Since the inhibition of in vitro fertilization by serine protease inhibitors was associated with blockage of sperm binding to and/or sperm penetration of the ZP [18–20, 34], we investigated the effect of rat caltrin on sperm-egg interaction.

Rat epididymal spermatozoa were incubated with zona-intact oocytes in the absence or presence of 0.1 mg/ml rat caltrin, and both the percentages of oocytes with ZP-bound sperm and the numbers of bound sperm per egg using a protocol for in vitro fertilization [24] were analyzed. In the absence of caltrin, the percentage of oocytes with sperm bound to the ZP increased during the first 6 h, reaching a maximum value of 45%, which remained almost unchanged (42%) for the next 2 h (Fig. 3A). When the gametes were incubated in the presence of caltrin, the percentage of oocytes with sperm bound to the ZP was approximately 90% at 4 and 6 h, and more than 75% at 8 h (Fig. 3A). In addition to this, the average number of sperm bound per egg was significantly higher in the presence than in the absence of caltrin at all incubation times (Table 1).

Although these results showed that the presence of caltrin in the incubation medium promoted a significant enhancement of the sperm-ZP binding, they could not discriminate whether this effect was supported by the caltrin bound to sperm or by the unbound molecules designated as "caltrin excess" in this paper.

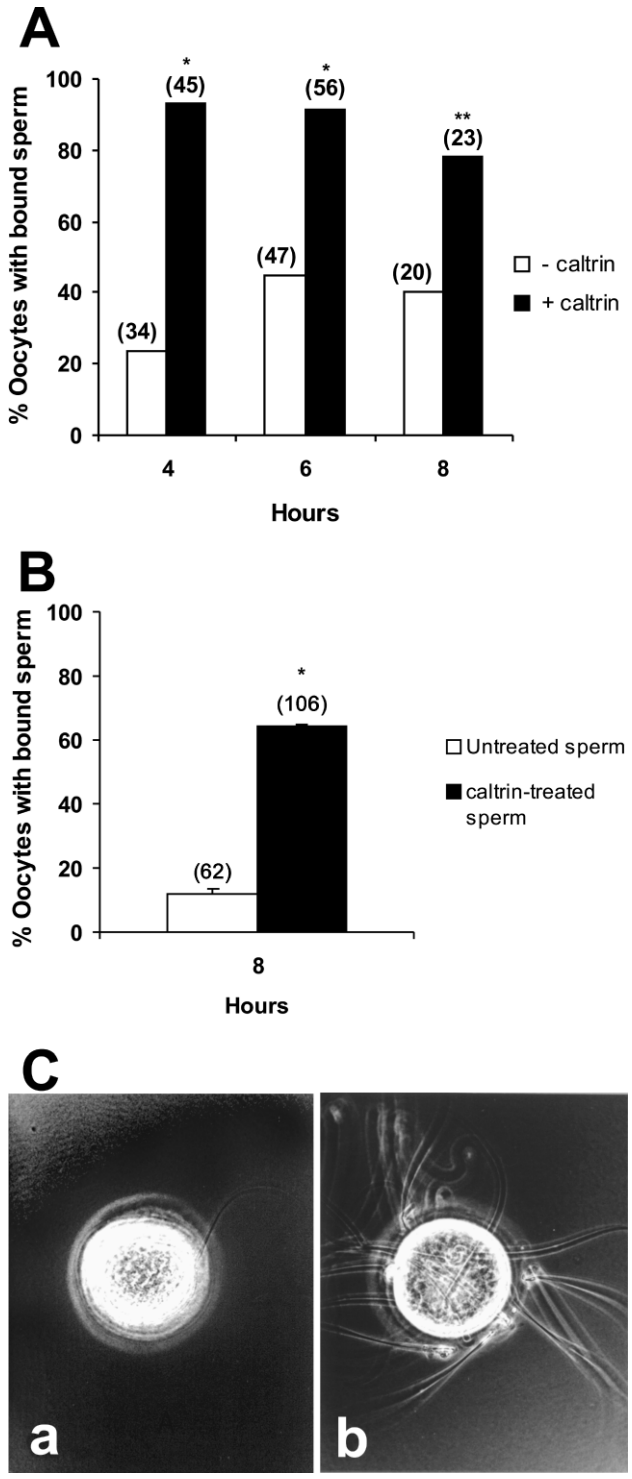

FIG. 3. **A**) Influence of rat caltrin in the incubation medium on sperm-egg interaction. Epididymal spermatozoa were coincubated with ZP-intact oocytes in the absence or presence of rat caltrin (0.1 mg/ml) for 4, 6, and 8 h. Then, the number of eggs with tightly bound sperm to the ZP was counted at each incubation time. Results show the percentage of oocytes with spermatozoa bound to the ZP calculated from six independent experiments. Total number of oocytes examined is indicated in parenthesis. Bars labeled with asterisks are significantly different from controls. *$P < 0.005$; **$P < 0.025$. **B**) Influence of sperm-bound caltrin on the sperm-egg interaction. Epididymal sperm incubated with 0.1 mg/ml of rat caltrin for 1 h (caltrin-treated) were washed to remove unbound protein, and then coincubated with ZP-intact oocytes in caltrin-free medium for 8 h. Then, the number of eggs with sperm tightly bound to the ZP was counted. Results show the percentage of oocytes with bound spermatozoa calculated from three independent experiments. Total number of oocytes examined is indicated in parenthesis. *Significantly different from control ($P < 0.005$). **C**) Influence of sperm-bound rat caltrin on sperm-ZP interaction. ZP-intact oocytes were coincubated with untreated (**a**) or caltrin-treated (**b**) epididymal sperm for 8 h. Original magnification ×400.

TABLE 1. Number of bound spermatozoa per egg (mean ± SD) in experiments performed under conditions for in vitro fertilization in the absence (–) or in the presence (+) of rat caltrin.

	Incubation time (h)		
Treatment	4	6	8
– Caltrin	1.0 ± 0.0	1.5 ± 0.5	1.3 ± 0.5
+ Caltrin	13.7 ± 6.0	21.6 ± 10.0	16.0 ± 5.0

To evaluate these possibilities, epididymal spermatozoa were preincubated with caltrin (0.1 mg/ml) for 1 h to allow its binding to the cells, unbound caltrin was removed by sperm centrifugation, and spermatozoa were coincubated with zona-intact oocytes for 8 h. Spermatozoa preincubated in the absence of caltrin were used as a control. Results showed both significantly higher percentages of oocytes with bound sperm (Fig. 3, B and C) and higher average numbers of bound sperm per egg (10.6 ± 1 vs. 1.5 ± 0.5) for caltrin-treated spermatozoa. Coincidentally, the rate of spontaneous AR after in vitro capacitation was significantly lower in these samples than in untreated sperm (37.3% ± 0.7% vs. 48.3% ± 0.3%, n = 3; $P < 0.001$).

Localization of Caltrin in Acrosome-Reacted Sperm

The observations described above indicate that caltrin might participate in gamete interaction by protecting the acrosomal integrity and facilitating the sperm binding to ZP. As another approach to explore the possible involvement of this protein in fertilization, we examined its distribution in the sperm head after induction of the acrosomal exocytosis.

Indirect immunofluorescence of cauda epididymal sperm fixed immediately after collection and then incubated with rat caltrin resulted in the labeling of both convex and concave surfaces (here designated as dorsal and ventral regions in accordance with Pikó's nomenclature [35]) of rat sperm head (Fig. 4A) as we previously reported [15]. When epididymal spermatozoa were incubated for 5 h in a capacitating medium containing caltrin and then were exposed to Ca^{2+} ionophore A23187, the immunofluorescence was localized exclusively on the ventral region of the head (Fig. 4C).

Spermatozoa from the same experiments were subjected to ultrastructural immunocytochemistry using protein A colloidal gold complex and then examined by electron microscopy. Different head sections of sperm fixed immediately after collection and then treated with caltrin showed intact plasma membrane decorated with gold particles (Fig. 5, A and B). A negative immunoreaction was observed when the primary antibody was omitted (Fig. 5C). Spermatozoa incubated with caltrin for 5 h showed the same immunogold distribution observed at the beginning of the incubation. However, few of the cells exhibited loose membrane apparently forming membrane vesicles around the head (Fig. 5, D and E). Sperm exposed to Ca^{2+} ionophore A23187 exhibited fewer immunogold particles bound to the sperm head, and most of them appeared to be associated with loose membrane distributed in a limited region (Fig. 5, F and G).

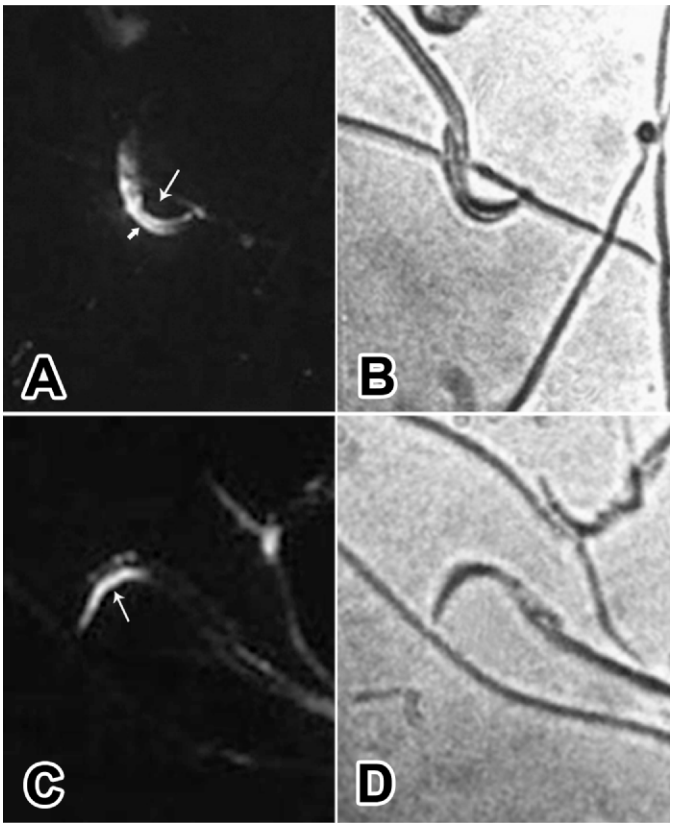

FIG. 4. Immunolocalization of sperm-bound caltrin. The fluorescence shows the specific binding of rat caltrin to epididymal spermatozoa distributed either on the dorsal (arrowhead) and ventral (arrow) surface of the head of sperm cells treated with the protein immediately after collection (**A**). After 5 h of incubation for capacitation and exposition to A23187 to induce acrosomal exocytosis, the immunofluorescence was only visualized on the ventral surface (**C**). **B**, **D**) Corresponding phase-contrast photomicrographs. Original magnification ×1000.

DISCUSSION

Structural and functional studies in bull and guinea pig suggested an important role for caltrin proteins in sperm physiology regulating Ca^{2+}-dependent processes associated with the fertilizing competence [7, 8, 13, 15, 36, 37]. Regarding the specific binding of caltrin to the head and tail of sperm cells at ejaculation, the unique caltrin molecule of bovine semen appears to be able to control Ca^{2+} influx required for both the acrosomal exocytosis and the hyperactivated motility [7]. In guinea pig, two different caltrin molecules purified from the seminal vesicle secretion are equally effective as inhibitors of sperm Ca^{2+} uptake, although they seem to participate in different cell processes [10]. The smaller molecule, designated caltrin I, binds to the head of epididymal sperm and prevents the release of acrosomal enzymes during in vitro capacitation; caltrin II, the bigger one, binds to the principal portion of the tail and delays sperm hyperactivation [13].

In this study, we have analyzed some functional properties of the caltrin protein purified from rat seminal vesicle secretion, which shares sequence identity with guinea pig and mouse caltrin I molecules [15]. To gain a better understanding of its biological role in sperm function, we have examined its effect on the spontaneous acrosomal exocytosis and protein tyrosine phosphorylation of epididymal sperm subjected to in vitro capacitation. In addition, we evaluated the effect of rat caltrin on the sperm ability to recognize and interact with the ZP that surrounds the eggs.

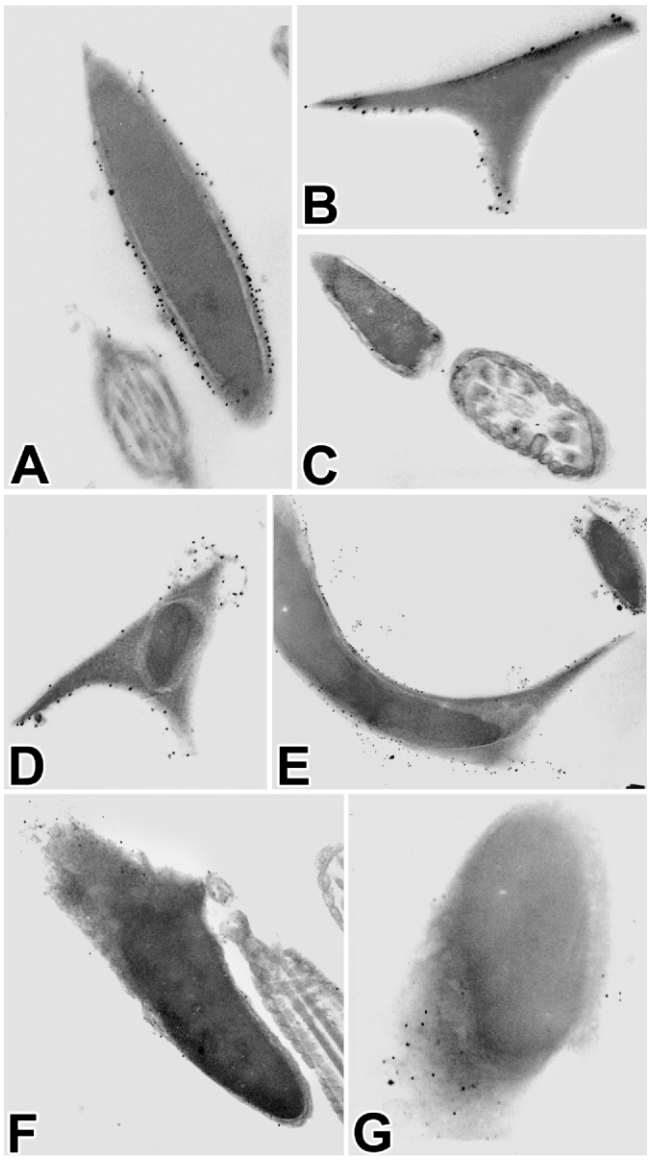

FIG. 5. Immunoelectron micrographs of rat epididymal spermatozoa incubated with rat caltrin under capacitation conditions. Transverse sections through the midregion (**A**; original magnification ×47 000) and the anterior portion (**B**; original magnification ×65 000) of rat sperm head show the plasma membrane decorated with gold particles, indicating the presence of bound rat caltrin protein. No gold labeling is shown in the transverse section through the midregion of a spermatozoon treated without anti-caltrin antibodies (**C**; original magnification ×33 000). After 5 h of incubation in capacitation medium, the sperm plasma membrane decorated with gold particles looks less preserved, and some vesicles become perceptible in a transverse section of the anterior portion (**D**; original magnification ×50 000) as well as in nearly media section (**E**; original magnification ×29 000) of the sperm head. A few immunogold particles associated with loose membrane and distributed in a limited region of the head surface are observed in the transverse sections through the midregion of the sperm head after acrosomal exocytosis induced by Ca^{2+} ionophore A23187 (**F**; original magnification ×35 000 and **G**; original magnification ×55 000).

To explore the influence of rat caltrin on the acrosomal integrity during sperm capacitation, we assessed the hyaluronan-hydrolyzing activity corresponding to the hyaluronidase enzyme released from sperm cells during the AR [38]. This hyaluronidase activity has recently been demonstrated to correspond to Hyal5, a 55-kDa protein that is distinguished from PH-20 in several aspects and appears to be present

exclusively in some rodents and guinea pig [38]. In agreement with previous studies [13, 27], the fast increment in the hyaluronidase activity detected in the absence of rat caltrin indicates that spontaneous acrosomal exocytosis in epididymal sperm starts sooner in the presence of calcium and increases greatly through time. Differently, when sperm cells were incubated with caltrin, the hyaluronidase activity was dramatically reduced at all of the times assessed, being more manifest at 90 min. Since caltrin proteins did not inhibit the enzyme [13, 39], the lower hyaluronidase activity recorded must be due to the protective effect of rat caltrin on the sperm acrosome, preventing the spontaneous exocytosis by inhibition of extracellular Ca^{2+} uptake. This hypothesis is strongly supported by the significantly lower percentages of spontaneous AR detected in spermatozoa incubated with rat caltrin. These results show that the changes in the acrosome produced by the spontaneous acrosomal exocytosis are more evident by means of the release of sperm enzymes.

Since the higher percentages of acrosomal integrity revealed in sperm incubated with rat caltrin could be a consequence of a decapacitating effect of this protein by its inhibitory activity on sperm Ca^{2+} uptake, we analyzed the patterns of protein tyrosine phosphorylation in sperm that were incubated for 5 h either in the absence or in presence of rat caltrin. Five hours of incubation allow sperm to get a full state of capacitation, as was previously observed by examining the sperm's ability to fuse with the egg [29]. Under these conditions, rat epididymal sperm underwent phosphorylation of tyrosine residues on numerous proteins in a range of 55 to 148 kDa. This pattern of tyrosine-phosphorylated proteins was not modified by the presence of rat caltrin during incubation, indicating that this signaling pathway was not affected by the protein. Contrarily, the spontaneous acrosomal exocytosis, which increased during capacitation, was significantly diminished in the presence of rat caltrin. Considering that rat caltrin inhibits around 50% of the sperm Ca^{2+} uptake [11] and that the levels of protein tyrosine phosphorylation in rat epididymal sperm are remarkably enhanced in the absence of extracellular calcium as reported by Baker et al. [40], our results suggest that the sperm calcium influx, which is inhibited by rat caltrin, is involved in the acrosomal exocytosis but does not operate in the signaling pathway of capacitation. These data sustain the hypothetic role of caltrin as a modulator of the acrosomal exocytosis without disturbing the capacitation process during sperm journey along the female reproductive tract.

In preliminary experiments, we observed that mouse epididymal sperm incubated under capacitating conditions showed lower percentages of spontaneous acrosomal exocytosis when mouse caltrin I protein was present in the medium. Nevertheless, these spermatozoa responded effectively to progesterone and reached the same rate of induced AR as those incubated in the absence of caltrin [41]. Since spermatozoa cannot be induced by natural or pharmacological agents to undergo AR unless they are capacitated [42], these results indicate that mouse caltrin I, which shares structural and functional properties with rat caltrin, also protects the sperm acrosome without affecting capacitation.

In vitro experiments in which sperm and eggs were coincubated in the presence of caltrin revealed that the protein enhances the binding of epididymal sperm to the ZP, as judged by the higher proportion of oocytes with spermatozoa bound to the ZP and the increased number of bound sperm per egg. The same results were observed when isolated ZPs were substituted for whole oocytes (data not shown). It is important to note that under these experimental conditions, free caltrin molecules were also present in the medium during the incubations. In ampulla, the oviductal region where the sperm-egg encounter occurs and fertilization begins, there is no free caltrin in the fluids because the exceeding unbound caltrin molecules and other seminal plasma components are retained in the lower portion of the female reproductive tract. Therefore, gamete coincubation experiments were also carried out using spermatozoa washed after their exposure to caltrin. The observation that under these conditions, sperm still exhibited an increased sperm-ZP binding ability and a lower rate of spontaneous acrosomal exocytosis indicate that the caltrin molecules bound to sperm are the ones involved in both acrosomal exocytosis inhibition and sperm-ZP binding enhancement. It is possible that the enhanced sperm-ZP binding observed occurred as a consequence of the protective effect of caltrin on the acrosomal integrity and sperm fertilizing competence.

Since rat caltrin molecules bound to sperm cells seem to be involved in cellular events associated with sperm-egg recognition and interaction, we examined by IIF its localization on the sperm surface after in vitro capacitation and acrosomal exocytosis. As previously reported [15], rat caltrin was localized on the dorsal (convex) and ventral (concave) regions of the head of capacitated spermatozoa, and the same distribution was also observed for rat ejaculated spermatozoa recovered from the female reproductive tract [41]. After treating with Ca^{2+} ionophore A23187, the fluorescence was visualized only on the ventral surface, suggesting the partial loss of caltrin as a consequence of the acrosomal exocytosis. This alternative was explored following the changes in the sperm plasma membrane as well as in the distribution of immunogold particles by electron microscopy. Capacitated spermatozoa showed immunogold particles distributed regularly around the sperm head, whereas in sperm exposed to the ionophore, the gold particles were remarkably reduced and observed just in a limited portion of the sperm head, which can probably be the ventral (concave) region observed by IIF. These images suggest that part of caltrin would be released from the sperm surface associated with the membrane vesicles generated by the acrosomal exocytosis.

Clark et al. [37] reported that bovine ejaculated spermatozoa were more effective than epididymal sperm to fertilize eggs in vitro. In agreement with this, the fertilizing ability of epididymal sperm was remarkably enhanced by addition of bovine caltrin to the medium. Together with a protective effect of caltrin on the acrosomal integrity and the inhibition of sperm Ca^{2+} uptake, these authors found a modulating action of the protein on fertilization through regulation of the sperm signal transduction pathways activated by ZP [37]. These results also show that the protective effect of caltrin on the sperm acrosome does not interfere with the AR induced by physiological agents.

Data presented provide evidence of caltrin involvement in sperm cell processes preceding fertilization. It reduces the rate of spontaneous AR, probably by inhibition of extracellular Ca^{2+} uptake and, consequently, more capacitated spermatozoa with intact and functional acrosome are available to interact with and bind to oocytes, as shown in our experiments. Spontaneous AR may have no importance in vitro, as each oocyte is exposed to thousands of sperm cells to guarantee successful fertilization, but it becomes tremendously significant in vivo, where only a few spermatozoa can reach the oviductal epithelium before finding the oocytes. Since calcium concentration in genital fluids is high enough to trigger the spontaneous acrosomal exocytosis, regulatory mechanisms controlling sperm Ca^{2+} influx should operate to protect the acrosomal integrity of sperm cells traveling across the female reproductive tract. In the oviduct, only a small number of acrosome-intact sperm are adhered to the epithelium, in the

distal portion of the isthmus [43, 44], where they reside under protective conditions until ovulation. Then, spermatozoa become hyperactivated, and when sperm-egg encounter occurs, the activity of physiologic inducers associated with the cumulus cells (progesterone) and the zona pellucida (ZP3 glycoprotein) promotes Ca^{2+} influx to initiate the signal transduction pathways involved in sperm AR [45, 46].

Our results show that rat caltrin is able to maintain the acrosomal integrity required for the interaction between spermatozoa and the oviductal epithelium as well as with the ZP. These findings suggest a modulatory effect of rat caltrin on sperm function in the female reproductive tract, assuring the proper compliance of critical stages leading to successful fertilization.

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