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SNAREs in Mammalian Sperm: Possible Implications for Fertilization

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Soluble *N*-ethylmalameide-sensitive factor attachment protein receptor (SNARE) proteins are present in mammalian sperm and could be involved in critical membrane fusion events during fertilization, namely the acrosome reaction. Vesicle-associated membrane protein/synaptobrevin, a SNARE on the membrane of a vesicular carrier, and syntaxin 1, a SNARE on the target membrane, as well as the calcium sensor synaptotagmin I, are present in the acrosome of mammalian sperm (human, rhesus monkey, bull, hamster, mouse). Sperm SNAREs are sloughed off during the acrosome reaction, paralleling the release of sperm membrane vesicles and acrosomal contents, and SNARE antibodies inhibit both the acrosome reaction and fertilization, without inhibiting sperm–egg binding. In addition, sperm SNAREs may be responsible, together with other sperm components, for the asynchronous male DNA decondensation that occurs following intracytoplasmic sperm injection, an assisted reproduction technique that bypasses normal sperm–egg surface interactions. The results suggest the participation of sperm SNAREs during membrane fusion events at fertilization in mammals.

Key Words: **sperm; fertilization; membrane; exocytosis; acrosome.**

INTRODUCTION

The acrosome is a large secretory vesicle localized as a cap-like structure on the heads of spermatozoa from most species. Acrosomal contents mediate sperm–egg interactions by aiding the sperm's penetration through the mammalian egg's zona pellucida (reviewed by Fawcett, 1975; Yanagimachi, 1994; Wassarman, 1999). Release of these contents takes place during the acrosome reaction, an exocytotic event resulting in the fusion of the acrosomal membrane and the sperm plasma membrane that primes the sperm for zona penetration and sperm–egg binding/fusion (Fawcett, 1975; Yanagimachi, 1994; Snell and White, 1996; Breitbart and Spungin, 1997; Wassarman, 1999). Unlike membrane fusion in other exocytotic systems that typically results in the formation (albeit temporary in many cases) of a single fused membrane, the acrosome reaction in mammals seems to involve partial destruction of the two fusing partners. Fusion of the outer acrosomal membrane with the overlaying sperm plasma membrane occurs at multiple points along the tip of the sperm head, forming mixed vesicles and fragments that are shed (Barros *et al.*, 1967; Franklin *et al.*, 1970; Yanagimachi and Noda, 1970). Recently, it has also been proposed that the acrosome reaction involves a series of continuous, but discrete, exocytotic fusion events and not an all-or-nothing immediate release of all acrosomal contents (Kim and Gerton, 1999). This model implies that acrosomal enzymes would be

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released in a time-controlled fashion, as the sperm passes through the zona pellucida.

In mammals, secretion of acrosome contents is preceded by an increase in intracellular calcium, similar to synaptic vesicle exocytosis (Calakos and Scheller, 1996; Breitbart and Spungin, 1997; Florman *et al.*, 1998; Wassarman, 1999). Although many details have surfaced on the signaling mechanisms involved in the release of acrosomal contents, the regulation of the ultimate step, i.e., contact between the acrosomal and the plasma membranes and fusion itself, has not yet been fully elaborated.

Intracellular membrane fusion consists of a complex set of tightly regulated membrane merging events that have been dissected at the molecular level in recent years (reviewed by Ferro-Novick and Jahn, 1994; Rothman, 1994; Hanson *et al.*, 1997; Gotte and von Mollard, 1998). Cytoplasmic regulators that seem to be involved in several fusion events, such as the ATPase *N*-ethylmalameide-sensitive factor and the several SNAPs (soluble *N*-ethylmalameide-sensitive factor attachment proteins) it associates with, have emerged (Ferro-Novick and Jahn, 1994; Rothman, 1994). But, if these elements are active in many events, what endows specificity to each fusion reaction? The SNARE hypothesis postulates that a SNAP receptor (SNARE) on the membrane of a vesicular carrier (termed a v-SNARE) specifically interacts with a complementary SNARE on the target membrane (t-SNARE) and that this interaction results in the recognition, docking, and/or fusion of the two membranes (Rothman, 1994). The established paradigm for the action of SNAREs during membrane fusion is derived from the exocytosis of synaptic vesicles in nerve terminals. In this system, vesicle-associated membrane protein (VAMP)/synaptobrevin, a v-SNARE present on the synaptic vesicle membrane, interacts with two t-SNAREs on the plasma membrane (syntaxin and SNAP-25; Calakos and Scheller, 1996). Although the SNARE hypothesis has been tentatively extended to other intracellular membrane fusion systems, including most exocytotic events, this model is undergoing revisions, with the likelihood of additional complexities (Hanson *et al.*, 1997; Gotte and von Mollard, 1998; see Discussion).

Although little functional evidence is available, homologues of VAMP/synaptobrevin and syntaxin were found in sea urchin sperm (Schulz *et al.*, 1997). These proteins are released following the secretion of acrosomal contents and the formation of the acrosomal process (Schulz *et al.*, 1997), an actin-based structure that does not exist in most mammals. SNARE homologues may therefore mediate exocytosis during the acrosome reaction and have also been suggested to play a role in sea urchin egg cortical granule exocytosis (Avery *et al.*, 1997; Conner *et al.*, 1997), although other studies dispute these findings (Coorsen *et al.*, 1998).

This report extends previous findings to several mammalian species and discusses functional data that support the idea that sperm SNARE proteins modulate exocytosis during the mammalian acrosome reaction and may therefore have an important role in fertilization. In addition, we have found a homologue of the calcium sensor synaptotagmin on the sperm head, suggesting that this protein may be involved in modulating exocytosis in this system.

Of clinical relevance, we also describe the abnormal behavior of SNAREs following the assisted reproduction technique (ART) of intracytoplasmic sperm injection (ICSI; Palermo *et al.*, 1992; Hewitson *et al.*, 1998, 1999; Van Steirteghem *et al.*, 1993). This technique is widely used in male factor infertility therapy because direct sperm injection into a mature oocyte bypasses any intrinsic deficiencies that might prevent the male gamete from penetrating the egg. Technically, acrosome-intact sperm are less sticky than acrosome-reacted ones and ICSI therefore introduces into the oocyte acrosome-intact sperm, including components normally removed prior to or during sperm–egg fusion, such as the sperm plasma membrane and the acrosome. In addition, the perinuclear theca overlaying the nuclear envelope, usually lost at the egg cortex at fertilization, is retained after ICSI (Sutovsky *et al.*, 1996, 1997; Hewitson *et al.*, 1999). We have found that a "collar" that includes VAMP overlaying the sperm nucleus seems to delay the decondensation of sperm DNA following ICSI. This information may be relevant in light of recent concerns related to the widespread application of ICSI in infertile patients, the nonrandom positioning of the X chromosome in the sperm nuclear apex (Luetjens *et al.*, 1999), and the increase in sex chromosomal anomalies observed in ICSI embryos (In't Veld *et al.*, 1995).

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies against VAMP/synaptobrevin, syntaxin, and synaptotagmin were generated against conserved sequences in these proteins. For VAMP, conserved sequences between rat cellubrevin, rat synaptobrevins I and II, and *Drosophila* synaptobrevin were used. For syntaxin, conserved sequences between syntaxin 1A from rat and from *Drosophila* were used. For synaptotagmin, conserved sequences between rat synaptotagmins I and II and homologues from *Drosophila, Caenorhabditis elegans*, and squid were used. For details see Conner *et al.* (1997).

To better characterize sperm SNAREs additional probes were used. Syntaxin antibodies were obtained from Sigma (St. Louis, MO; clone HPC-1), antibodies specific for syntaxin 1A and syntaxin 1B were obtained from Synaptic Systems (Göttingen, Germany), and antibodies against syntaxin 4 and syntaxin 6 were from Transduction Laboratories (Lexington, KY). Anti-synaptobrevin was also obtained from Serotec (Raleigh, NC, clone MCA 1433), and anti-VAMP 1 and anti-VAMP 2 were from Synaptic Systems. Anti-synaptotagmin I was obtained from both Sigma and Transduction Laboratories (clone 41) and anti-acrosin was a kind gift from Dr. Claudio Barros, Pontifical Catholic University of Chile (Valdivia *et al.*, 1994).

Sperm

Frozen bull semen samples were obtained from the American Breeders Service (DeForest, WI). The thawed semen samples were centrifuged (10 min, 700*g*) through a two-layer Percoll gradient to remove dead spermatozoa, and the final sperm pellet was washed and resuspended in TALP medium (modified Tyrode–lactate medium with pyruvate and albumin: 114 mM NaCl, 3.2 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 10 mM sodium lactate, 6.5 IU penicillin, 25 μg/ml gentamicin, 6 mg/ml fatty acid-free bovine serum albumin, 0.2 mM pyruvate), buffered with 10 mM Hepes at pH 7.4 (Sutovsky *et al.,* 1998). Bovine sperm was incubated at 39°C for 1–2 h before use. Sperm from rhesus macaques was obtained by penile electroejaculation, washed in TALP–Hepes, and resuspended in TALP (Bavister *et al.,* 1983) at a final concentration of 20×10^6 sperm/ml.

For *in vitro* fertilization (IVF) purposes, rhesus sperm was cultured at 37°C in TALP containing caffeine and dibutyryl cAMP (1 mM each) to ensure capacitation prior to use (Wu *et al.,* 1996). Frozen human sperm was purchased from a sperm bank (Follas Laboratories, OH) and used in accordance with the Oregon Health Sciences University Human Subjects Institutional Review Board. Samples were thawed at room temperature, centrifuged through a two-layer Percoll gradient, washed, and resuspended in TALP. Mouse and hamster sperm were obtained by mincing the epididymides into TALP–Hepes. Sperm were cultured for 2–4 h at 37°C before use. Rhesus and bovine sperm mitochondria were labeled by a 10-min incubation with a 400 nM solution of the fluorescent dye MitoTracker Green FM (Molecular Probes, Eugene, OR; Sutovsky *et al.,* 1997, 1998).

Triggering the Acrosome Reaction

Although the acrosome reaction can take place spontaneously following prolonged sperm incubation, hamster, rhesus, and bull sperm were incubated for 20 min (at 37°C for hamster and rhesus, at 39°C for bull) with the calcium ionophore ionomycin (5–10 mM), to increase the percentage of acrosome-reacted spermatozoa (Cummins *et al.,* 1991). Bull and rhesus sperm incubated with ionomycin were then further incubated with the FITC-conjugated peanut agglutinin PNA [*Arachis hypogaea* (peanut) lectin; EY Laboratories, San Mateo, CA] at a final concentration of 1 mg/ml, for 15 min at 39 and 37°C, respectively (Cross and Meizel, 1989; Thomas *et al.,* 1997). Sperm were washed twice by centrifugation and then fixed and processed for immunocytochemistry (see below).

To monitor the acrosome reaction in live cells, bull sperm was labeled with the vital membrane dye L-lissamine–rhodamine phosphatidylethanolamine (Rh-PE; Molecular Probes). For this purpose, a sperm suspension in TALP was incubated with 5 μM Rh-PE (stock solution in ethanol, less than 1% v/v of ethanol in the incubation medium) for 30 min at 39°C. The sperm was then washed twice in fresh medium and the DNA labeled with Hoescht 34443 (1 mg/ml). This protocol adapts established methods for labeling the membrane of lipid-enveloped viruses (Arbuzova *et al.,* 1994). To assess the behavior of the sperm plasma membrane during the acrosome reaction, samples were observed by fluorescence microscopy before and after exposure to ionomycin.

The effect of SNARE antibodies on the acrosome reaction was assessed by incubating bull sperm in the presence or absence of 4 μg of azide-free syntaxin and VAMP antibodies (both separately and in a 1:1 mixture). This protocol adapts the experimental procedure described previously to determine the effect of rab3 antibodies and peptides on the acrosome reaction (Garde and Roldan, 1996; Iida *et al.,* 1999). Two different sets of experiments were carried out. In the first set sperm was incubated at 39°C for 30 min (spontaneous acrosome reaction), in the second ionomycin was included in the medium and the sperm incubated for 10 min at 39°C. Hoescht 34443 and FITC–PNA, both at a final concentration of 1 mg/ml, were then added, and, following a 10-min incubation, the sperm was attached to poly-L-lysine-coated coverslips. The coverslips were then placed in PBS containing 2% formaldehyde and fixed for 1 h. Acrosome reaction was quantified as a function of PNA-labeled sperm in each case.

IVF and ICSI

IVF in the bovine system was carried out as described elsewhere (Sutovsky *et al.,* 1997). Metaphase II-arrested oocytes were obtained from Bomed, Inc. (Madison, WI). Bull sperm was prepared as described above and added to 50-μl drops of TALP medium with oocytes under mineral oil, at a final concentration of 10^6 sperm/ml. Oocytes were fertilized at 39°C in a humidified atmosphere containing 5% CO_2. Fertilization was carried out in the presence or absence of 4 μg of azide-free syntaxin and VAMP antibodies (both separately and in a 1:1 mixture) and also in the presence of 4 μg of the preimmune serum. In some experiments zona-free bovine oocytes were used. In this case the zona pellucida was removed by treating the oocytes with 1 mg/ml Pronase (Sigma) prior to fertilization. Oocytes were incubated at 39°C for 8–12 h following the addition of sperm. They were then transferred to TALP medium containing Hoescht 34443 (1 mg/ml) for 5 min, extensively washed in fresh TALP, incubated in Pronase-containing medium (1 mg/ml) to remove the zona pellucida (in the case of zona-intact eggs), attached to poly-L-lysine coverslips, fixed in PBS containing 2% formaldehyde, and scored under fluorescence microscopy. To avoid confusion between fertilization and parthenogenetic activation bull sperm was prelabeled with MitoTracker, as described above. Thus, only oocytes with one pronucleus adjacent to a MitoTracker-labeled sperm tail were scored as fertilized eggs, while only oocytes with an intact metaphase II spindle were judged to be unfertilized. In control experiments fertilization was monospermic when zona-intact oocytes were used, while fertilized zona-free oocytes were typically penetrated by three to five spermatozoa.

IVF and ICSI in the rhesus macaque were performed following the protocols of Wu *et al.* (1996) and Hewitson *et al.* (1996, 1998, 1999). Females exhibiting normal menstrual cycles were hyperstimulated by a regimen of exogenous gonadotropic hormones. Females were down-regulated by daily subcutaneous injections of a gonadotropin-releasing hormone antagonist (Serono, Randolph, MA) at 0.5 mg/kg body weight for 6 days during which recombinant human follicle-stimulating hormone (R-hFSH; Organon Inc., NJ) was administered twice daily (30 IU, im). This was followed by 1–3 days of R-hFSH + recombinant human luteinizing hormone (30 IU each, im twice daily). Ultrasonography was performed on day 7, and when there were at least four follicles equaling 4 mm in diameter, a final im injection of 1000 IU recombinant human chorionic gonadotropin (Serono) was administered. Follicles were aspirated by laparoscopy 27 h post-human chorionic gonadotropin injection, and the collected oocytes assessed for maturity. Mature, metaphase II-arrested oocytes were cultured for up to 6 h in preequilibrated TALP containing 3 mg/ml BSA at 37°C in 5% CO_2 under mineral oil (Sigma) prior to insemination/injection.

IVF was carried out by incubating mature rhesus oocytes with

capacitated sperm at a final concentration of 20×10^4 sperm/ml in TALP (37°C in 5% CO_2 under mineral oil; Wu *et al.,* 1996).

Fertilization by ICSI was accomplished by injecting a single sperm into a mature rhesus oocyte. A holding pipette with external and internal diameters of 100 and 20 μm, respectively, and an injection pipette with an outer diameter of 6–7 μm and an internal diameter of 4–5 μm, beveled at 50°, were used (Hewitson *et al.,* 1996, 1998, 1999). Washed sperm was diluted 1:50 in polyvinylpyrrolidone (Sigma) to reduce motility and aspirated tail first into the injecting pipette. Injection was carried out in TALP–Hepes, and injected oocytes were returned to culture in TALP (37°C in 5% CO_2 under mineral oil). Oocytes were fixed at several time points following insemination by either IVF or ICSI (see Results).

Immunocytochemistry

Samples were attached to poly-L-lysine-coated coverslips. In the case of fertilized eggs, the zona pellucida was removed with a short incubation in Pronase-containing TL–Hepes (1 mg/ml) prior to attachment. The coverslips were then placed in PBS containing 2% formaldehyde and fixed for 1 h. Following fixation, the samples were permeabilized for 30–60 min in PBS containing 1% Triton, and nonspecific reactions were blocked by further incubation in PBS containing 2 mg/ml bovine serum albumin and 400 mM glycine. For labeling, the antibodies were solubilized in this blocking solution and incubated with the coverslips for 1–2 h at the appropriate dilutions. After extensive washing in PBS containing 0.1% Triton, the samples were sequentially labeled with either TRITC- or FITC-conjugated appropriate secondary antibodies (Zymed, San Francisco, CA) for 40 min and the DNA stain DAPI (Molecular Probes) for 10 min. Following these incubations, coverslips were mounted in VectaShield mounting medium (Vector Laboratories, Burlingame, CA). Samples were examined with a Zeiss Axiophot epifluorescence-equipped microscope operated with Metamorph software or with a Leica TCS NT confocal microscope. The sperm tail was imaged by phase-contrast microscopy and by the fluorescent label MitoTracker to identify male pronuclei following IVF or ICSI (Sutovsky *et al.,* 1997, 1998).

SDS–PAGE and Western Blot

The presence of SNARE antigens in bovine sperm extracts was determined by one-dimensional polyacrylamide gel electrophoresis (SDS–PAGE), followed by Western blotting of sperm extracts prepared by overnight incubation of bull sperm in extraction buffer (1 M NaCl, 1 mM EDTA, 10 μg/ml PMSF, 1% v/v Triton X-100, 20 mM Tris–Cl, pH 7.0) and collection of the clear supernatant following centrifugation. As a control, sea urchin sperm extracts were also used. In this case, male gametes were obtained from *Strongylocentrotus purpuratus,* and extracts were prepared (Schulz *et al.,* 1997).

Samples for analyses were run on SDS–PAGE under reducing and denaturing conditions and then transferred to Hybond sheets (Amersham, Buckinghamshire, England) using a dry system at 0.8 mA/cm^2. Hybond sheets were blocked with 2% PBS–BSA for 1 h and then incubated overnight at 4°C with the antibodies against either syntaxin or VAMP/synaptobrevin at a dilution of 1:200. In additional assays the commercially available anti-syntaxin (Sigma), anti-synaptobrevin/VAMP (Serotec), and anti-synaptotagmin I antibodies (Sigma and Transduction Laboratories)were employed. The anti-VAMP antibody reacts with both VAMP-1 and VAMP-2, two vesicular proteins involved in exocytosis in the nervous system (Elferink *et al.,* 1989; Archer *et al.,* 1990). After extensive washing, the blots were incubated with anti-rabbit or anti-mouse goat IgG tagged with horseradish peroxidase. The bands were developed using the ECL plus system (Amersham).

Immunogold Labeling and Electron Microscopy

For electron microscopy, frozen bull sperm was prepared as described above. Following centrifugation, sperm pellets were fixed in 2% formaldehyde, blocked, and incubated with the primary antibody as described above. FITC- or TRITC-conjugated secondary antibody was replaced with 10-nm gold-conjugated goat anti-rabbit antibody (Amersham) diluted 1:10. After being washed in PBS, the labeled sperm were pelleted, fixed overnight in 0.6% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer, postfixed for 1 h in 1% osmium tetroxide, dehydrated by an ascending ethanol series (30–100%), and embedded in Epon 812. Ultrathin sections were cut using a Sorvall MT2B ultramicrotome and collected onto Formvar-coated copper grids. Grids with sections were stained with uranyl acetate for 10 min and examined in a Phillips 300 transmission electron microscope. Controls (secondary-only incubations) were routinely performed to discount nonspecific binding.

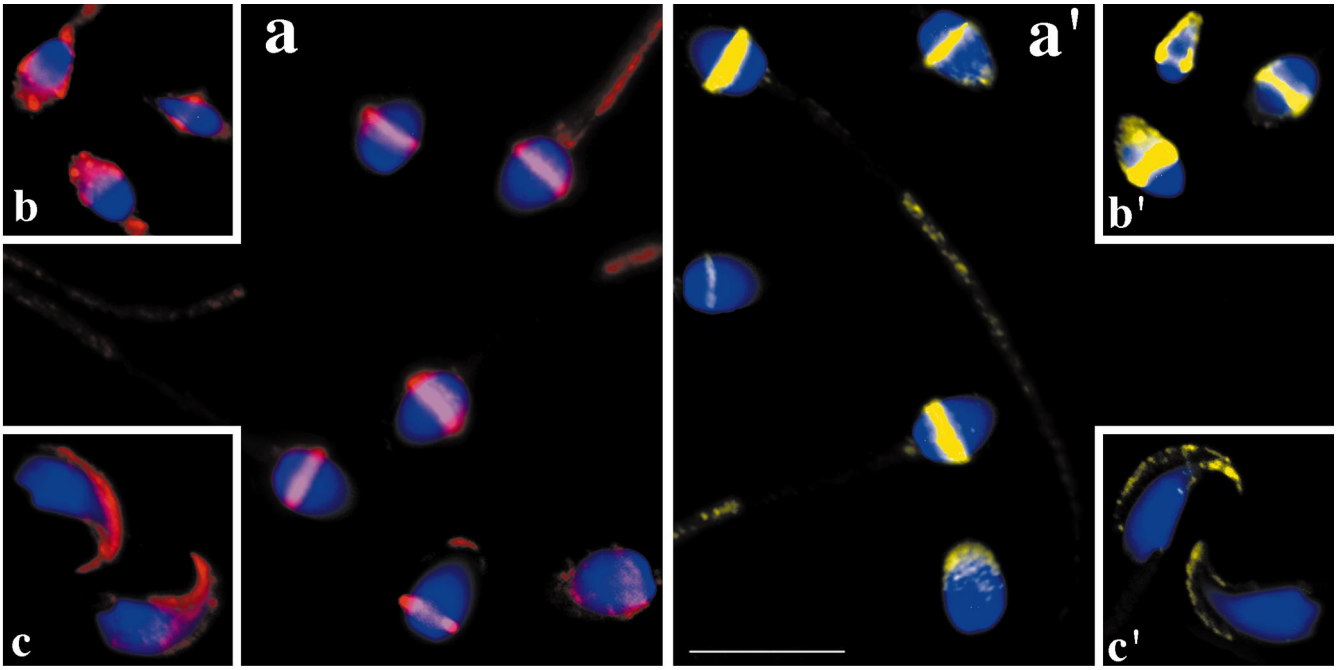
RESULTS

SNAREs and the Acrosome Reaction in Mammalian Sperm

Homologues of the t-SNARE syntaxin and the v-SNARE VAMP/synaptobrevin are identified on mouse, human, rhesus monkey (Fig. 1), hamster (Fig. 2), and bull (Figs. 3 and 5) sperm. Although there was some variability, in all cases the proteins were present in both the acrosomal region and the sperm equatorial segment. This stain was found to be specific to the anti-SNARE antibodies because it was not visible when either the preimmune serum or the secondary antibodies alone were employed (data not shown). However, use of the preimmune serum did result in a faint label of the sperm tail's principal piece, thus suggesting that this particular staining pattern is not specific (data not shown). Using freshly ejaculated rhesus monkey sperm, we were able to determine that labeling of the sperm head with anti-syntaxin or anti-VAMP antibodies was dependent on detergent permeabilization following fixation (data not shown), implying that these SNARE homologues are on the inside of the sperm plasma membrane in intact, non-acrosome-reacted spermatozoa and therefore inaccessible to antibodies under these conditions (data not shown).

The presence of SNAREs on mammalian sperm heads suggests that these proteins could be involved in membrane fusion during the acrosome reaction, similar to what has been proposed in sea urchin sperm (Schulz *et al.,* 1997).

Hamster sperm is employed as a distinctive model system of the acrosome reaction. There are two advantages: the hamster acrosome is considerably larger than that of other mammals, and this structure is discarded almost intact into the medium following the acrosome reaction in the form of an "acrosomal ghost" (Barros *et al.,* 1967; Franklin *et al.,* 1970; Yanagimachi and Noda, 1970). It is



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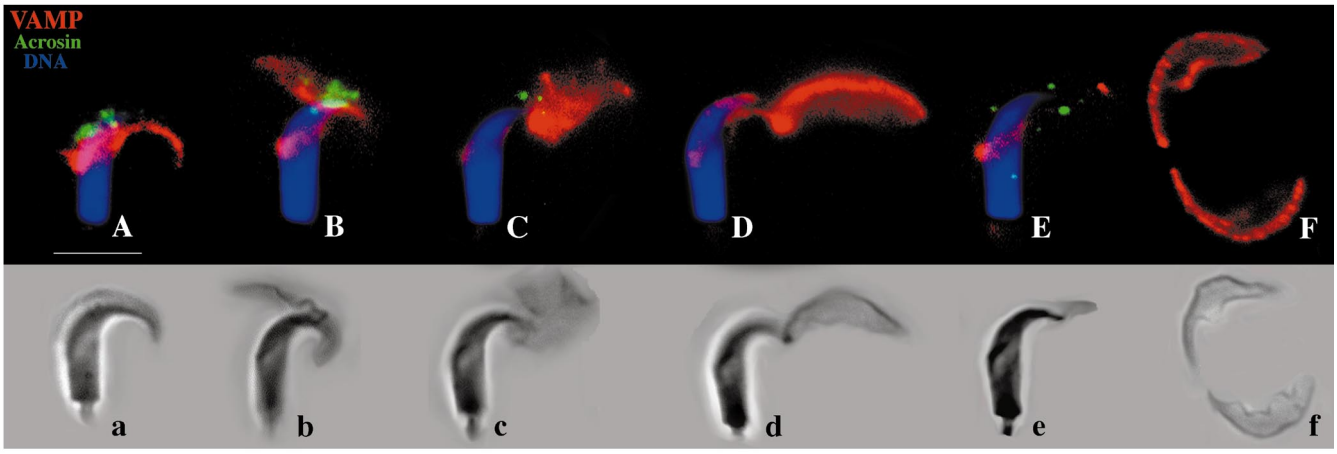

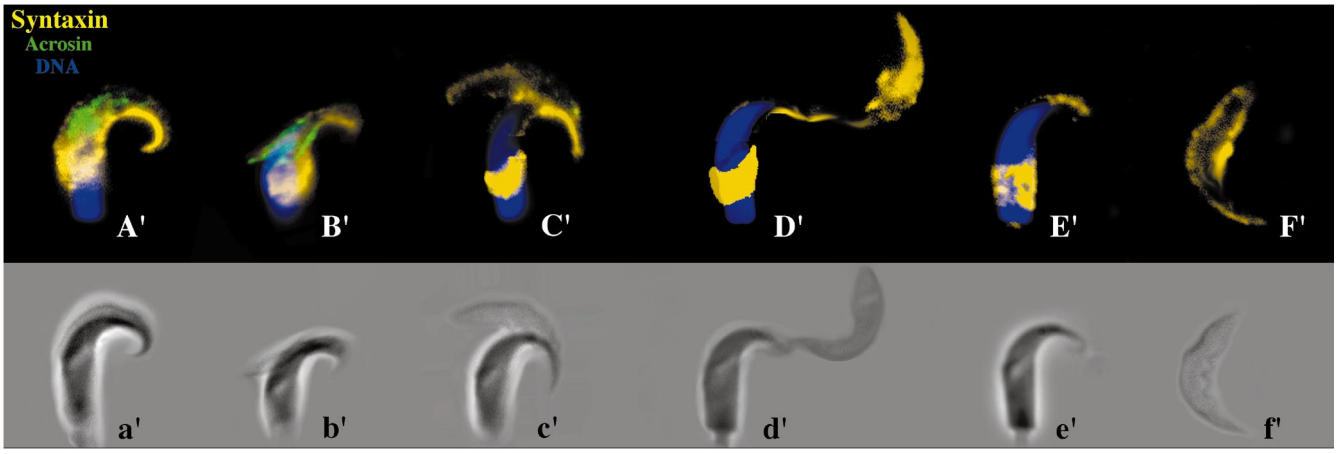

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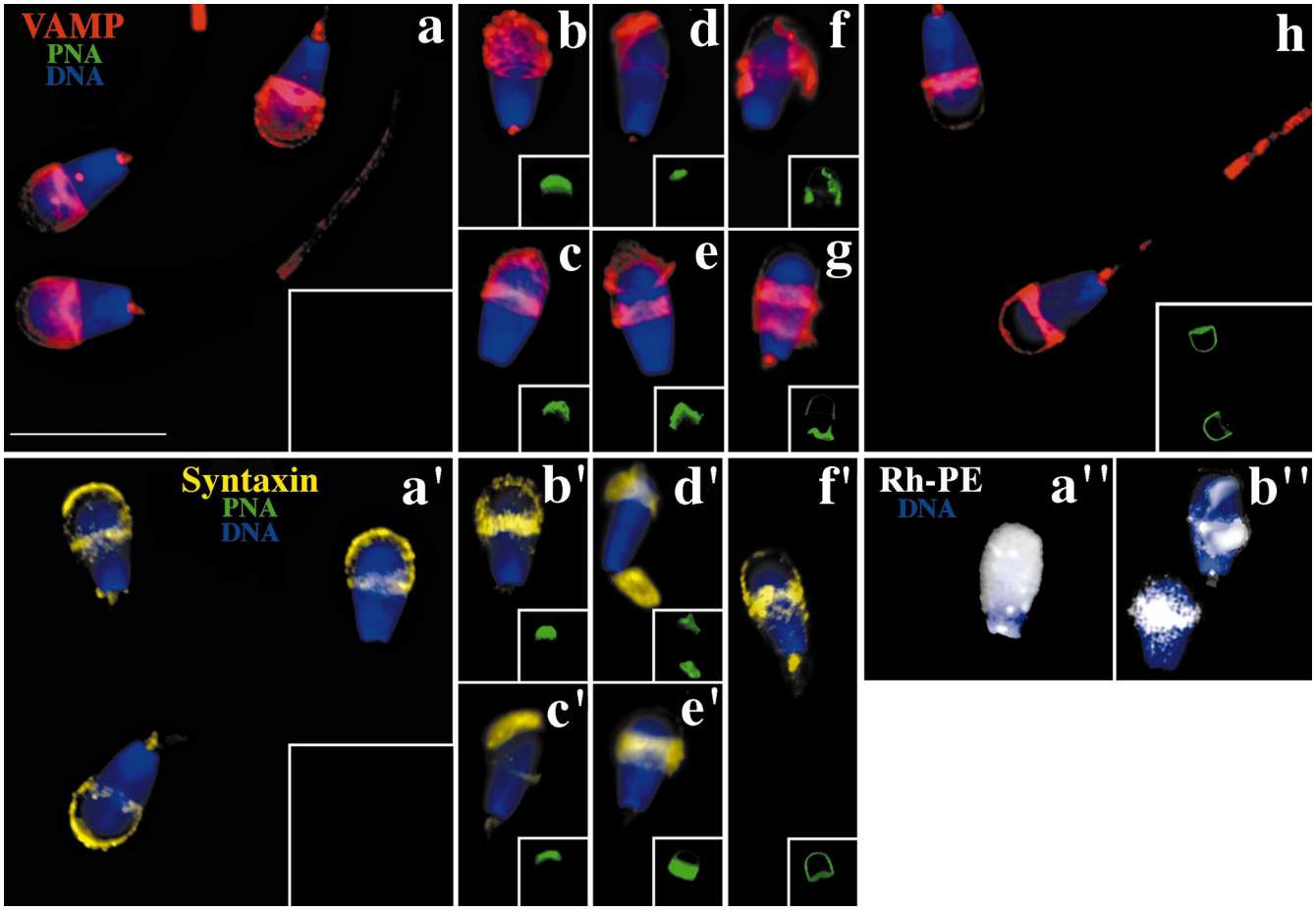


FIG. 3. SNAREs and the acrosome reaction in bull sperm. VAMP is detected as two layers, possibly corresponding to the inner and outer acrosome membranes (a), while only one syntaxin layer is apparent, possibly corresponding to the plasma membrane (a′). The acrosome reaction starts with an initial "ruffling" of the sperm head, perhaps corresponding to multiple plasma membrane–acrosome fusion events, and results in FITC-labeled PNA access to acrosomal contents (b, c, and b′). Following this, the fused membranes are peeled off from the sperm head, concomitant with the release of acrosomal contents (d–g, c′–e′). Acrosome-reacted sperm show traces of PNA (i.e., acrosomal contents) on the sperm head and SNARE staining on both the head and the equatorial segment (h and f′). Live sperm were imaged with the vital membrane probe Rh-PE during the acrosome reaction. Initially sperm show a homogeneous head label (a″). Later the labeled membrane is almost exclusively on the equatorial segment (b″), demonstrating that the shedding of SNAREs during the acrosome reaction parallels the partial removal of sperm membranes. VAMP/synaptobrevin (a–f, red), syntaxin (a′–f′, yellow), DNA: DAPI (blue). Bar, 10 μm. For clarity, insets depict PNA staining (green). Control samples (a, a′) are not labeled by PNA, which can interact only with exposed acrosomal contents following the acrosome reaction.

FIG. 1. VAMP and syntaxin homologues in mammalian sperm. VAMP/synaptobrevin (a–c, red) and syntaxin (a′–c′, yellow) are detected in rhesus monkey (a, a′), human (b, b′), and mouse (c, c′) sperm. SNAREs are localized in or over the acrosomal region of the sperm head with brilliant detection at the equatorial segment. DNA: DAPI (blue). Bar, 10 μm.

FIG. 2. SNARE redistribution during the acrosome reaction in hamster sperm. Hamster sperm was incubated with ionomycin and processed for immunocytochemistry as described. Using phase-contrast microscopy, the stages of the acrosome reaction are well displayed in hamster sperm (a–f, a′–f′), and the corresponding SNARE images at different stages of the acrosome reaction are presented (A–F, A′–F′). The sequence begins with acrosome-intact spermatozoa, with SNARE detection in the acrosomal region and intact acrosomal contents (A, A′). The acrosome reaction begins by a "peeling off" of the acrosome (typically from the back of the sperm head), resulting in the release of acrosomal contents (B, B′). The removal of the acrosome proceeds until the empty vesicle is discarded from the sperm head (C and D, C′ and D′). Acrosome-reacted sperm retain strong SNARE signals on the equatorial segment (E and E′), and most of the labeling previously found on the sperm head is now included in acrosomal ghosts (F and F′). VAMP/synaptobrevin (A–F, red), syntaxin (A′–F′, yellow), acrosin (green). DNA: DAPI (blue). Bar, 10 μm.

therefore straightforward to determine the status of the acrosome in this species (Figs. 2a–2f, 2a′–2f′′). In addition, we used the enzyme acrosin as a marker for acrosomal contents. Acrosin is thought to aid sperm–egg penetration after being released from the acrosome following the acrosome reaction (Valdivia *et al.*, 1994), although its exact relevance is still debated (Wassarman, 1999). In all cases spermatozoa with a clear, intact acrosome were crossreacted with acrosin antibodies (Figs. 2A, 2a, 2A′, and 2a′). However, acrosin staining was lost as the acrosome acquired a ruffled appearance and began to detach from the sperm head, typically by peeling off from the posterior side and hanging loosely to the tip of the head before being released into the medium (Figs. 2B, 2b, 2F, 2f, 2B′, 2b′, 2F′, and 2f′). Throughout this process, the SNARE VAMP/synaptobrevin (Fig. 2, red) and syntaxin (Fig. 2, yellow) antibodies strongly labeled the acrosome and could be clearly detected in discarded acrosomal ghosts (Figs. 2F and 2F′).

The acrosome reaction in the hamster is somewhat unusual in that fusion between the sperm plasma membrane and the outer acrosomal membrane takes place at different points of contact, allowing acrosomal contents to leak out, but leaving the structure morphologically intact due to the cohesiveness provided by the acrosomal matrix (Barros *et al.*, 1967; Yanagimachi and Noda, 1970). In other mammals, vesicles and fragments derived from the two fusing membranes are shed or "sloughed" (Fawcett, 1975; Yanagimachi, 1994; Wassarman, 1999). The behavior of SNARE homologues during this type of acrosome reaction was investigated using bull sperm (Fig. 3). The approach was slightly different from that in the case of the hamster in that the FITC-tagged peanut-derived lectin PNA was used as a marker for acrosomal contents (Cross and Meizel, 1989; Thomas *et al.*, 1997). Following exposure to ionomycin, the sperm were incubated with PNA, washed, and only then fixed, permeabilized, and further processed for immunocytochemistry using anti-SNARE antibodies (see Materials and Methods). This ensures that the fluorescent lectin has access to the acrosomal contents exposed to the culture medium following the acrosome reaction and that PNA labeling is not due to compromised sperm integrity following fixation/permeabilization. In separate experiments, we also determined that both PNA- and SNARE-staining patterns could be observed independently, thus precluding any nonspecific interactions between the antibodies and the fluorescent lectin (data not shown). The results obtained were very similar for both VAMP (Fig. 3, red) and syntaxin (Fig. 3, yellow). Two layers of SNAREs, notably in the case of VAMP, were detected in non-acrosome-reacted bovine sperm heads (inaccessible to PNA), perhaps corresponding to plasma membrane/outer acrosomal membrane staining and inner acrosomal membrane staining (Figs. 3a and 3a′). The acrosome reaction proceeds by an initial "ruffling" of the sperm plasma membrane with strong PNA labeling on the tip of the sperm head (corresponding to the still virtually intact acrosomal contents, Figs. 3b and 3b′), followed by the peeling off of both VAMP- and syntaxin-positive membranes and closely paralleling the release of acrosomal contents (Figs. 3c–3g, 3c′–3e′). Sperm in which the acrosome reaction was complete showed vestigial PNA staining on the sperm head (corresponding to acrosomal contents that remain attached to the sperm head), including the sperm equatorial segment. The sequence presented in Figs. 3a–3h and 3a′–3f′) is in accordance with phases established by Cummins *et al.* (1991). A similar peeling off of membranes could also be observed during the ionomycin-triggered acrosome reaction in live bull sperm, using the membrane probe Rh-PE incorporated in the sperm plasma membrane (Fig. 3, white). While acrosome-intact spermatozoa presented a uniform head label (Fig. 3a″), only remnants of the fluorescent lipid could be detected on the sperm head (concentrating notably on the equatorial segment) following ionomycin treatment (Fig. 3b″). This result confirms that the shedding of VAMP- and syntaxin-positive structures from the sperm head during the acrosome reaction corresponds to the loss of SNARE-containing membranes.

Similar events occurred in rhesus monkey sperm (data not shown). The use of ionomycin resulted in 70–90% acrosome-reacted sperm, as judged by morphological criteria (hamster) or PNA staining (bull, rhesus); control samples, incubated in the absence of the ionophore, were found to include 15–30% acrosome-reacted spermatozoa. Syntaxin and VAMP/synaptobrevin persist on the sperm head in hamster, rhesus, and bull sperm, with a brilliant pattern at the sperm equatorial segment, even after the acrosome reaction (Figs. 2E, 2E′, 3h, and 3f′). This is in contrast to sea urchin sperm, in which the SNARE protein homologues are released in the sloughed acrosome/plasma membrane vesicles and cannot be detected in acrosome-reacted sperm (Schulz *et al.*, 1997).

Effect of SNARE Antibodies on the Acrosome Reaction and on Fertilization

The behavior of sperm SNAREs during the acrosome reaction suggests a possible role for these proteins in this event. But, as in the case of sea urchin (Schulz *et al.*, 1997), the localization of SNAREs alone does not imply any physiological relevance. Therefore, we attempted to block the acrosome reaction using SNARE antibodies. Since any blocking effect should be observed only if the antibodies have access to the intracellular environment (see above), bovine sperm was permeabilized with digitonin, saponin, or Triton X-100. However, following these treatments we were unable to adequately judge the acrosomal status of the permeabilized sperm and thus quantify any antibody-specific effects (data not shown). We therefore decided to add the antibodies to intact sperm, without any prior treatment, and monitor their effect on the acrosome reaction using the PNA method, both in the absence (spontaneous acrosome reaction) and in the presence of ionomycin. This approach adapts previous protocols (Garde and Roldan, 1996; Iida *et al.*, 1999), and the rationale is that SNARE

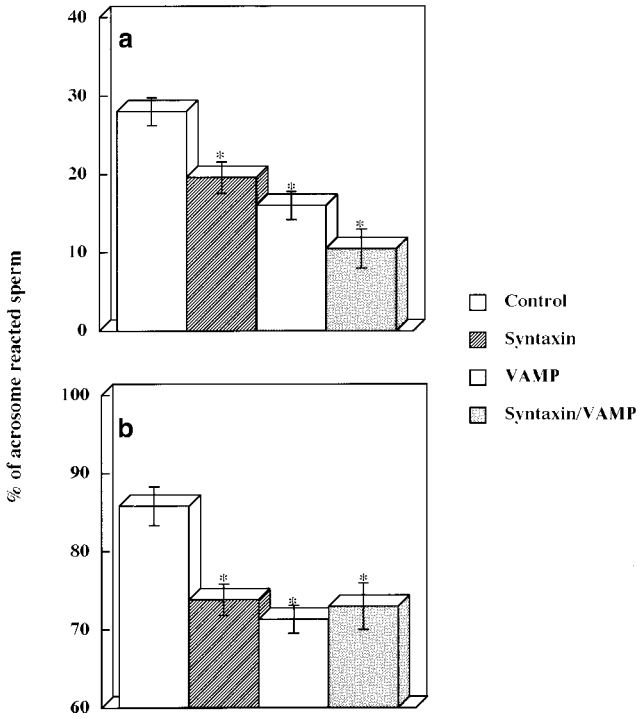


FIG. 4. Inhibition of the bovine acrosome reaction by SNARE antibodies. The acrosome reaction was quantified using FITC–PNA as described under Materials and Methods (see also Fig. 3). Bull sperm was incubated in the presence or absence of 4 μg of azide-free syntaxin and VAMP antibodies (both separately and in a 1:1 mixture). (a) Experiments carried out in the absence of ionomycin (spontaneous acrosome reaction). (b) Experiments carried out in the presence of ionomycin (ionophore-triggered acrosome reaction). Results represent the average ± SD of six sets of independent assays. *$P < 0.001$ compared with the control (Student's t test).

antibodies could penetrate the sperm at the initial stages of the acrosome reaction and halt its progress before extensive contents release (i.e., PNA labeling) takes place.

Using this approach we were able to detect a statistically significant inhibition of the acrosome reaction, both in the absence (Fig. 4a) and in the presence (Fig. 4b) of ionomycin, thus suggesting that sperm SNARE homologues do play a role in the release of acrosomal contents. Although combining both antibodies seemed to potentiate the effect in spontaneous acrosome reaction (Fig. 4a), the difference was not significant. The inhibition observed in these experiments is comparable to the modulatory effects of rab3A peptides and antibodies on the acrosome reaction using the same protocol (Garde and Roldan, 1996; Iida *et al.*, 1999). The fact that total inhibition is not observed could stem from either low antibody penetration into the sperm or the fact that, similar to what takes place during exocytosis in nerve terminals, the complementary SNAREs are tightly associated in the sperm head and therefore less accessible to the blocking action of antibodies or to toxins that specifically promote their cleavage (Almeida *et al.*, 1997; Hayashi *et al.*, 1994; Pellegrini *et al.*, 1994). Similar observations were made in the case of membrane fusion during the exocytosis of sea urchin egg cortical granules (Avery *et al.*, 1997). Additionally, these experiments seem to validate the recently proposed model that considers the acrosome reaction a series of continuous, but discrete, exocytotic events that can therefore be modulated by extracellular agents (Kim and Gerton, 1999).

The fact that SNARE antibodies inhibit the acrosome reaction suggested the possibility that they could also therefore inhibit sperm–egg interactions. Although antibodies against syntaxin and VAMP inhibited fertilization when assayed separately, the effect was not statistically significant (data not shown). However, bovine IVF was inhibited in the presence of a mixture of both probes (Table 1). The appropriate control using the preimmune serum also had a inhibitory effect, but SNARE antibodies almost completely abolished fertilization. This effect was found when either zona-intact (Table 1) or zona-free (data not shown) oocytes were used. In addition, the presence of the antibodies did not inhibit sperm binding to either the zona pellucida (zona-intact oocytes) or the egg plasma membrane (zona-free oocytes, data not shown), suggesting that the effect of the antibodies is not solely related to steric inhibitions of sperm–egg contact.

It is likely that inhibitions of both the acrosome reaction and fertilization by SNARE antibodies are functionally connected, but we cannot be sure if the former is directly responsible for the latter. The higher inhibition detected with the fertilization experiments could be due to the fact that the antibodies are incubated with the sperm for a longer period of time, under physiologically relevant condi-

TABLE 1
Inhibition of Bovine Fertilization by SNARE Antibodies

	Number of fertilized oocytes	Number of unfertilized oocytes
Control	12.5 ± 2.1 (81)	3 ± 1.7 (19)
Preimmune serum	14 ± 3 (63)	8 ± 1.9 (37)
Syntaxin/VAMP antibodies	3 ± 2.9 (17)[a]	14.7 ± 4 (83)[a]

Note. Fertilization of zona-intact bovine oocytes was carried out as described under Materials and Methods. Fertilization media contained 4 μg of preimmune serum or 4 μg of a 1:1 mixture of anti-syntaxin and anti-VAMP antibodies. Successful fertilization was distinguished from parthenogenic activation by prelabeling the sperm with Mitotracker (see Materials and Methods). The numbers in parentheses are the percentages of fertilized and unfertilized oocytes. Results represent the averages ± SD of at least four sets of independent assays.

[a] $P < 0.01$ compared with preimmune serum controls (Student's t test).

tions. Alternatively, it is also possible that the binding of SNARE antibodies to their epitopes on the sperm would somehow prevent correct sperm–egg contact, without inhibiting binding per se, which could also help explain why only a mixture of both probes has a statistically relevant effect. Nevertheless, taken together these results suggest a possible role for SNARE proteins during fertilization.

Characterization of Sperm SNAREs

Sea urchin egg and sperm syntaxin and VAMP/synaptobrevin are similar to their counterparts involved in synaptic vesicle exocytosis (Avery *et al.*, 1997; Conner *et al.*, 1997; Schulz *et al.*, 1997). Characterization of mammalian sperm SNAREs by Western blot, using sea urchin sperm as a control (Fig. 5a) was performed. Bull sperm syntaxin is similar to the sea urchin form, both having molecular weights in the 29–33 kDa range. Minor bands of higher molecular weight were also detected in both samples, possibly due to protein aggregates. The acrosome of both mouse and bull sperm was labeled with the same pattern as the one shown in Figs. 1 and 3, respectively, using antibodies against syntaxin 1A and 1B (data not shown). Western blots using these antibodies revealed a doublet in the 30 kDa range, as well as some higher molecular weight bands, similar to Fig.5a (data not shown). Since the manufacturer mentions that there might be some crossreactivity between the anti-syntaxin 1A antibody and syntaxin 1B (and vice versa), we presume that this doublet corresponds to the two forms of the protein. Syntaxin 1 is the member of the syntaxin family implicated in synaptic vesicle exocytosis (Calakos and Scheller, 1996). On the other hand, antibodies against syntaxin 4 (active in basolateral exocytosis) and syntaxin 6 (active in Golgi trafficking) did not appreciatively label the acrosome (data not shown).

Sea urchin VAMP was confirmed to have the expected molecular weight for this protein (17–18 kDa), and the major crossreactivity with mammalian samples was detected at this level, with a minor higher molecular weight band (Fig. 5a). Similar results were obtained with an antibody that recognizes VAMP-1 and VAMP-2 (clone MCA 1433), the two forms predominantly present in neurons. In addition, immunocytochemistry labeling patterns described in Figs. 1 and 3 could be reproduced using antibodies specific for both VAMP-1 and VAMP-2, again suggesting that the sperm VAMP is similar to proteins involved in exocytosis at the synapse.

In addition, we should also note that the anti-SNARE antibodies used in this study did not crossreact with the plasma membrane or cortical granule of mammalian (mouse, cow, rhesus) oocytes (data not shown). Although antibody reactivity was lower, rhesus sperm extracts gave results similar to those shown for bovine samples (data not shown).

Immunocytochemistry of acrosome-intact bull sperm results in the detection of two layers of VAMP staining on the tip of the sperm head (Fig. 3a). This may correspond to the inner and outer acrosome membranes. Syntaxin is detected only in the outermost limit of the sperm head, possibly the sperm plasma membrane (Fig. 3a′). To confirm this putative localization, immunogold electron microscopy was performed. Although both syntaxin (Fig. 5b) and VAMP (Fig. 5c) are detected on the sperm head surface using this technique, notably on the equatorial segment, it is not possible to assign specific antigens to either the plasma membrane or the acrosomal membranes.

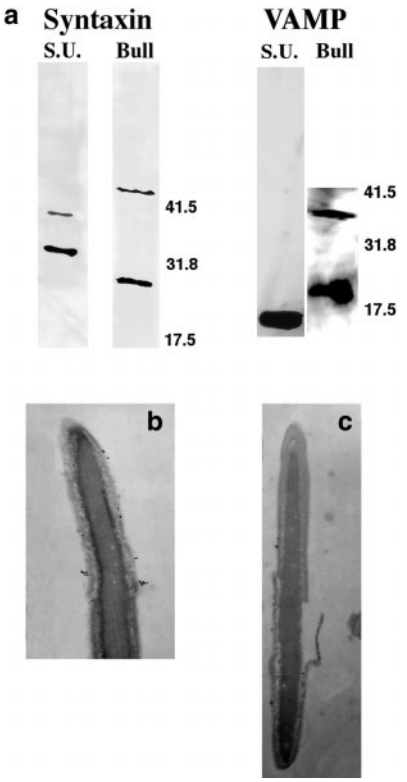
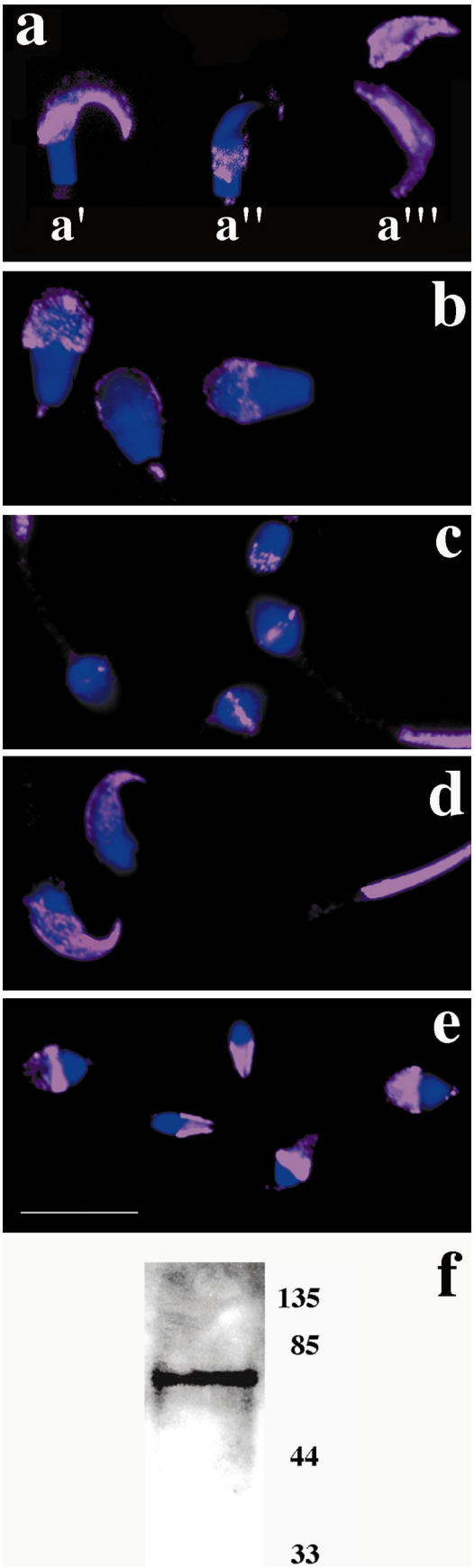


FIG. 5. Identification and localization of SNAREs on bull sperm by Western blot and immunogold labeling. (a) SNAREs were detected in bull sperm extracts by Western blotting. Sea urchin sperm extracts were used as controls. Syntaxin is found to have similar molecular weights in both species (30–33 kDa), with slightly higher molecular weight bands. Bull VAMP also showed two bands, an expected major band similar to sea urchin VAMP and also a higher molecular weight band. It is possible that the higher molecular weight bands represent protein aggregates. Each lane contains 5 μg (sea urchin) or 30–50 μg (bull) of extract protein, and the numbers represent the positions of standard molecular weight markers. (b and c) Immunogold labeling and electron microscopy of transverse sections of bull sperm. Both syntaxin (b) and VAMP (c) are detected at the ultrastructural level on the sperm surface.

Presence of Synaptotagmin I in Mammalian Sperm

The synaptic vesicle integral membrane protein, synaptotagmin, is found on all the mammalian sperm samples



tested (Fig. 6). The localization of synaptotagmin is similar to that of SNAREs and, in the case of hamster sperm, synaptotagmin is also discarded in the acrosomal ghosts (Fig. 6a‴). Using antibodies specific for synaptotagmin I this protein is detected at the expected molecular weight (≈65 kDa) in bull sperm extracts (Fig. 6f), and the antibodies also labeled the acrosome with the same pattern shown in Figs. 6a–6e (data not shown). This finding again stresses possible similarities between the acrosome reaction and synaptic vesicle exocytosis, since synaptotagmin I is present on the vesicle membrane and seems to play a role in secretion at the synapse (Calakos and Scheller, 1996; Schiavo *et al.*, 1998). However, our results do not exclude the possibility that other members of the growing synaptotagmin family, and indeed other (possibly even sperm-specific) SNAREs, might also be present in sperm.

Removal of SNARE Proteins from the Sperm Head Following IVF or ICSI

The partial retention of SNARE homologues on acrosome-reacted mammalian sperm, as well as their presence on the sperm head following the acrosome reaction, forces questions regarding their involvement during IVF and ICSI. These questions can be addressed using the rhesus monkey model because fertilization events in this model closely resemble those in humans (Simerly *et al.*, 1995; Hewitson *et al.*, 1998, 1999).

During IVF the v-SNARE VAMP/synaptobrevin, although initially present on sperm at the egg surface (Fig. 7a), was lost from the sperm head prior to egg penetration (Fig. 7b). It could no longer be found associated with the male pronucleus inside the egg (Figs. 7c–7e), even at the earliest stages of DNA decondensation and pronuclear formation (Fig. 7c).

Alternatively, ICSI introduced the sperm SNARE proteins, together with other sperm components, directly into the egg cytoplasm. VAMP was detectable on sperm heads a

FIG. 6. Synaptotagmin on mammalian sperm in the hamster (a), the bull (b), the rhesus monkey (c), the mouse (d), and the human (e). Synaptotagmin is detected on the acrosomal region of the sperm head with conspicuous labeling in the equatorial segment. In hamsters, synaptotagmin closely parallels the patterns observed for both VAMP and syntaxin during the acrosome reaction (see Fig. 2). Acrosomal labeling was seen in intact sperm (a′), the equatorial segment labeling was retained in acrosome-reacted spermatozoa (a″), and empty acrosomal ghosts stained heavily for synaptotagmin (a‴). Synaptotagmin (purple), DAPI (blue). Bar, 10 μm. (f) Identification of synaptotagmin I as a single ≈65-kDa band in 30 μg of bull sperm extract by Western blot using an antibody from Sigma. Results with another probe (see Materials and Methods) were identical (data not shown). Numbers represent the positions of standard molecular weight markers. Both anti-synaptotagmin I antibodies labeled the acrosome with the same pattern shown in a–e (data not shown).

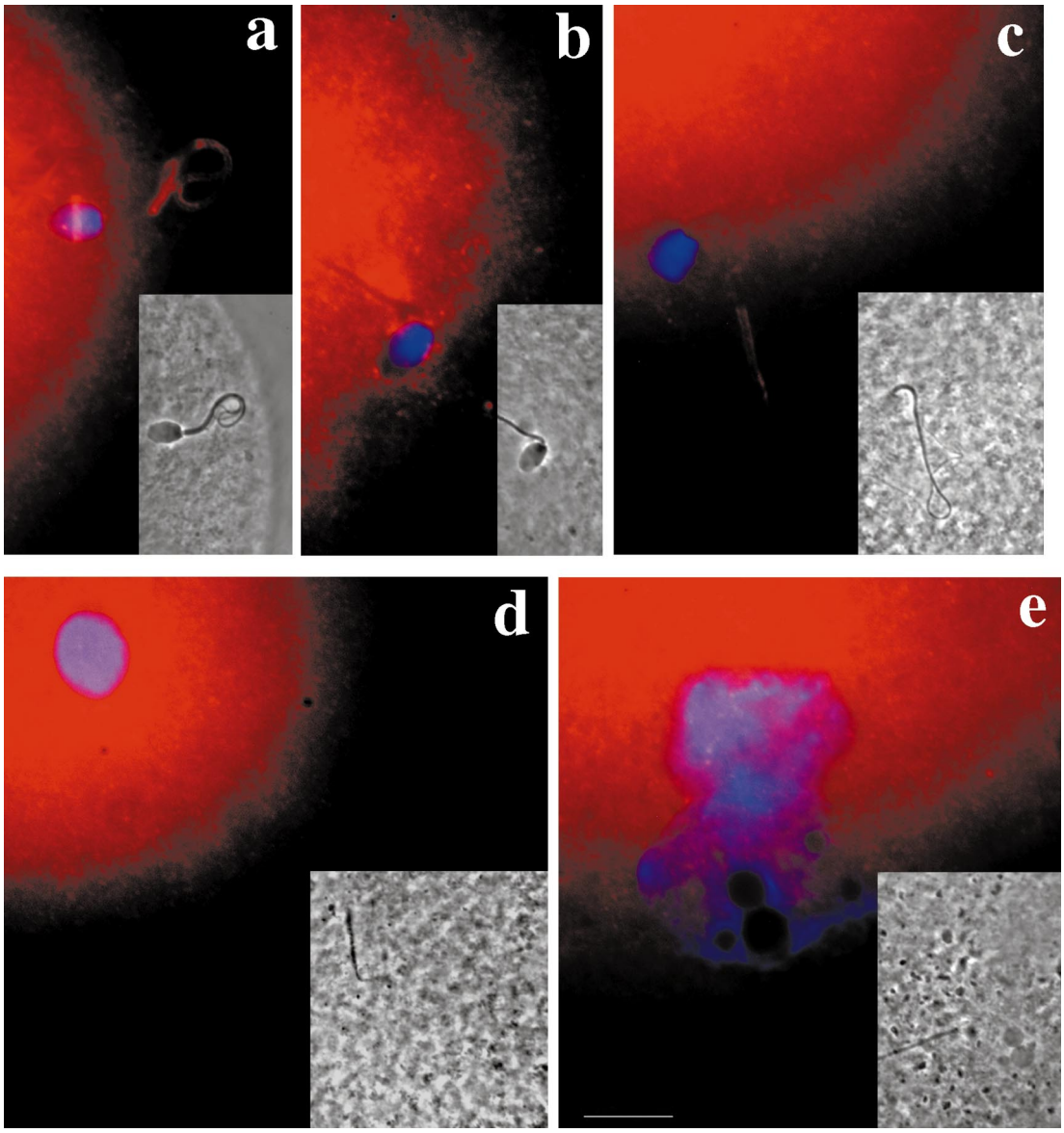
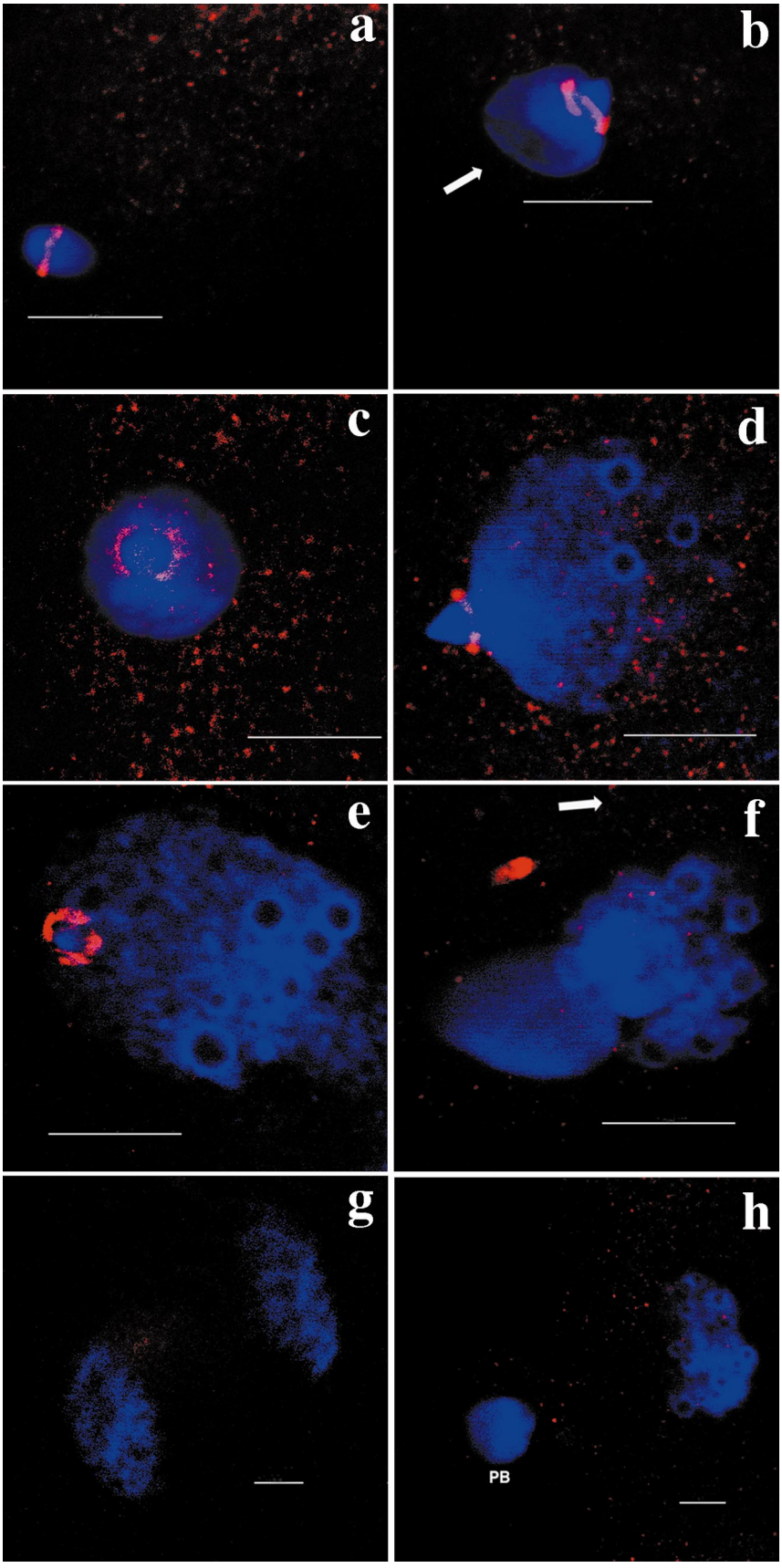


FIG. 7. Removal of VAMP during rhesus IVF. In early stages of fertilization (2 h postinsemination) VAMP (red) is observed at the egg surface, either depicting the pattern typical for sperm (a, see Fig. 1) or showing only traces of reactivity (b). VAMP was not detected on the decondensing sperm nucleus inside the egg (c–e), even at the earliest stages of sperm incorporation (c, 4 h postinsemination). Note the presence of the sperm tail, identifying the male pronucleus, as observed by phase-contrast microscopy (insets). DNA: DAPI (blue). Bar, 10 μm.

FIG. 8. Confocal imaging of the persistence of VAMP on the sperm head following rhesus ICSI. (a) Sperm with a typical VAMP (red) pattern (30 min postinjection). (b–d) DNA decondensation at the anterior portion of the sperm head seems to be prevented or retarded by a VAMP two-piece "collar" 4 h postinjection (b and d, side views; c, top view). Eventually the male DNA decondenses completely, forming a pronucleus with VAMP remnants in the vicinity (e, 8 h postinjection), sometimes persisting up to the stage of pronuclear apposition (f, 20 h postinjection). VAMP became undetectable by first mitosis (g, first mitotic anaphase). Arrows denote the presence of the sperm tail as observed by phase-contrast microscopy and MitoTracker labeling. Nucleoli are detected as dark regions within the labeled male chromatin (DAPI: blue). VAMP is not observed associated with female pronuclei nor the polar body (h, PB, polar body). Bars, 10 μm.



short time after sperm injection (Fig. 8a). Because some sperm head structures, totally or partially removed at the egg surface and cortex during normal fertilization (e.g., the acrosome, the perinuclear theca), were still present following ICSI, sperm DNA decondensation after ICSI occurred asynchronously. The apical (i.e., acrosomal) portion of the sperm head remained condensed for an unusually long time, until the aforementioned structures were later removed by the egg cytoplasm (Sutovsky *et al.*, 1996, 1997; Bourgain *et al.*, 1998; Yanagimachi, 1998; Hewitson *et al.*, 1999). We have extended these observations here to show that a VAMP two-piece "collar," probably marking the sperm equatorial segment, sharply separates the condensed apical DNA from the decondensed posterior DNA (Figs. 8b–8d; compare with Figs. 7c and 7d).

Although DNA decondensation is retarded in the apical portion of the sperm head, complete pronuclear formation eventually occurs, and the VAMP-containing collar is discarded in the vicinity of the decondensed male-derived DNA (Fig. 8e). VAMP remnants could, in some cases, be detected at the stage of pronuclear apposition (Fig. 8f), but they were no longer found at first mitosis (Fig. 8g) and are not associated with the female pronucleus (Fig. 8h) nor metaphase spindles of unfertilized oocytes (not shown). This observation is consistent with the reported success of ICSI in this model system (Hewitson *et al.*, 1999).

DISCUSSION

The morphological aspects of membrane fusion during the sperm acrosome reaction have been described in great detail (Dan, 1952, 1954; Dan and Wada, 1955; Austin and Bishop, 1958a,b; Barros *et al.*, 1967; Franklin *et al.*, 1970; Yanagimachi and Noda, 1970), and there is already much information on how this process is regulated (Yanagimachi, 1994; Breitbart and Spungin, 1997; Florman *et al.*, 1998; Wassarman, 1999). We have set out to identify possible components of the membrane recognition/contact/fusion machinery active during the acrosome reaction, using the framework of the SNARE hypothesis for intracellular membrane traffic as a possible working model.

Although SNARE proteins are recognized as important elements in membrane trafficking/fusion events, their exact role (membrane recognition/docking, "priming" of membranes for fusion, membrane merging) and importance remain controversial, especially because the SNARE hypothesis has been applied to an array of fusion processes studied in various experimental models, few of which have been characterized in the same detail as synaptic vesicle exocytosis (Ferro-Novick and Jahn, 1994; Rothman, 1994; Calakos and Scheller, 1996; Hanson *et al.*, 1997; Gotte and von Mollard, 1998). Despite very recent reports suggesting that SNAREs involved in synaptic vesicle exocytosis can mediate membrane fusion in a reconstituted system, and might therefore constitute a "minimal fusion machinery," i.e., the necessary and sufficient components of the membrane fusion reaction (Weber *et al.*, 1998; Nickel *et al.*, 1999; Parlati *et al.*, 1999), this issue is still under vigorous debate, inasmuch as it relates to other fusion events (e.g., Coorssen *et al.*, 1998). The unexpected distribution of particular v- and t-SNAREs in both fusing membranes in some systems must also be clarified and suggests that the elegantly simple initial models might warrant revision (Hanson *et al.*, 1997; Gotte and von Mollard, 1998).

A possible role for SNAREs in the regulation/triggering of membrane fusion during fertilization was first proposed in the case of cortical granule exocytosis in sea urchin eggs (Avery *et al.*, 1997; Conner *et al.*, 1997). This research identified homologues for the t-SNARE syntaxin and v-SNARE VAMP/synaptobrevin on the egg plasma membrane and on cortical granule membranes, respectively. Functional studies suggested that synaptobrevin might play a critical role in the release of cortical granule contents into the extracellular medium following fertilization (Avery *et al.*, 1997), a notion that is, however, disputed by other studies in the same system (Coorsen *et al.*, 1998). SNARE antibodies used in this study did not crossreact with the plasma membrane or cortical granules of mammalian (mouse, cow, rhesus) oocytes. In addition to SNAREs, homologues of rab3, a small GTPase involved in the regulation of exocytosis, and of synaptotagmin, a putative calcium sensor for secretion at the synapse, were also described in sea urchin eggs (Avery *et al.*, 1997; Conner *et al.*, 1997).

Despite present controversies, parallels can be drawn between cortical granule exocytosis in fertilized eggs and the acrosome reaction in sperm, two secretory events essential for natural fertilization to take place. Homologues for syntaxin and VAMP/synaptobrevin were indeed found in the acrosome of sea urchin sperm (Schulz *et al.*, 1997). Furthermore, these proteins were discarded from the sperm following the acrosome reaction.

Although the acrosome reaction in sea urchin sperm results in the formation of an actin process that aids sperm penetration into the egg, a structure that does not exist in mammals (Schulz *et al.*, 1997), we have nevertheless extended these previous findings to demonstrate the presence of the SNARE proteins syntaxin and VAMP/synaptobrevin in several mammalian species. That these proteins are recognized by probes directed against synapse SNAREs (syntaxin 1 and VAMP 1 and 2), and that they are shed from the sperm head during the acrosome reaction, strongly suggests that they might play a role in this event, by bridging the outer acrosomal membrane and the sperm plasma membrane (i.e., recognition and docking of the fusion partners) and/or by triggering membrane merging itself. Additionally, assays in the presence of antibodies provide the first functional evidence that SNAREs could modulate secretion during the acrosome reaction. The fact that SNARE antibodies also block fertilization without interfering with sperm–egg binding suggests that the inhibitory effect on the acrosome reaction is physiologically

relevant and that sperm SNAREs play a role in sperm–egg interactions.

The detection of syntaxin and VAMP/synaptobrevin at the equatorial segment of acrosome-reacted mammalian spermatozoa, in contrast to sea urchin sperm (Schultz *et al.*, 1997), is not unexpected. This portion of the acrosome is not normally involved in early secretion and persists on the sperm head even after the acrosome reaction has occurred (Fawcett, 1975; Yanagimachi, 1994; Wassarman, 1999). Although the exact relevance is not yet understood, it is the equatorial segment that initially contacts the egg plasma membrane, and thus it is the domain of the sperm plasma membrane first involved in sperm–egg fusion (Yanagimachi, 1994; Wassarman, 1999). Interestingly, studies with artificial lipid vesicles (liposomes) have shown not only that protein-dependent liposome–sperm fusion occurs exclusively at the sperm equatorial segment, but also that this region becomes fusogenic only after the acrosome reaction has taken place (Arts *et al.*, 1993, 1997).

The parallels between secretory granule exocytosis and the acrosome reaction, as far as the molecular mechanisms involved are concerned, have been stressed further by the recent report identifying a homologue to SNAP-25 (a plasma membrane t-SNARE that interacts with both syntaxin and VAMP/synaptobrevin) in sea urchin sperm (Schulz *et al.*, 1998). In addition, we report here that the synaptic vesicle protein synaptotagmin I, or a very similar homologue, can also be found in mammalian sperm. Although the precise requirements and roles for this protein in synaptic secretion remain in question, most models propose that it may participate both in SNARE complex formation (possibly interacting with syntaxin) and as a calcium sensor, modulating exocytosis in response to an increase in the intracellular calcium concentration (Calakos and Scheller, 1996; Schiavo *et al.*, 1998). Since the acrosome reaction is also triggered by an increase in sperm intracellular calcium (Breitbart and Spungin, 1997; Florman *et al.*, 1998; Wassarman, 1999), it is conceivable that synaptotagmin I could also function as a calcium sensor relevant for membrane merging in this system. More recently rab3A, a small GTPase that has a regulatory role in many exocytotic fusion events, was also found in mammalian sperm (Iida *et al.*, 1999; Ward *et al.*, 1999), and some evidence was presented regarding its possible modulation of the acrosome reaction (Garde and Roldan, 1996; Iida *et al.*, 1999).

SNARE proteins should also be included in the list of sperm proteins/structures normally discarded during the initial stages of sperm–egg interactions, but that are introduced in the egg following ICSI. These include components of the acrosome and the sperm perinuclear theca (Bourgain *et al.*, 1998; Yanagimachi, 1998; Hewitson *et al.*, 1999; Sutovsky *et al.*, 1996, 1997). Strikingly, a VAMP-containing collar, probably representing the sperm equatorial segment, seems to separate decondensing DNA at the posterior end of the sperm head from the condensed anterior portion. This asynchronous decondensation of male DNA, although overcome in most cases, may be related to the reported higher rate of sex chromosome disorders in embryos and fetuses conceived using this ART (In't Veld *et al.*, 1995). Indeed, the X chromosome shows a preference for the apical portion of the sperm head (Luetjens *et al.*, 1999), which is precisely the region with delayed decondensation, and this delay could therefore be involved in inappropriate chromosomal behavior during first interphase and/or improper positioning at first mitosis. Removal of sperm components, including SNARE-containing structures, prior to ICSI should be considered by clinical researchers in order to reduce the possible risks of this very successful and widely employed ART.

Overall, our results suggest that SNARE proteins possibly similar to those involved in other exocytotic events modulate secretion during the acrosome reaction in several mammalian species, as well as in marine invertebrates, and thus could play an important role during fertilization. The differences encountered between mammals and sea urchins may prove to be relevant, and readjustments in the SNARE paradigm are anticipated (Hanson *et al.*, 1997; Gotte and von Mollard, 1998). Nonetheless, this work underscores the conservation of membrane fusion effectors/regulators throughout evolution and development/differentiation (Ferro-Novick and Jahn, 1994; Rothman, 1994).

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