The Exocytosis Regulatory Proteins Syntaxin and VAMP Are Shed from Sea Urchin Sperm during the Acrosome Reaction

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Syntaxin is a cytoplasmically oriented plasma membrane protein and VAMP (vesicle-associated membrane protein; synaptobrevin) is a protein associated with the secretory vesicle membrane. These two proteins form part of a complex which is thought to mediate the fusion of plasma and vesicle membranes during exocytosis. This paper reports the identification of syntaxin and VAMP homologues in sea urchin sperm. During fertilization, sea urchin sperm release the contents of a single vesicle, the acrosomal vesicle, exposing the membrane destined to fuse with the egg. During acrosomal exocytosis, the plasma membrane over the acrosomal vesicle fuses at multiple points with the acrosomal membrane (vesiculation) and syntaxin and VAMP are shed with the resulting membrane vesicles. Sea urchin sperm syntaxin and VAMP are associated in a complex as detected by immunoprecipitation. Following acrosomal exocytosis, syntaxin and VAMP cosediment to denser fractions on sucrose gradients showing that they have undergone associative changes during or after the acrosome reaction. Syntaxin and VAMP localization and loss during acrosomal exocytosis support a role for these proteins in regulating the acrosome reaction.

INTRODUCTION

Sperm of many animals, including sea urchins and mammals, contain a single exocytotic vesicle, the acrosomal vesicle, in close association with the plasma membrane. When sea urchin sperm encounter the outer investments of the egg, a sulfated fucan (Alves et al., 1997) in the egg jelly activates a receptor in the sperm plasma membrane (Moy et al., 1996). Occupancy of the egg jelly receptor activates ion channels (Darszon et al., 1996) which regulate exocytosis of the acrosomal vesicle (the acrosome reaction). The acrosome reaction is unique in that the plasma membrane and acrosomal vesicle membrane fuse at multiple points (Epel and Vacquier, 1978; Yanagimachi, 1988; Barros et al., 1967). These membrane fusions result in the formation of acrosome reaction vesicles (ARVs) which are shed from sperm. This event exposes the gamete recognition protein bindin (Vacquier et al., 1995) from the acrosomal vesicle and is followed by elongation of the acrosomal process (Tilney and Inoue, 1985). Acrosomal exocytosis requires influx of Ca2+ and can be induced by ionophores (Podell and Vacquier, 1984) or by addition of Ca2+ to permeabilized sperm cells (Castellano et al., 1995).

When treated with egg jelly or ionophore, an entire population of sea urchin sperm undergoes acrosomal exocytosis simultaneously, making these cells a useful model for studying Ca2+-triggered exocytosis. The molecular details underlying the signaling events from sperm receptor for egg jelly to acrosomal exocytosis remain unknown (Darszon et al., 1996; Moy et al., 1996). Similarities between sea urchin and mammalian sperm, such as the presence of voltage-dependent Ca2+ channels with the same biophysical properties, suggest that conserved regulatory mechanisms underlie the acrosome reaction (Beltran et al., 1994; Darszon et al., 1996).

Isolation and characterization of proteins on the presynaptic and synaptic vesicle membranes of neurons have increased our understanding of the regulation of exocytosis. Two of these proteins are syntaxin, an intracellular protein integral to the plasma membrane (Bennett et al., 1992,
Gametes and Preparation of Sperm Proteins

Sea urchins, Strongylocentrotus purpuratus, were spawned by intracoelomic injection of 0.5 M KCl. Isolation of sperm heads and flagella was as described (Vacquier, 1975). Isolated sperm heads were incubated at 30°C for 1 hr. Incoelomic complexes were collected with prewashed protein A–Sepharose beads (Sigma Chemical Co.; 20 μl packed beads), washed five times in SB, and resuspended in Laemmli sample buffer. Samples were immunoblotted and primary antibodies were detected with HRP-conjugated protein A (Sigma).

Sucrose Density Gradients of Solubilized Sperm Proteins

Solubilized sperm proteins were prepared as described above. All aliquots of 200 μg were layered on 7.5 to 25% sucrose gradients.

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FIG. 1. Alignment of deduced partial amino acid sequences of sea urchin (**Strongylocentrotus purpuratus**) syntaxin and VAMP homologues (**SpSYN** and **SpVAMP**) with the highest scoring non-sea urchin sequences using BLASTP 1.4.9MP (Altschul et al., 1990). Periods indicate amino acid identity between aligned sequences. Letters above the aligned **Sp** testis sequences indicate the four amino acid differences with the sea urchin (**S. purpuratus**) ovary forms (Conner et al., 1997). Underlining of the sea urchin sequences indicates regions used to generate polyclonal antibodies against syntaxin and VAMP (Conner et al., 1997). (A) Alignment of the sea urchin testis syntaxin sequence (**SpSYN**; GenBank Accession Number AF015486) to positions 21–220 of **Aplysia californica** syntaxin (**AcSYN**; GenBank Accession Number U03123). The aligned sequences are 72% identical. Also included in the alignment are positions 19–218 of **Rattus norvegicus** syntaxin 1A (**RnS1A**; Genbank Accession Number A38141) which is 71% identical to the sea urchin syntaxin sequence. The sea urchin testis partial sequence differs from the ovary sequence at four positions. (B) Alignment of the sea urchin testis VAMP (**SpVAMP**) sequence to positions 35–98 of mouse VAMP 1 (**mVAMP1**; GenBank Accession Number U61751). The aligned sequences are 89% identical. There is a single amino acid difference between the sea urchin testis and ovary sequences (F at position 50).

(4.4 ml), prepared in SB, and centrifuged at 300,000g for 20 hr at 4°C in a SW60 rotor (Beckman Instruments). Fractions of 190 µl were collected and 50 µl was analyzed by SDS–PAGE and immuno-blotting.

RESULTS

Determination of Sequences Encoding Syntaxin and VAMP Homologues in Sea Urchin Testes

Using degenerate and exact match primers, multiple overlapping PCR products were amplified from a sea urchin testis cDNA library encoding a single form of syntaxin. The partial sea urchin sequence of 200 amino acids aligns with Aplysia syntaxin (**AcSYN**) positions 21–220 (full length = 289 residues); where the two sequences are aligned they are 72% identical (Fig. 1A), confirming that the two are homologues. For comparison, sea urchin syntaxin and rat syntaxin 1A (**RnS1A**) are 71% identical over this region (Fig. 1A), indicating a high degree of sequence conservation. The 200 residues of the sea urchin testis syntaxin are 98% identical to the syntaxin homologue expressed in the ovary of this species of sea urchin (Conner et al., 1997). The differences between ovary and testis forms are localized to a region of high variability between AcSYN residues 93 and 115 (Fig. 1A). In addition to these amino acid differences, there are only two silent substitutions between the aligned sea urchin syntaxin testis and ovary DNA sequences, suggesting that these testis and ovary syntaxins are allelic and not different protein isoforms.

Using degenerate primers, a single PCR product was obtained from a testis cDNA library encoding 64 residues of the sea urchin VAMP homologue, which aligns to residues 35–98 (full length = 118 residues) of mouse VAMP 1 (Fig. 1B). Where they align the two VAMP sequences are 89% identical. The sea urchin testis VAMP homologue differs from the recently identified sea urchin ovary homologue (Conner et al., 1997) at only one position (F at position 50), suggesting that the same VAMP isoform is expressed in both testis and ovary.

Identification of Syntaxin in Sea Urchin Sperm

Using antibodies generated against the underlined sequence shown in Fig. 1A (Conner et al., 1997), syntaxin was identified in sperm which comigrated with syntaxin of sea urchin egg cortical granule lawns (Fig. 2, lanes 1 and 2). To determine if syntaxin is selectively localized, sperm were...
Exocytosis of the Sperm and Release during Acrosomal Localization of Syntaxin to the Acrosomal Region

Inhibitors of trypsin and other eukaryotic proteases did not block the appearance of this syntaxin cleavage product. With the knowledge of the association of syntaxin and VAMP in neurons (Calakos et al., 1994), we were interested in determining if the two proteins were associated in sperm. Immunoprecipitation of detergent-solubilized sperm proteins with syntaxin antibodies coprecipitated VAMP in unreacted sperm as shown by an immunoblot of the syntaxin immunoprecipitate with anti-VAMP serum (Fig. 6, lane 5). Similarly, syntaxin is present in VAMP immunoprecipitates as detected on anti-syntaxin immunoblots (Fig. 6, lane 5).
FIG. 3. Indirect immunofluorescence localizes syntaxin exclusively to the acrosomal region of the sperm (A). Micrographs at the top are rhodamine (TRITC) fluorescence of the secondary antibody (Fl). Micrographs at the bottom are the corresponding phase-contrast images (Ph). Schematic representation of a sea urchin sperm cell indicates the positions of the flagellum (F), midpiece (M), nucleus (N), and acrosomal vesicle (AV). Syntaxin localization is lost from acrosome reacted sperm (B). Controls using normal rabbit serum, the secondary antibody alone, or nonpermeabilized sperm are also devoid of the immunofluorescence signal. Bar, 2 μm.

8). The lack of this complex in acrosome-reacted sperm (Fig. 6, lane 4) and the absence of VAMP in VAMP immunoprecipitates of reacted sperm (Fig. 6, lane 3) support the loss of both proteins during acrosomal exocytosis. A normal rabbit serum control did not react with solubilized sperm proteins (Fig. 6, lane 1). In lane 6, a similar normal rabbit serum control precipitated a minor reacting band on anti-syntaxin immunoblots. When compared to lanes 7 and 8, this cross-reactivity is minor. Lane 2 shows that antibodies to VAMP immunoprecipitate VAMP from unreacted sperm.

To investigate the association of VAMP and syntaxin, detergent-solubilized sperm proteins were separated by sucrose gradient velocity sedimentation. Syntaxin and VAMP had overlapping but nonidentical sedimentation patterns (Fig. 7, a and b) with estimated sedimentation coefficients of 2.1S and 0.7S, respectively. Boiling the sample with SDS in SB resulted in shifts of both syntaxin and VAMP to fractions of lower density (Fig. 7, c and d). Enough ARV material was obtained from acrosome-reacted sperm to fractionate the detergent-solubilized membrane proteins on sucrose gradients. In contrast to the sedimentation pattern from unreacted sperm (Fig. 7, a and b), syntaxin and VAMP from ARVs had overlapping patterns of sedimentation and sedimented farther than the unreacted samples with an estimated sedimentation coefficient of 6.2 S (Fig. 7, e and f).

Syntaxin and VAMP cosedimentation was labile to boiling immunoblots. When compared to lanes 7 and 8, this cross-reactivity is minor. Lane 2 shows that antibodies to VAMP with SDS in SB resulted in patterns of sedimentation identical to those in Fig. 7, c and d (data not shown). The different patterns of syntaxin and VAMP sedimentation before and after the acrosome reaction indicate that larger complexes of these proteins have formed.

**DISCUSSION**

In this report, we have identified syntaxin and VAMP homologues in sea urchin sperm. These are the first protein...
Syntaxin and VAMP Are Shed from Sperm

FIG. 4. Syntaxin is shed with membrane vesicles (ARVs) during acrosomal exocytosis. The 10,000g supernatants from acrosome-reacted sperm and mock-reacted sperm were pelleted at 180,000g to collect the ARVs. Equal volumes of the resuspended ARVs from the acrosome-reacted (lane 1) and mock-reacted control (lane 2) were immunoblotted using the syntaxin antiserum along with 12 µg of solubilized protein from the mock-reacted (lane 3) and acrosome-reacted (lane 4) sperm. Asterisk indicates apparent syntaxin degradation product (34 kDa) in the ARVs (lane 1).

Components of the vesicle fusion machinery to be identified in animal sperm. Immunoblotting demonstrates that both proteins are present in the sperm head, but are absent from the flagellum. Immunofluorescence localizes syntaxin to the acrosomal region, presumably to the plasma membrane covering the acrosomal vesicle. Immunoprecipitation with syntaxin and VAMP specific antibodies shows that the two are associated in a complex. Membrane vesiculation during the acrosome reaction releases both proteins in shed ARVs.

ARVs were pelleted by ultracentrifugation and analyzed by immunoblotting and sucrose density gradient sedimentation. The qualitative absence of both proteins on immunoblots of adequately loaded gel lanes (Figs. 4 and 5) and from immunoprecipitates (Fig. 6) supports the claim that most, if not all, of both proteins are released from acrosome-reacted sperm. These data support the involvement of both proteins in the acrosome reaction. The release of both of these exocytosis regulatory proteins from acrosome-reacted sea urchin sperm is similar to the shedding of the IP3 receptor with the outer acrosomal membrane of mammalian sperm during the acrosome reaction (Walensky and Snyder, 1995). This receptor potentially regulates Ca2+ which triggers acrosomal exocytosis.

Sucrose gradient analysis of the ARV proteins solubilized by nonionic detergents showed that syntaxin and VAMP increased in complex size following the acrosome reaction. It is not known if the increases in the size of the complexes are significant to the exocytotic process or only a consequence. In either case, identification of the mechanism of complex formation of syntaxin and VAMP in ARVs, as well as identification and characterization of the other proteins in ARVs, is important for future studies of the mechanism of the acrosome reaction and exocytosis in general. The demonstration that sea urchin sperm acrosomes and egg cortical granules (Conner et al., 1997) contain the same forms of syntaxin and VAMP (Figs. 1, 2, and 4) argues for the same exocytotic mechanism being operative in both types of gametes. The loss of these two proteins from sperm does not support a role for them in mediating membrane

FIG. 5. VAMP (17 kDa; synaptobrevin) also localizes to the sea urchin sperm head and is shed from sperm during the acrosome reaction. Sperm fractionated into heads (lane 2) and flagella (lane 3) as well as mock-reacted (lane 4) and acrosome-reacted (lane 5) sperm were immunoblotted using antisera to sea urchin VAMP along with cortical granule lawn protein as a control (lane 1). Each lane contains 10 µg of detergent-solubilized protein. As in Fig. 4, the 10,000g supernatants from acrosome-reacted sperm and mock-reacted sperm were pelleted at 180,000g to collect the ARVs. Equal volumes of the resuspended ARVs from reacted (lane 6) and mock-reacted control (lane 7) sperm were immunoblotted with the VAMP antiserum.

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ARVs may be an important material for obtaining post-acrosome-reacted SNAP-25.

In animal sperm, the acrosome reaction involves the influx of Ca²⁺ and Na⁺, efflux of H⁺ and K⁺, and depolarization of the plasma membrane, the culmination being the Ca²⁺ triggering of acrosomal vesicle exocytosis (Arnoult et al., 1996; Espinosa and Darszon, 1995; Florman, 1994; Florman et al., 1992; Gonzalez-Martinez et al., 1992). Recent evidence has linked syntaxin, SNAP-25, and VAMP with the influx of Ca²⁺-triggered synaptic vesicle exocytosis at the presynaptic membrane (Martin et al., 1996). In neurons, syntaxin binds directly to a voltage-dependent Ca²⁺ channel in the presynaptic membrane thereby directly linking regulation on fluxes to the fusion machinery (Martin et al., 1996; Sheng et al., 1994, 1996). Sperm syntaxin could associate with an acrosome-specific voltage-dependent Ca²⁺ channel during the final steps preceding vesicle exocytosis. This association would provide a link between the upstream transmembrane signaling events and acrosomal vesicle exocytosis. Such a Ca²⁺ channel protein might be found in the shed ARVs. Further study of the exocytosis regulatory proteins before and after the acrosome reaction will lead to a deeper understanding of this developmentally important event necessary for animal fertilization.

FIG. 7. Syntaxin and VAMP sediment as larger complexes following acrosomal exocytosis. Detergent-solubilized proteins of unreacted sperm (U: a–d) or shed ARVs (ARV: e, f) were resolved on 7.5–25% sucrose by ultracentrifugation. Gradients were run in the presence (c,d) or absence (a,b,e,f) of 0.1% SDS. Collected fractions were immunoblotted using anti-syntaxin (S: a,b,e) and anti-VAMP (V: b,d,f) sera. In unreacted sperm, syntaxin (a) and VAMP (b) sediment with an estimated sedimentation coefficient of 2.1S and 0.7S, respectively. When the samples are boiled and run in the presence of 0.1% SDS, both syntaxin (c) and VAMP (d) remain in lighter fractions. Following acrosomal exocytosis and collection of the ARVs, syntaxin and VAMP cosedimented to heavier fractions (e,f) with peak amounts having an estimated sedimentation coefficient of 6.2S. Sedimentation coefficients were estimated from the sedimentation patterns of standard proteins and samples on both 7.5–25% and 5–55% sucrose gradients (not shown).

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