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Isolation of a calcium-binding protein of the acrosomal membrane of bovine spermatozoa

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Abstract

The mammalian sperm acrosome reaction is a calcium-dependent exocytotic event characterized by extensive fusion between the plasma and the outer acrosomal membrane. The mechanisms by which elevation of cytosolic calcium initiates the membrane fusion process are not understood and the present study was undertaken to identify calcium-binding proteins in the acrosomal membrane (AM) of bovine spermatozoa. Sperm heads, purified from sonicated spermatozoa, were used to isolate an acrosomal membrane-enriched fraction on Percoll density gradients. Using SDS-PAGE and a ⁴⁵Ca²⁺-blot overlay assay, calcium-binding proteins of 64, 45, 43, and 39 kDa were identified in the AM enriched fraction. Phase separation analysis with Triton X-114 identified the 64 kDa polypeptide as an integral membrane protein. The 64 kDa polypeptide was purified and utilized to prepare a polyclonal antiserum. Both light and electron microscopic immunocytochemistry demonstrated that the protein was distributed throughout all domains of the acrosomal membrane. These results identify a 64 kDa calcium-binding integral membrane protein of the mammalian acrosome. Its potential function in calcium-dependent membrane fusion events of the acrosome reaction and in fertilization is discussed.

Keywords

Bovine spermatozoa; Acrosome; Acrosomal calcium-binding protein; Acrosomal membrane

1. Introduction

The mammalian sperm acrosome reaction involves the vesiculation and the fusion of the outer acrosomal membrane (OAM) with the overlying plasma membrane and the subsequent release of the acrosomal contents (Barros et al., 1967; Russell et al., 1979; Kopf and Gerton, 1991; Yanagimachi, 1993). The acrosome reaction requires extracellular calcium (Yanagimachi and Usui, 1974; Yanagimachi, 1982; Thomas and Meizel, 1988; Kopf and Gerton, 1991) and in capacitated spermatozoa the acrosome reaction is initiated by sperm attachment to the zonapellucida (Wassarman, 1987; Kopf and Gerton, 1991; Wassarman, 1995) or by exposure to progesterone (Melendrez et al., 1994; Roldan and Murase, 1994;

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O'Toole et al., 1996). Several studies have provided insights into the biochemical mechanisms which regulate calcium influx and cytosolic calcium levels in spermatozoa. Both contact with zona pellucida and/or exposure to progesterone promotes an elevation of spermatozoan cytosolic calcium (Lee and Storey, 1985; Florman et al., 1989, 1992; Bailey and Storey, 1994; Arnoult et al., 1996; Meizel et al., 1997). Calcium channels located in the spermatozoan plasma membrane are activated in response to the zona pellucida (Florman et al., 1992; Fraser, 1993; Arnoult et al., 1996), but conflicting data has been obtained for a role of these channels in the progesterone-stimulated acrosome reaction (Shi and Roldan, 1995; Aitken et al., 1996; Meizel et al., 1997). It has been proposed that sperm-zona interaction promotes an influx of Na^+ and that Na^+-H^+ exchange elevates intracellular pH which in turn is responsible for activating a plasma membrane calcium channel (Fraser, 1993). In addition both the ZP-dependent acrosome reaction and the elevation of cytosolic calcium are inhibited by a voltage-sensitive channel (Florman et al., 1992; Fraser, 1993; Florman, 1994). G-proteins, specifically Gi1 and/or Gi2 , also have been implicated in signaling pathways leading to calcium elevations as pertussis toxin (PTx) inhibits the ZP3-induced cytosolic changes in pH and calcium levels (Endo et al., 1988; Florman et al., 1989). It has been shown that both solubilized ZPs and purified ZP3 depolarize mouse and bovine sperm membrane potential by activating a pertussis toxin (PTx)-insensitive pathway (Arnoult et al., 1996). The same investigators demonstrated that ZP3 also activates a PTx-sensitive pathway which leads to increased cytosolic pH and calcium levels followed by the acrosome reaction, thereby demonstrating the participation of two distinct calcium channels located in the spermatozoan plasma membrane (Arnoult et al., 1996). Brenker et al. (2012) showed that the human sperm CatSper channel is directly activated by progesterone and prostaglandins female factors that stimulate calcium influx.

The mechanism of calcium action on the acrosome is less well understood. Several ultrastructural studies have demonstrated calcium-binding sites on the outer acrosomal membrane (Roomans, 1975; Berruti et al., 1986; Watson and Plummer, 1986; Watson et al., 1995) but their composition and function remain to be identified. It was shown that thapsigargin, which acts specifically on the intercellular calcium release system, stimulates the acrosome reaction (Casey, 1995; Walensky and Snyder, 1995), and that inositol 1,4,5-trisphosphate (IP3) promotes calcium release from digitonin-permeabilized rat sperm (Walensky and Snyder, 1995), suggesting that modulation of intracellular calcium stores also play a role in acrosomal exocytosis. Ultrastructurally the IP3 receptors were localized to the dorsal region of the acrosome (Walensky and Snyder, 1995), consistent with a function in the acrosome reaction. Also, calcium has been demonstrated to promote dispersion of the acrosomal matrix (Noland et al., 1989); in guinea pig spermatozoa a calcium-binding matrix protein has been identified and characterized at the molecular level (Noland et al., 1994). Sukardi et al. (2001) demonstrated that 33 and 39 kDa polypeptides of the acrosomal membrane fraction of ram ejaculated spermatozoa bind $^{45}\text{Ca}^{2+}$. Internal amino acid sequence data of a 39 kDa polypeptide obtained after cyanogen bromide cleavage showed 68% homology with SP-10 protein precursor and 64–72% homology with various annexins. However, the mechanism by which calcium promotes vesiculation and/or fusion of the outer acrosomal membrane during the acrosome reaction remains unresolved.

In Ca^{2+} -dependent exocytosis of somatic cells, calcium-binding proteins of the secretory vesicle membrane play central roles in the regulation of membrane fusion (Burgoyne and Morgan, 1995). We hypothesized that calcium-binding proteins located in the acrosomal membrane may function in an analogous manner in the calcium-dependent membrane fusion events of the acrosome reaction. In the present study we present the isolation, characterization and localization of a 64-kDa calcium-binding, integral membrane protein of the acrosomal membrane of bovine spermatozoa.

2. Methods

2.1. Sperm fractionation

Bovine epididymides were purchased from Martin's Abattoir in Godwin, North Carolina. Epididymides were stored at 4°C during transit and utilized for sperm preparation within 1 h of retrieval. To facilitate sperm release the cauda epididymal region was removed from the organ, minced, and incubated for 5 min at 37 °C in Hank's balanced saline solution, pH 7.4, containing 5 mM HEPES, 2 mM benzamidine, and 0.05% sodium azide. To evaluate cellular viability, sperm were examined by phase-contrast microscopy. Sperm suspensions were centrifuged at $100 \times g$ for 1 min to sediment epididymal tubule fragments. Supernatants were centrifuged at $1500 \times g$ for 10min at 4°C using an Eppendorf Centrifuge 5403 (Brinkman Instruments, Inc, Westbury, New York). Pellets were washed 3 times by resuspension in Hank's balanced saline solution, as stated above, following which they were resuspended in a Tris-saline-protease inhibitor solution (TNI) containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 2 mM benzamidine, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 0.05% sodium azide and centrifuged at $1500 \times g$ for 10min at 4°C. Resulting pellets were washed two more times in TNI as stated above.

Sperm heads possessing an associated acrosomal membrane were isolated as described by NagDas et al. (1996). Sperm were suspended in 35 mL of TNI, sonicated four times for 10 s at the medium power setting and examined by phase contrast microscopy to insure that >90% of the sperm exhibited head-tail separation. The suspension was centrifuged at $1000 \times g$ for 10 min and the pellet was then washed three times by resuspension in TNI followed by centrifugation as above. The sonicated sperm pellet was resuspended with 60 mL TNI and 10 mL aliquots were layered on discontinuous sucrose gradient composed of 10mL 55% sucrose, 5 mL 70% sucrose and 5 mL 75% sucrose; all sucrose solutions contained 25 mM Tris-HCl, pH 7.5. The gradients were centrifuged at $60,000 \times g$ for 60 min in a Beckman SW28 rotor. The pellet fraction, containing the sperm heads was collected and washed once in TNI.

Mature male rats and mice were housed in the Benedict College, South Carolina, animal care facility. Animals were asphyxiated with CO₂ and the cauda epididymides were collected. Mouse and rat sperm heads were isolated by sucrose density gradient centrifugation following the bovine protocol as described above in the laboratory facility of Benedict College.

An acrosomal membrane enriched fraction was isolated by slight modification of protocol previously utilized to isolate a stable acrosomal matrix fraction (NagDas et al., 1996). The sperm heads were resuspended in 10 mL of 25 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 2 mM benzamidine, 1 µg/mL leupeptin, 1 µg/mLpepstatin and 0.05% sodium azide and extracted overnight with constant agitation. The suspension was then homogenized with 30–40 strokes of a glass-Teflon homogenizer to release membranes associated with the sperm heads (Zahler and Doak, 1975; Parks et al., 1987). The homogenate was mixed with 40 mL of 50% percoll, 0.25 M sucrose and 0.05 M Tris-HCl, pH 7.5, and then centrifuged at $65,000 \times g$ for 30min in a Beckman 70Ti rotor. The acrosomal membrane containing fraction banded near the top of the gradient; the band was collected and diluted with TNI and pelleted by centrifugation at $100,000 \times g$ for 60 min in a Beckman SW40 rotor. Protein was estimated by the procedure of Bradford (1976).

2.2. Phase separation analysis with Triton X-114

The acrosomal membrane containing fraction, at a protein concentration of 0.5–0.7 mg/mL, was extracted in 1% Triton X-114, 25 mM Tris-HCl, pH 7.5, 0.6 M KCl, 2 mM benzamidine, 1 mM DTT for 1 h at 4°C and then centrifuged at $10,000 \times g$ for 10min to

pellet detergent insoluble material. The supernatant fluid, containing detergent soluble proteins, was warmed for 3 min at 37°C to induce clouding and centrifuged at $1500 \times g$ for 5 min to obtain a detergent pellet, containing integral membrane proteins, and the aqueous supernatant, containing hydrophilic proteins (Bordier, 1981). Fractions were dialyzed against 25 mM Tris-HCl, pH 7.5, 150mM NaCl, 2 mM benzamidine and used for SDS-PAGE (Laemmli, 1970).

2.3. ^{45}Ca overlay assay

A ^{45}Ca overlay assay was performed following the method of Maruyama et al. (1984). Samples were separated by non-reducing SDS-PAGE (Laemmli, 1970) and transferred onto a PVDF membrane (Towbin and Gordon, 1984). The blots were washed four times for 30 min each in overlay buffer of 10 mM HEPES, pH 6.8, 60 mM KCl and 5 mM MgCl_2 and then incubated for 10 min at room temperature in overlay buffer containing 1 $\mu\text{Ci}/\text{mL}$ $^{45}\text{CaCl}_2$. The blots were rinsed in 50% ethanol for 5 min, dried overnight and exposed to KODAK XAR-5 film for 2–5 days at -70°C .

2.4. Protein purification and antibody preparation

Isolated sperm heads were resuspended into 10 mL TNI containing 0.1% Triton X-100, extracted for 1 h at 4°C, and centrifuged at $40,000 \times g$ for 30 min in a Beckman 70Ti rotor. The supernatant was dialyzed against TED buffer (20 mM Tris-HCl, pH 7.5, 2 mM EGTA, 4 mM PMSF, 0.5 $\mu\text{g}/\text{mL}$ leupeptin) overnight at 4 °C and then centrifuged at $40,000 \times g$ for 30 min in a Beckman 70Ti rotor. The supernatant was applied to a 1.6×5.0 cm DEAE-Sephadex column equilibrated with TED Buffer. The column was washed extensively with TED buffer and bound protein was eluted with a step gradient of 0.1 M, 0.2 M, and 0.4 M KCl in TED buffer. Fractions containing the 64 kDa calcium-binding protein were pooled, dialyzed against 10mM Tris-HCl, pH 7.5, containing 2mM benzamidine, passed through an Extracti-gel D column (Pierce, Rockford, IL) to remove detergent and then lyophilized. The 64 kDa polypeptide was subjected to final purification by preparative SDS-PAGE and visualized by CuCl_2 staining (Lee et al., 1987).

Rabbits were housed in the Benedict College Animal Care Facility under the supervision of University Veterinarians of Benedict College, SC. Care and use of animals conformed to NIH guidelines for humane animal care and use in research, and all protocols were approved by the institutional animal care committee. Animals received a primary injection of purified protein emulsified in Freund's adjuvant followed by two booster injections at 2-week intervals. Animals were anesthetized with Nembutal and blood was collected by cardiac puncture. Specific antibodies were purified by blot-affinity purification against the 64 kDa antigen immobilized on PVDF membranes (Talian et al., 1983; Olmsted, 1986).

2.5. Gel electrophoresis and Western blot analysis

SDS-PAGE was performed on 12.5% acrylamide gels (Laemmli, 1970). Polypeptide bands were visualized either by Coomassie blue (Fairbanks et al., 1971) or silver (Wray et al., 1981) staining. Western blots were prepared on PVDF membranes (Towbin et al., 1979). Two-dimensional PAGE was performed using a Bio-Rad precast immobilized pH (3–10) gradient gel ready for isoelectric focusing (IEF). IEF was done in a Bio-Rad Protean IEF cell following the conditions described in the Bio-Rad manual.

Immunoblots were washed with PBS (150 mM NaCl and 20 mM sodium phosphate buffer, pH 7.6) and then blocked by overnight incubation at 4°C in PBS containing 0.1% Tween-20, 2.5% BSA and 5% heat inactivated normal goat serum (NGS). Blots were incubated with blot affinity purified antibody (either immune or pre-immune serum) diluted in PBS containing 0.1% Tween and 1% NGS (PBS-NGS). After three washes in PBS-NGS, the

blots were incubated in an affinity-purified peroxidase-conjugated goat anti-rabbit IgG secondary antibody (KPL Inc., Gaithersburg, MD) diluted in PBS-NGS; following several PBS washes immunoreactive bands were visualized either by color development with diaminobenzidine and H_2O_2 or by chemiluminescence using SuperSignal (Pierce) and detection on Kodak BioMax film.

2.6. Immunocytochemistry

For immunofluorescence microscopy intact and sonicated sperm fractions, prepared as described above, were fixed at 4°C in 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.6, for at least 30 min, attached to poly-L-lysine coated coverslips, washed with PBS, and permeabilized by incubation for 10 min in -20 °C acetone. After three rinses in PBS, nonspecific protein-binding sites were blocked in PBS containing 0.1% Tween-20, 2.5% BSA and 5% heat inactivated normal goat serum (NGS) as described above. Coverslips were then incubated with equal dilutions of preimmune or immune serum in PBS-NGS, washed and incubated with FITC-conjugated goat anti-rabbit IgG (KPL Inc., Gaithersburg, MD) in PBS-NGS. Coverslips were washed with PBS and the cells were examined by phase contrast and epifluorescence microscopy.

Both pre- and post-embedding immunolabeling were utilized for ultrastructural localization of the 64 kDa polypeptide. For pre-embedding labeling, 1–2 mL aliquots of spermatozoa were sonicated at maximum power with a microtip probe to maximize disruption and to provide antibody access to internal sperm structures. Spermatozoa were sequentially incubated in blocking solution, immune or preimmune serum diluted in PBS-NGS and finally in 10 nm gold conjugated affinity purified goat anti-rabbit IgG (Amersham Inc., Arlington Heights, IL) diluted in PBS-NGS. Sperm were centrifuged for 1 min in a microcentrifuge tube and the pellets washed three times in PBS-NGS between all incubation steps. The final pellets were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed with 2% OsO_4 in cacodylate buffer, then dehydrated and embedded in epon (Olson and Winfrey, 1985). Thin sections were stained with uranyl acetate and lead citrate. For post-embedding labeling both intact and sonicated spermatozoa were fixed with 4% formaldehyde, 0.25% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated through an ethanol series and embedded in LR white resin. Sections were immunostained as described for immunofluorescence except that the secondary antibody was conjugated to 10 nm gold.

3. Results

3.1. Identification and characterization of calcium-binding proteins in a sperm head membrane fraction

We previously demonstrated that sperm heads purified from sonicated spermatozoa retained an associated acrosomal membrane but lacked the plasma membrane (NagDas et al., 1996). Purified sperm heads (Fig. 1A, panel b) were homogenized and centrifuged on Percoll gradients to obtain an acrosomal membrane enriched fraction for identification of calcium-binding proteins. SDS-PAGE of the membrane fraction revealed an array of polypeptides (Fig. 1B). A $^{45}Ca^{2+}$ -overlay assay was performed to identify calcium-binding polypeptides in the membrane fractions and phase separation analysis with Triton X-114 was utilized to identify integral membrane proteins with calcium-binding activity. The total membrane fraction was first solubilized at 4 °C in 1% Triton X-114 containing 0.6 M KCl and then centrifuged to obtain a detergent soluble fraction. The detergent soluble fraction contained calcium-binding polypeptides of 64, 45, 42 and 30 kDa (Fig. 2, lane 1). Phase separation analysis demonstrated that the 64 kDa polypeptide is fractionated to the detergent phase (Fig. 2, lane 2) and was not seen in the aqueous phase (Fig. 2, lane 3). The major polypeptides of 45 kDa and 42 kDa were present in both detergent and aqueous phases. This

experiment indicates that 64 kDa calcium-binding polypeptide is an integral membrane protein.

3.2. Purification and characterization of the 64 kDa protein

The 64 kDa calcium-binding protein was purified from the Triton X-100 soluble fraction of sperm heads. The detergent-soluble fraction exhibited a complex polypeptide pattern by SDS-PAGE (Fig. 3, lane 1) and was fractionated by ion-exchange chromatography on DEAE-Sephadex. The 0.4 M KCl-eluted fraction contained the 64 kDa protein and polypeptides in this fraction were separated by preparative SDS-PAGE. Following staining with CuCl_2 , the 64 kDa polypeptide band was excised from the gels and electroeluted; the purified material showed a single band of 64 kDa by SDS-PAGE (Fig. 3, lane 2).

To demonstrate the binding efficacy of 64 kDa polypeptide to calcium, the variable amount of isolated 64 kDa polypeptide (0.25 μg -2 μg protein) was analyzed by a $^{45}\text{Ca}^{2+}$ -overlay assay at a 1 $\mu\text{Ci/mL}$ $^{45}\text{CaCl}_2$ in the overlay buffer (Fig. 4). The purified 64 kDa polypeptide specifically bound calcium in a dose-dependent fashion as evidenced by the increase in intensity of the calcium-binding band. The calcium binding reaches the saturation point at a 1 μg 64 kDa polypeptide and the intensity remains the same at a 2 μg 64 kDa polypeptide, as was observed at a 1 μg 64 kDa polypeptide.

3.3. Characterization of anti-64 kDa calcium-binding protein

A polyclonal antiserum against the 64 kDa polypeptide was prepared for use in Western blotting and immunocytochemistry. The affinity purified antibody recognized a 64 kDa polypeptide on Western blots of the Triton X-100 soluble sperm head fraction (Fig. 5A, lane 1). Identical blot lanes stained with preimmune serum displayed no immunoreactive bands (Fig. 5A, lane 2). Immunoblots of total sperm head lysate analyzed by two-dimensional PAGE showed the presence of one isoform of the 64 kDa calcium-binding protein exhibiting $pI \sim 6.5$ (Fig. 5B). No spot was seen when an identical blot was stained with preimmune rabbit IgG (data not shown). To reevaluate the phase separation analysis studies, an acrosomal membrane enriched fraction was extracted in high salt solution 1 M (NaCl) for 1 hat 4°C and then centrifuged at $100,000 \times g$ for 10 min to obtain high salt solubilized and insolubilized fractions. Western blots of high salt soluble and insoluble fractions stained with anti-64 kDa immune serum exhibited the presence of 64 kDa band in the high salt insoluble fraction (data not shown). This experiment reconfirms that the 64 kDa calcium-binding polypeptide is an integral membrane protein.

Western blots of rat (Fig. 6, lanes 1 and 3) and mouse (Fig. 6, lanes 2 and 4) Triton X-100 soluble sperm head fractions stained with a blot affinity purified 64 kDa bovine calcium-binding polypeptide immune serum exhibited the presence of a 64 kDa polypeptide both in the rat (lane 1) and mouse (lane 2) head lysates. Identical blot lanes stained with preimmune serum displayed no immune reactive bands (lanes 3 and 4).

3.4. Immunolocalization of the 64 kDa calcium-binding protein

Cauda epididymal spermatozoa which were fixed, permeabilized and immunostained with antiserum to the 64 kDa polypeptide exhibited intense fluorescence of the entire acrosomal segment and of a narrow circumferential band at the base of the head (Fig. 7a and b); no staining was noted over the anterior portion of the postacrosomal segment or over the flagellum. Sperm immunostained with identical dilutions of preimmune serum displayed no fluorescence (Fig. 7c and d). The isolated sperm heads prepared from sonicated spermatozoa exhibited the same immunostaining pattern as noted in the permeabilized intact spermatozoa, with staining of both the acrosomal segment and the narrow band at the base of the head (Fig. 7e and f).

Immunoelectron microscopy was utilized to identify the specific membranes possessing the 64 kDa calcium-binding protein. Intact spermatozoa immunolabeled with antibody to the 64 kDa polypeptide by the post-embedding protocol exhibited specific labeling of the acrosomal segment. Gold particles were present on the outer and the inner acrosomal membrane and no labeling of the acrosomal matrix was noted (Fig. 8a); parallel sections incubated with preimmune serum exhibited no specific binding of gold particles (Fig. 8b). Some sperm suspensions were sonicated, using maximum power with a microtip probe, prior to preparation for post-embedding immunolabeling. Frequently the outer acrosomal membrane, as well as the acrosomal matrix, was released from the head, but the inner acrosomal membrane and both the outer and inner acrosomal membrane of the equatorial segment remained associated with the underlying nucleus. Labeling of the exposed inner acrosomal membrane and of both the outer and inner acrosomal membrane of the equatorial segment was noted with the immune serum. No label was noted in the postacrosomal segment (Fig. 8c). Sonicated sperm samples were also prepared for pre-embedding labeling. As with the post-embedding protocol specific labeling of both the outer and inner acrosomal membrane was noted with the immune serum (Fig. 8d and e).

4. Discussion

During the fertilization process spermatozoa require extracellular calcium for the development of hyperactive motility, to undergo the acrosome reaction and to fuse with the egg plasma membrane (Yanagimachi, 1982, 1993). Moreover, different levels of cytosolic calcium have been demonstrated in activated spermatozoa, in hyperactivated spermatozoa and in acrosome-reacted spermatozoa supporting a regulatory role for intracellular calcium in these processes (Suarez and Dai, 1995). An early event of both the zonapellucida- and progesterone-promoted acrosome reaction is an elevation of spermatozoan cytosolic calcium (Lee and Storey, 1985; Florman et al., 1989, 1992; Bailey and Storey, 1994; Aitken et al., 1996; Arnoult et al., 1996; Meizel, 1997). Both voltage-sensitive and voltage-insensitive calcium channels of the spermatozoa plasma membrane participate in the ZP-dependent elevation of sperm cytosolic calcium (Arnoult et al., 1996) but the specific channels functioning in the progesterone-mediated acrosome reaction are unresolved (Aitken et al., 1996; Meizel, 1997; Meizel et al., 1997). In addition to an influx of extracellular calcium, several studies have also demonstrated that the release of intracellular calcium stores may contribute to the acrosome reaction (Meizel and Turner, 1993; Walensky and Snyder, 1995) and IP₃ receptors, localized to the dorsal acrosome in rat sperm, appear to function in the release of acrosomal calcium stores (Walensky and Snyder, 1995; Bernabo et al., 2012). Recently it has been shown the presence of transient receptor potential vanilloid 1 (TRPV1) channel plays a key role in capacitation. TRPV1 allows a rapid increase in intracellular calcium concentration that will interrupt the F-actin network linked between the plasma membrane and the outer acrosomal membrane, leading to their fusion and finally to the acrosome reaction (Bernabo et al., 2012). Despite the considerable progress in understanding the molecular regulation of sperm cytosolic calcium levels, the mechanism by which elevated cytosolic calcium promotes the onset of fusion between the peracrosomal plasma membrane and the outer acrosomal membrane is not well understood. This is in contrast to somatic cells where calcium-binding proteins associated with the secretory vesicle membrane have been demonstrated to play critical functions, in calcium-dependent exocytosis (Burgoyne and Morgan, 1995; Sudof, 1995; Mehta et al., 1996). For example, synaptotagmin is an integral membrane protein of synaptic vesicles and is a high affinity calcium-binding protein proposed to function as calcium sensor with a major role in the regulation of fusion between the synaptic vesicle membrane and presynaptic membrane (Brose et al., 1992; Sudof, 1995). Several members of the synaptotagmin family have been identified in various tissues and they contain a calcium-binding motif termed the C₂ domain which is present in many proteins participating in signal transduction or membrane

trafficking (Shao et al., 1996). Hutt et al. (2002) demonstrated the presence of synaptotagmin VIII to the acrosomal crescent and the loss of synaptotagmin VIII occurs during the acrosome reaction, suggesting that synaptotagmin VIII is a candidate for the calcium sensor that regulates acrosomal exocytosis in mammalian sperm.

Previous ultrastructural studies on several mammalian species have demonstrated calcium-binding sites on the spermatozoan outer acrosomal membrane (Roomans, 1975; Berruti et al., 1986; Watson and Plummer, 1986; Watson et al., 1995); however the biochemical nature of Ca^{2+} -binding sites were not determined. In the present study, we have identified a 64 kDa calcium-binding, integral membrane protein which is distributed throughout all domains of the acrosomal membrane of bovine spermatozoa suggesting the multiple functions in acrosomal exocytosis. Other investigators have identified sperm polypeptides with relationships to neurotransmitter proteins of the nervous system (Meizel et al., 1997). Also a specific antagonist of the muscarinic class of acetylcholine receptor, 3-quinuclidinyl benzilate (QNB) inhibits in vitro fertilization of mouse eggs; although it does not affect sperm binding to the zona pellucida. QNB blocks the zona-induced calcium influx and acrosome reaction (Bailey and Storey, 1994; Ward et al., 1994a). A candidate QNB-binding polypeptide of 72 kDa has been identified in a mouse sperm membrane fraction, and is proposed to represent a plasma membrane polypeptide (Ward et al., 1994b) although its specific localization has yet to be defined. The acrosome reaction is initiated by several biochemical events, including tyrosine kinases and phosphatases, and fusion-related proteins such as Rab3A, α -SNAP, N-ethylmaleimide-sensitive factor (NSF), SNAREs, complexin and synaptotagmin VI (Zarelli et al., 2009). Bennett et al. (2012) reported that synaptotagmin VI phosphorylated at the polybasic region of the C2B domain and showed that a calcium-dependent dephosphorylation of synaptotagmin VI occurred at early stages of the acrosomal exocytosis. Other investigators showed the role of protein tyrosine phosphatase 1B in the human sperm exocytosis; this phosphatase dephosphorylates NSF to disassemble SNARE complexes (Zarelli et al., 2009). The functional relationship of the 64 kDa acrosomal membrane calcium-binding protein identified in the present study to calcium-binding proteins of the secretory vesicle should be clarified by future studies to obtain its full length sequence.

Presently we do not know if the 64 kDa calcium-binding protein functions in acrosomal calcium transport or instead represents a protein whose functional activity is regulated by calcium. Its distribution throughout all domains of the acrosomal membrane suggests several potential functions during fertilization. It is possible that it represents a component of the membrane fusion machinery of the outer acrosomal membrane and may be regulated by the ZP- or progesterone-induced elevation of cytosolic calcium. Alternatively, in the equatorial segment and/or in the inner acrosomal membrane of the principal segment it could function in calcium-regulated events related to the initial binding or fusion of the sperm and egg plasma membranes. These possibilities will be addressed in future studies.

References

- Aitken RJ, Buckingham DW, Irvine DS. The extragenomic action of progesterone on human spermatozoa: evidence for a ubiquitous response that is rapidly down-regulated. *Endocrinology*. 1996; 137:3999–4009. [PubMed: 8756577]
- Arnoult C, Zeng Y, Florman HM. ZP3-dependent activation of sperm cation channels regulates acrosomal secretion during mammalian fertilization. *Journal of Cell Biology*. 1996; 134:637–645. [PubMed: 8707844]
- Bailey JL, Storey BT. Calcium influx into mouse spermatozoa activated by solubilized mouse zona pellucida, monitored with the calcium fluorescent indicator, fluo-3. Inhibition of the influx by three inhibitors of the zona pellucida induced acrosome reaction: tyrphostin A48, pertussis toxin, and 3-

- quinuclidinyl benzilate. *Molecular Reproduction and Development*. 1994; 39:297–308. [PubMed: 7888169]
- Barros C, Bedford JM, Franklin LE, Austin CR. Membrane vesiculation as a feature of the mammalian acrosome reaction. *Journal of Cell Biology*. 1967; 34:C1–C5. [PubMed: 6050956]
- Bennett CRC, Mancifesta FE, Mayorga LS. Calcineurin-mediated dephosphorylation of synaptotagmin VI is necessary for acrosomal exocytosis. *Journal of Biological Chemistry*. 2012; 285:26269–26278.
- Bernabo N, Palestini P, Chiarini M, Maccarrone M, Mattioli M, Barboni B. Endocannabinoid-binding CB1 and TRPV1 receptors as modulators of sperm capacitation. *Communicative & Integrative Biology*. 2012; 5:68–70. [PubMed: 22482014]
- Berruti G, Franchi E, Camatini M. Ca^{2+} localization in boar spermatozoa by the pyroantimonate technique and X-ray microanalysis. *Journal of Experimental Zoology*. 1986; 237:257–262. [PubMed: 3950567]
- Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. *Journal of Biological Chemistry*. 1981; 256:1604–1607. [PubMed: 6257680]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 1976; 72:248–254. [PubMed: 942051]
- Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Krahling M, Muller A, Kaupp UB, Strunker T. The CatSper channel: a polymodal chemosensor in human sperm. *EMBO Journal*. 2012; 31:1654–1665. [PubMed: 22354039]
- Brose N, Petrenko AG, Sudhof TC, Jahn R. Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science*. 1992; 256:1021–1025. [PubMed: 1589771]
- Burgoyne RD, Morgan A. Ca^{2+} and secretory-vesicle dynamics. *Trends in Neuro-sciences*. 1995; 18:191–196.
- Casey PJ. Protein lipidation in cell signaling. *Science*. 1995; 268:221–225. [PubMed: 7716512]
- Endo Y, Lee MA, Kopf GS. Characterization of an islet-activating protein-sensitive site in mouse sperm that is involved in the zona pellucida-induced acrosome reaction. *Developmental Biology*. 1988; 129:12–24. [PubMed: 3137110]
- Fairbanks G, Steck TL, Wallach DFH. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. 1971; 10:2606–2617. [PubMed: 4326772]
- Florman HM, Tombes RM, First NL, Babcock DF. An adhesion-associated agonist from the zona pellucida activates G protein-promoted elevations of internal Ca^{2+} and pH that mediate mammalian sperm acrosomal exocytosis. *Developmental Biology*. 1989; 135:133–146. [PubMed: 2504631]
- Florman HM, Corron ME, Kim TD-H, Babcock DF. Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Developmental Biology*. 1992; 152:304–314. [PubMed: 1379559]
- Florman HM. Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. *Developmental Biology*. 1994; 165:152–164. [PubMed: 8088433]
- Fraser LR. Calcium channels play a pivotal role in the sequence of ionic changes involved in initiation of mouse sperm acrosomal exocytosis. *Molecular Reproduction and Development*. 1993; 36:368–376. [PubMed: 8286120]
- Hutt DM, Cardullo RCA, Baltz JM, Ngsee JK. Synaptotagmin VIII is localized to the mouse sperm head and may function in acrosome exocytosis. *Biology of Reproduction*. 2002; 66:50–56. [PubMed: 11751263]
- Kopf, GS.; Gerton, GL. The mammalian sperm acrosome and the acrosome reaction. In: Wassarman, PM., editor. *Elements of mammalian fertilization*. Boston: CRC Press; 1991. p. 153–203.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
- Lee C, Levin A, Branton D. Copper staining: a five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. *Analytical Biochemistry*. 1987; 166:308–312. [PubMed: 2449094]

- Lee MA, Storey BT. Evidence for plasma membrane impermeability to small ions in acrosome-intact mouse spermatozoa bound to mouse zona pellucidae, using an aminoacridine fluorescent pH probe. Time course of the zona-induced acrosome reaction monitored by both chlortetracycline and pH probe fluorescence. *Biology of Reproduction*. 1985; 33:235–246. [PubMed: 3840700]
- Maruyama K, Nujawa T, Ebashi S. Detection of calcium binding protein proteins by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *The Journal of Biochemistry*. 1984; 95:511–519.
- Mehta PP, Battenberg E, Wilson MC. SNAP-25 and synaptotagmin involvement in the final Ca^{2+} -dependent triggering of neurotransmitter exocytosis. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:10471–10476. [PubMed: 8816825]
- Meizel S. Amino acid neurotransmitter receptor/chloride channels of mammalian sperm and the acrosome reaction. *Biology of Reproduction*. 1997; 56:569–574. [PubMed: 9046998]
- Meizel S, Turner KO. Initiation of the human sperm acrosome reaction by thapsigargin. *Journal of Experimental Zoology*. 1993; 267:350–355. [PubMed: 8228870]
- Meizel S, Turner KO, Nuccitelli R. Progesterone triggers a wave of increased free calcium during the human sperm acrosome reaction. *Developmental Biology*. 1997; 182:67–75. [PubMed: 9073447]
- Melendrez CS, Meizel S, Berger T. Comparison of the ability of progesterone and heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction in vitro. *Molecular Reproduction and Development*. 1994; 39:433–438. [PubMed: 7893492]
- NagDas SK, Winfrey VP, Olson GE. Proacrosin-acrosomal matrix binding interactions in ejaculated bovine spermatozoa. *Biology of Reproduction*. 1996; 54:111–121. [PubMed: 8838007]
- Noland TD, Davis LS, Olson GE. Regulation of proacrosin conversion in isolated guinea pig sperm acrosomal apical segments. *Journal of Biological Chemistry*. 1989; 264:13586–13590. [PubMed: 2503502]
- Noland TD, Friday BB, Maulit MT, Gerton GL. The sperm acrosomal matrix contains a novel member of the pentaxin family of calcium-dependent binding proteins. *Journal of Biological Chemistry*. 1994; 269:32607–32614. [PubMed: 7798265]
- O'Toole CMB, Roldan ERS, Fraser LR. Role for Ca^{2+} channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. *Molecular Reproduction and Development*. 1996; 45:204–211. [PubMed: 8914078]
- Olmsted JB. Analysis of cytoskeletal structures using blot-purified monospecific antibodies. *Methods in Enzymology*. 1986; 134:467–472. [PubMed: 3547038]
- Olson GE, Winfrey VP. Structure of membrane domains and matrix components of the bovine acrosome. *Journal of Ultrastructure Research*. 1985; 90:9–25. [PubMed: 2415711]
- Parks JE, Arion JW, Foote RH. Lipids of plasma membrane and outer acrosomal membrane from bovine spermatozoa. *Biology of Reproduction*. 1987; 37:1249–1258. [PubMed: 2832008]
- Roldan ERS, Murase T. Polyphosphoinositide-derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phospholipase C during exocytosis of the ram sperm acrosome. Effect is not mediated by protein kinase C. *Journal of Biological Chemistry*. 1994; 269:23583–23589. [PubMed: 8089126]
- Roomans GM. Calcium binding to the acrosomal membrane of human spermatozoa. *Experimental Cell Research*. 1975; 96:23–30. [PubMed: 1193170]
- Russell L, Peterson R, Freund M. Direct evidence for formation of hybrid vesicles by fusion of plasma and outer acrosomal membranes during the acrosome reaction in boar spermatozoa. *Journal of Experimental Zoology*. 1979; 208:41–56. [PubMed: 381567]
- Shao X, Davletov BA, Sutton RB, Sudhof TC, Rizo J. Bipartite Ca^{2+} -binding motif in C2 domains of synaptotagmin and protein kinase C. *Science*. 1996; 273:248–251. [PubMed: 8662510]
- Shi Q, Roldan ERS. Evidence that a GABA_A -like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. *Biology of Reproduction*. 1995; 52:373–381. [PubMed: 7536051]
- Suarez SS, Dai X. Intracellular calcium reaches different levels of elevation in hyper-activated and acrosome-reacted hamster sperm. *Molecular Reproduction and Development*. 1995; 42:325–333. [PubMed: 8579847]

- Sudof TC. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature*. 1995; 375:645–653. [PubMed: 7791897]
- Sukardi S, Elliott RMA, Withers JO, Fontaine U, Millar JD, Curry MR, Watson PF. Calcium-binding proteins from the outer acrosomal membrane of ram spermatozoa: potential candidates for involvement in the acrosome reaction. *Reproduction*. 2001; 122:939–946. [PubMed: 11732989]
- Talian JC, Olmstead JB, Goldman RD. A rapid procedure for preparing fluorescein-labeled specific antibodies from whole antiserum: its use in analyzing cytoskeletal architecture. *The Journal of cell Biology*. 1983; 97:1277–1282. [PubMed: 6413513]
- Thomas P, Meizel S. An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. *Gamete Research*. 1988; 20:397–411. [PubMed: 3235048]
- Towbin H, Gordon J. Immunoblotting and dot immunoblotting—current status and outlook. *Journal of Immunological Methods*. 1984; 72:313–340. [PubMed: 6206159]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from poly-acrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*. 1979; 76:4350–4354. [PubMed: 388439]
- Walensky LD, Snyder SH. Inositol 1,4,5-triphosphate receptors selectively localized to the acrosomes of mammalian sperm. *Journal of Cell Biology*. 1995; 130:857–869. [PubMed: 7642703]
- Ward CR, Kopf GS, Storey BT. Solubilization and partial purification from mouse sperm membranes of the specific binding activity for 3-quinuclidinyl benzilate, a potent inhibitor of the zona pellucida-induced acrosome reaction. *Molecular Reproduction and Development*. 1994a; 39:423–432. [PubMed: 7893491]
- Ward CR, Storey BT, Kopf GS. Selective activation of G_{i1} and G_{i2} in mouse sperm by the zona pellucida, the egg's extracellular matrix. *Journal of Biological Chemistry*. 1994b; 269:13254–13258. [PubMed: 8175755]
- Wassarman PM. The biology and chemistry of fertilization. *Science*. 1987; 235:553–560. [PubMed: 3027891]
- Wassarman PM. Towards molecular mechanisms for gamete adhesion and fusion during mammalian fertilization. *Current Opinion in Cell Biology*. 1995; 7:6658–6664.
- Watson PF, Plummer JM. Relationship between calcium binding sites and membrane fusion during the acrosome reaction induced by ionophore in ram spermatozoa. *Journal of Experimental Zoology*. 1986; 238:113–118. [PubMed: 3086485]
- Watson PF, Plummer JM, Jones PS, Bredl JCS. Localization and intracellular calcium during the acrosome reaction in ram spermatozoa. *Molecular Reproduction and Development*. 1995; 41:513–520. [PubMed: 7576619]
- Wray W, Boulikas T, Wray VP, Hancock R. Silver staining of proteins in polyacrylamide gels. *Analytical Biochemistry*. 1981; 118:197–203. [PubMed: 6175245]
- Yanagimachi R. Requirement of extracellular calcium ions for various stages of fertilization and fertilization-related phenomena in the hamster. *Gamete Research*. 1982; 5:323–344.
- Yanagimachi R. Mammalian fertilization. In: Knobil, E.; Neill, JD., editors. *The physiology of reproduction*. New York: Raven Press; 1993. p. 189–317.
- Yanagimachi R, Usui N. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Experimental Cell Research*. 1974; 89:161–174. [PubMed: 4435057]
- Zahler WL, Doak GA. Isolation of the outer acrosomal membrane from bull sperm. *Biochimica et Biophysica Acta*. 1975; 406:479–488.
- Zarelli VEP, Ruete MC, Roggero CM, Mayorga LS, Tomes CN. PTP1B dephosphorylates N-ethylmaleimide-sensitive factor and elicits SNARE complex disassembly during human sperm exocytosis. *Journal of Biological Chemistry*. 2009; 284:10491–10503. [PubMed: 19208619]

Fig. 1.

(A) Phase contrast photomicrograph showing intact ejaculated bovine sperm (panel a) and enriched sperm head fraction isolated from sonicated spermatozoa (panel b). (B) Silver-stained SDS-polyacrylamide gel (7.5–15% gradient) of acrosomal membrane-enriched fraction isolated from the sperm head fraction exhibits a complex array of polypeptides.

Fig. 2. Identification of calcium-binding polypeptides in the acrosomal membrane-enriched fraction using a $^{45}\text{Ca}^{2+}$ blot-overlay assay. The membrane fraction was solubilized in 1% Triton X-114 containing 0.6 M KCl and exhibited calcium-binding polypeptides of 64, 45, 42 and 39 kDa (lane 1). Phase separation analysis showed that the 64 kDa polypeptide partitions to the detergent phase (lane 2). The other polypeptides of 45, 42, and 39 kDa, were present in both the detergent (lane 2) and the aqueous (lane 3) phases.

Fig. 3.

Purification of the 64 kDa calcium-binding protein. The sperm head fraction was extracted with the Triton X-100 and the detergent soluble proteins fractionated by SDS-PAGE, several polypeptides were present (lane 1). The detergent-soluble fraction was then fractionated on a DEAE-Sephadex column and 0.4 M KCl eluted fraction was subjected to preparative SDS-PAGE; the 64 kDa polypeptide was electroeluted from the excised band. The purified fraction showed a single band of 64 kDa (lane 2).

Fig. 4. Dose-dependent analysis of a calcium-binding ability of the purified 64 kDa polypeptide by a $^{45}\text{Ca}^{2+}$ blot-overlay assay. Western blots of a variable amount of the 64 kDa polypeptide (0.25–2 μg protein) were incubated in overlay buffer containing 1 $\mu\text{Ci/mL}$ $^{45}\text{CaCl}_2$. The amount of calcium binding increases in a dose-dependent manner.

Fig. 5.

(A) Western blot analysis using the anti-64 kDa serum. Triton X-100 extracts of the sperm head fraction were separated by SDS-PAGE for immunoblot analysis. Blot affinity purified 64 kDa antiserum specifically recognized the 64 kDa polypeptide in the Triton X-100 extract (lane 1). Lane 2 was stained with preimmune serum and reveals no specifically stained polypeptide bands. Each lane contained 10 μ g protein. (B) Immunoblot, stained with anti-64 kDa serum, of total Triton X-100 extracts of sperm head fraction (10 μ g protein), exhibited the presence of one isoform of 64 kDa calcium-binding polypeptide (pI ~6.5).

Fig. 6. Immunoblots of Triton X-100 extracts of rat sperm heads (lanes 1 and 3) and mouse sperm heads (lanes 2 and 4) fractionated by reducing SDS-PAGE. Immunostaining with a blot affinity purified 64 kDa bovine calcium-binding polypeptide immune serum (lanes 1 and 2) revealed the presence of a 64 kDa band. Lanes 3 and 4 were stained with preimmune serum; no band was seen. Each lane contained 10 μ g protein.

Fig. 7. Immunofluorescence localization of the 64kDa calcium-binding protein. Matched phase contrast and fluorescence photomicrographs of permeabilized bovine spermatozoa immunostained with 64 kDa antiserum (panels a and b). Staining was present on the apical, principal and equatorial segments of the acrosome (ac); a faint ring of fluorescence was also noted at the base of the sperm head (arrowheads). Inset shows at high magnification view of an immunostained spermatozoon. Sperm stained with preimmune serum exhibited no fluorescence (panels c and d). Sonicated sperm suspensions stained with immune serum exhibited the same staining pattern (panels e and f) as the permeabilized spermatozoa (compare to panels a and b). Bar represents 10 μ m.

Fig. 8.

Ultrastructural immunolocalization of the 64 kDa calcium-binding polypeptide. (a) shows a cross section of the acrosomal segment of an intact spermatozoon immunolabeled with the anti-64kDa antibody by the post-embedding protocol. Gold particles are associated with the outer (oa) and inner (ia) acrosomal membranes and the acrosomal membranes of the equatorial segment (es). $\times 33,000$. (b) shows a cross section of the acrosomal segment of an intact spermatozoon immunolabeled with preimmune serum by the post-embedding protocol. No gold particles are evident ($\times 29,000$). (c) shows a sonicated sperm preparation immune labeled with 64 kDa antibody using the post-embedding protocol. Gold particles are specifically associated with the inner acrosomal membrane (ia) and the acrosomal

membranes of the equatorial segment (es). $\times 34,000$. (d) and (e) show sonicated spermatozoa immunolabeled with the immune serum using pre-embedding protocol. Both the inner acrosomal (ia) and the outer acrosomal membrane-matrix complex (arrowheads) exhibit bound gold particles (d = $\times 63,000$ and e = $\times 48,000$). pa=postacrosomal segment; n = nucleus; pp = principal piece; and mp = midpiece.