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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 $^{45}Ca^{2+}$-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.

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; 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

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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 $^{45}Ca^{+2}$. 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 × *g* for 1 min to sediment epididymal tubule fragments. Supernatants were centrifuged at 1500 × *g* for 10 min 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 × *g* for 10 min 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 × *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 10 mL 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 × *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 CO_2 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 ug/mL leupeptin, 1 ug/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 × *g* for 30 min 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 × *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 × *g* for 10 min 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 × *g* for 5 min to obtain a detergent pellet, containing integral membrane proteins, and the aqueous supernatant, containing hydrophillic proteins (Bordier, 1981). Fractions were dialyzed against 25 mM Tris–HCl, pH 7.5, 150 mM 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 μCi/mL $^{45}CaCl_2$. 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.