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Functional distribution of synapsin I in human sperm

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ABSTRACT

Proteins known to function during cell–cell communication and exocytosis in neurons and other secretory cells have recently been reported in human sperm. Synapsins are a group of proteins that have been very well characterized in neurons, but little is known about synapsin function in other cell types. Based upon previous findings and the known function of synapsin, we tested the hypothesis that synapsin I was present in human sperm. Washed, capacitated, and acrosome induced sperm preparations were used to evaluate the functional distribution of synapsin I using immunocytochemistry. Protein extracts from mouse brain, mouse testis/epididymis, and human semen were used for protein blotting techniques. Immunolocalization revealed synapsin I was enriched in the sperm equatorial segment. Protein extracts from mouse brain, mouse testis/epididymis, and human semen were positive for synapsin I using several different antibodies, and dot blot results were confirmed by Western blot analyses. Finally, treatment of capacitated and acrosome reaction induced samples with anti-synapsin antibodies significantly reduced sperm motility. Localization of synapsin I in human sperm is a novel finding. The association of synapsin I with the sperm equatorial segment and effects on motility are suggestive of a role associated with capacitation and/or acrosome reaction, processes that render sperm capable of fertilizing an oocyte.

1. Introduction

Successful fertilization involves species-specific communication between the sperm and oocyte. *In vivo*, as sperm travel through the female reproductive tract, a series of calcium dependent biochemical changes, known collectively as capacitation, occur. Changes include sterol oxidation and efflux from the membrane, movement of lipid rafts, increase in intracellular cAMP, tyrosine phosphorylation, and hyperactivated motility [1]. Upon interaction with the oocyte zona pellucida, sperm undergo acrosome reaction (AR); an exocytotic process whereby the contents of the acrosome are released. Previous studies have shown that both capacitation and acrosome reaction are necessary for sperm to acquire the ability to fertilize an oocyte [2,3]. Vesiculation of the sperm membrane and simultaneous release of the acrosomal contents could be likened to the release of signaling substances as a means of cell–cell communication in other cell types. This communication must be efficient, reliable, and carefully regulated. Exocytosis and release of signaling substances in neurons have been studied in detail. One group of proteins implicated in these processes are synapsins. The synapsins arise from three separate genes in vertebrates, and multiple isoforms exist due to alternative splicing [4–6]. Synapsin I has two isoforms, Ia and Ib, with molecular weights of 86 and 80 kDa respectively [7]. Synapsin I was discovered as a phosphorylation target dependent on cAMP [7–9], and later as a phosphorylation target of Ca^{2+}-calmodulin dependent protein kinases [10–12]. Synapsin I is highly abundant in both peripheral and central nervous systems. It is associated with small synaptic vesicles within presynaptic terminals but dissociates upon calcium dependent phosphorylation [13–18]. Several studies have shown that synapsin I interacts with actin, which changes upon phosphorylation [19–23]. Synapsin I is thought to create a reserve pool of vesicles by binding to synaptic vesicles and actin. Upon phosphorylation following calcium influx, synapsin I dissociates from synaptic vesicles and actin, allowing vesicle mobilization and subsequent release. Additionally, synapsins play roles in neuronal growth, development, and synapse formation [24–28]. Early studies suggested synapsin proteins were neuron specific [7,9,29,30], and little is known about their function in non-neuronal cells. Synapsin I has been characterized in cell lines of non-neuronal origin, such as AtT-20 cells [31] and PC12 cells treated with NGF [32,33]. Synapsin I has been implicated in insulin

Abbreviations: AR, acrosome reaction; Cap, capacitated; HSM, human sperm medium; T/E, testis/epididymis

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exocytosis, although whether it is associated with secretory granules or other vesicular structures is unclear [34–37]. Synapsin I has also been reported in cultured astrocytes [38], liver, NRK epithelial cells [39,40], and osteoblasts [41].

Rab3a and Munc13, whose respective functions in synaptic vesicle targeting and priming in neurons are well known [42,43] also regulate secretion in bovine chromaffin cells [44–46] and insulin release [47,48]. The interaction and functions of several key secretory regulators have been investigated in acrosomal exocytosis in human sperm [49–51]. Recently, Rab3a, Munc13, and RIM have been reported in human sperm with functions during acrosomal exocytosis [49,51]. These findings led to our hypothesis that synapsin proteins may be present in human sperm. The purpose of this study was to investigate the localization and functional distribution of synapsin I in washed, capacitated, and acrosome induced human sperm. Additionally, we evaluated sperm motility following capacitation and acrosome reaction in the presence and absence of anti-synapsin antibodies. Results reported herein revealed synapsin I enrichment in the sperm equatorial segment. Inclusion of anti-synapsin antibodies during capacitation and acrosome reaction significantly reduced sperm motility.

2. Materials and methods

2.1. Semen sample collection

Human semen samples were collected with written consent from volunteers 18 years of age and older. All experimental procedures involving human samples were approved by the Bloomsburg University Institutional Review Board (approved protocol # 2013-4). All samples were de-identified such that volunteer confidentiality was maintained, and samples were assigned a random study identification code. Whole semen was analyzed within 1–2 h of collection. Total sample volume, pH, total cell count, and percent motility were recorded.

2.2. Sperm sample preparation

Washed human sperm were prepared by placing 1.5 mL of whole, human semen into a 15 mL conical tube with 5 mL of human sperm medium (HSM) [52]. The sample was then centrifuged for 10 min, 500×*g* at room temperature (RT). The supernatant was carefully decanted and the sperm pellet was resuspended in 1.5 mL HSM. For capacitation, 1 mL of washed sperm was mixed with 1 mL of HSM with 7% BSA and incubated 3–4 h at 37 °C with 5% CO_2. Following capacitation, the sample was microscopically observed for the presence of hyperactivation. Only samples with at least 50% motility were utilized for acrosome reaction induction. Capacitated sperm were induced to undergo acrosome reaction by incubation with 10 μM bromo-A23187 ionophore (EMD Millipore Corporation) for 30 min at 37 °C with 5% CO_2.

2.3. Protein extract preparation

2.3.1. Extraction buffer

Protein extraction was conducted using chilled RIPA buffer. Briefly, RIPA buffer was prepared in sterile 1X PBS and contained 1% Nonidet-P40 (Fisher Scientific), 0.5% deoxycholic acid sodium salt (Fisher Scientific), 0.1% sodium dodecyl sulfate (SDS) (Thermo Scientific), and 1X protease inhibitor cocktail (Sigma–Aldrich).

2.3.2. Protein extracts from mouse samples

All experimental procedures involving animal samples were approved by the Bloomsburg University Institutional Animal Care and Use Committee (protocol # 106). All tissues were extracted post-mortem from male mice (Harlan Laboratories, Hsd:ICR, CD-1) that were rapidly euthanized with an overdose of the inhalant anesthetic halothane, in accordance with the IACUC protocol. Protein extracts for dot blot and Western blot analyses were prepared from mouse brain and mouse testis/epididymis (T/E). Tissue was harvested from six different mice on separate occasions. For each mouse, extracts were prepared individually and samples were not pooled. Brain and T/E extracts from three different mice were utilized for the experiments presented herein and representative results are shown. The entire brain or T/E dissected from each mouse was weighed and 10 μL of RIPA buffer were added per mg of tissue. Samples were homogenized using a Polytron tissue homogenizer for 10 s. Following homogenization, samples were centrifuged for 20 min, 16,000×*g* at 2 °C. The supernatant was decanted and placed into sterile cryovials and used for dot and Western blot analyses. Total protein was quantified by bicinchoninic acid (BCA) assay. Samples were diluted with 1X PBS. Ten microliters of each sample were placed into a well of a micro-plate, mixed with 200 μL Pierce™ BCA working reagent (Thermo Scientific), and incubated for 60 min at 37 °C with shaking. Samples were run in triplicate. Absorbance was quantified at 570 nm using a Tecan GENios Plus plate reader. Total protein was determined by comparison to bovine serum albumin (BSA) standard curve.

2.3.3. Protein extracts from human semen

Human sperm and seminal plasma extracts were prepared by pooling whole semen samples from the same donor collected on separate days. For each sample, 1.5 mL of whole semen (approximately 62,000,000 sperm cells) were placed into a sterile microfuge tube and centrifuged at 6000×*g* for 15 min. The top 500 μL of supernatant were decanted and placed into a new microfuge tube containing 500 mL of RIPA buffer with 1X protease inhibitor. This sample was labeled as seminal plasma and frozen at −80 °C. The remaining supernatant was discarded and the pellet was resuspended with 250 μL of RIPA buffer with 1X protease inhibitor. This sample was labeled as sperm pellet and frozen at −80 °C. The second semen sample from the same donor was prepared following the above protocol. The first two samples were thawed and added to either the previous seminal plasma or sperm pellet extracts. Samples were then referred to as pooled seminal plasma or pooled sperm cell. Each pooled sample was sonicated at 9 V for 45 s. The samples were centrifuged at 15,000×*g* for 10 min. The top 400–500 μL of supernatant were reserved, placed into a new cryovial, and used for subsequent dot and Western blot analysis. Total protein was quantified using BCA assay as previously described.

2.3.4. Dot blot

Protein extracts from mouse brain, mouse T/E, human sperm cells, and human seminal plasma were spotted three times onto a piece of 0.20 μm nitrocellulose membrane (Thermo Scientific) in 2 μL aliquots, with drying time between applications. Vertical pencil lines were hand-drawn on each blot to delineate the area in which each sample was applied. All samples were applied to a single, continuous blot. The membrane was wet in 1X PBST (1X PBS containing 0.1% Tween-20 (Fisher Scientific)), placed into a 10 cm petri dish, and blocked for 1 h with 10 mL of 5% milk in 1X PBST on a shaker at RT. Following blocking, milk was poured off and 10 mL of primary polyclonal goat anti-synapsin Ia/b A-15 antibody (Santa Cruz Biotechnology Cat# sc-55774), polyclonal rabbit anti-synapsin Ia/b H-170 antibody (Santa Cruz Biotechnology Cat# sc-20780), or monoclonal mouse anti-synapsin Ia/b B-11 antibody (Santa Cruz Biotechnology Cat# sc-376622) were added. A-15 was raised against a peptide near the C-terminus of human synapsin Ia, H-170 was raised against amino acids 491–660 at the C-terminus of human synapsin Ia/b, and B-11 was raised against amino acids 2–29 at the N-terminus of human