Changes in Immunocytochemical Localization of Cytoskeletal Proteins in Boar Spermatozoa after Acrosome Reaction Induced by Specific Cytoskeletal Inhibitors

(spermatozoa / acrosome reaction / cytoskeleton / γ-tubulin / colcemide / cytochalasine B / nocodazole / vinblastine)

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Abstract. Certain morphological changes such as rearrangement of cytoskeletal proteins of the mammalian spermatozoa are detectable during the AR. The type of changes differs according to the studied sperm species and follows the course of AR. Relocation of cytoskeletal structures was previously observed especially in the case of actin-, α-, γ-tubulin- and spectrin-containing structures. To prove these findings we used specific inhibitors of cytoskeletal proteins (eg. colcemide, cytochalasine B, nocodazole and vinblastine). It has been shown that the AR is influenced by cytoskeletal inhibitors, but the obtained results also document that cytoskeletal proteins actin, tubulin and spectrin play a significant role in the course of AR *in vitro*. Our results of confocal and electron microscopy also demonstrate visible changes of actin-, tubulin- and spectrin-containing structures after the AR. Our data indicate that specific cytoskeletal inhibitors influence the AR and they prove the role of cytoskeletal proteins in this process.

Primary binding of sperm surface proteins to the glycoprotein net of zona pellucida (ZP) leads to activation of the sperm membrane and a receptor-mediated exocytotic event leads to activation and release of the acrosomal contents. The acrosome reaction (AR) is required for secondary binding of sperm to ZP. The AR is characterized by multiple fusions between the outer acrosomal mebrane and the overlying plasma membrane, which exposes the acrosomal contents of the spermatozoon and its resistant inner acrosomal membrane (Yanagimachi, 1994, Brucker and Lipford, 1995). Mammalian spermatozoa without an acrosome do not bind (during secondary binding) to the ZP and consequently fuse with the oocyte plasma membrane *in vitro* (von Bernhardi et al., 1990; Bourne et al., 1995). A number of cytoskeletal proteins are involved in this complex of exocytotic process of the AR. It is known that actin plays an important role in the events concerning the plasma membrane in human spermatozoa (Vogl, 1989) during capacitation and the AR (Paleček et al., 1999). In the recent study by Liu et al. (1999), the authors confirm that actin is present in the acrosomal region of human spermatozoa and that inhibition of actin polymerization by cytochalasine B and D blocks the ZP-induced AR. On the other hand, inhibition of actin depolymerization with phalloidin has no effect on ZP-induced AR (Liu et al., 1999). Nevertheless, there might be other proteins that are involved in the AR, such as tubulins (α, β, γ) and spectrin. Unlike α- and β-tubulins, which represent the major structural subunits of microtubules, γ-tubulin is a minor tubulin species. In most cells inspected so far, detectable levels of γ-tubulin were very low, the protein being usually restricted to discrete microtubule organizing centers (MTOCs). Immunofluorescence studies have shown the presence of γ-tubulin in centrosomes and spindle poles of animal cells (Zheng et al., 1991), as well as in basal bodies of ciliated epithelia, mouse sperms (Palacios et al., 1993; Liang et al., 1996). On the basis of these findings, it has been widely accepted that γ-tubulin is a MTOC-specific protein. Moreover, its localization in MTOCs of animal and fungal cells has led to the hypothesis that the protein is essential for nucleation of microtubules in MTOCs. But no data are available about the role of γ-tubulin during the process of fertilization. The field of our investigations was to find out the role of

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Abbreviations: ACR.2 – monoclonal antibody against intra-acrosomal protein, AR – acrosome reaction, FITC – fluorescein isothiocyanate, MTOC(s) – microtubule organizing center(s), PBS – phosphate-buffered saline, SB – Sörensen buffer, TBM – Tris-buffered medium, TBS – Tris-buffered saline, ZP – zona pellucida.

cytoskeletal proteins (actin, α-, γ-tubulin and spectrin), which are included in structures responsible for the process of the AR. We were also interested in whether the individual proteins participate in the AR or whether there is a whole complex of cytoskeletal protein-containing structures that is involved in the AR. For that task we used different specific inhibitors of cytoskeletal proteins such as colcemide, cytochalasine B, nocodazole and vinblastine.

Material and Methods

Cells

Ejaculates of boar spermatozoa were obtained from the Insemination Station, Nové Zámky, Czech Republic.

Gamete preparation

Boar semen samples were washed twice in Tris-buffered saline ((TBS): 130 mM NaCl, 20 mM Tris, pH 7.4). The pellet of spermatozoa was resuspended in Tris-buffered medium ((TBM); pH 7.7; Berger and Horton, 1988), and centrifuged 40 min in 40–80% discontinuous Percoll (Sigma, Prague, Czech Republic) gradient (Berger and Horton, 1988). The fraction of spermatozoa from 70% Percoll was washed in TBM. After the washing, spermatozoa were diluted in TBM to a final concentration of 2.5×10^7 cells ml^{-1}. The acrosome reaction was induced in freshly ejaculated boar spermatozoa by addition of calcium ionophore A-23187 (Sigma, Prague, Czech Republic) to a final concentration of 2 μM, followed by incubation for 30 min at 37°C in 5% CO_2, either with or without inhibitors. The incubation of spermatozoa with specific inhibitors took 30–60 min at 37°C in 5% CO_2, either with or without calcium ionophore. We used a specific dilution for each drug: cytochalasine B (No. 85.777 – 7.5 mg, Sigma Aldrich, Prague) – 2.5 μg/ml in DMSO solution (dimethylsulfoxide 99+% Hybri-Max; D2650; 5 × 5 ml), vinblastine (RG – 5 mg, Sigma, Prague) – 2×10^{-3} M in TBS solution, colcemide (demecolcine; D7385; 5 mg, Sigma, Prague) – 2×10^{-3} M in TBS solution, nocodazole (M-1404, 2 mg, Sigma, Prague) – 20, 50, 100 μg/ml in TBS solution. Sperm cells were air-dried on slides, fixed by different fixative techniques and prepared for immunocytochemical detection. Simultaneously, aliquots with spermatozoa were taken for detection of viability, motility and state of the acrosome using monoclonal antibody (ACR.2) against acrosin (Pěknicová and Moos, 1990). Similar sperm samples were prepared for confocal and electron microscopy observation.

Fixation of samples

1. methanol (10 min) – acetone (8 min); (both pre-cooled to –20°C) (Bacceti et al., 1989) – fixation solution for α-, γ-tubulin and spectrin.
2. 3.7% formaldehyde in phosphate-buffered saline (PBS) at 22°C (45 min) – fixation solution for actin.
3. acetone at 22°C (10 min) – fixation solution for the intra-acrosomal protein (acrosin) (Pěknicová and Moos, 1990).

The summary of the used methods was published in the review article by Paleček and Ubbels (1997).

Specific antibodies and indirect immunofluorescence

We used a specific monoclonal antibody against intra-acrosomal protein (ACR.2) (Pěknicová and Moos, 1990). For immunofluorescence labeling, the following primary antibodies were used: A-2668 against actin (Sigma, Prague, Czech Republic), T9026 against α-tubulin (Sigma, Prague, Czech Republic), Tu-30 and Tu-31 against γ-tubulin (provided from the Laboratory of Dr. Pavel Dráber, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Czech Republic), S-1515 against spectrin (Sigma, Prague, Czech Republic).

Slides were rinsed 3 × 10 min with PBS (130 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 105 mM KH_2PO_4, pH 7.4 buffer). Incubation with an appropriate primary antibody (diluted in 3% bovine serum albumin (BSA) in PBS) took place in a 37°C thermostat for 90 min. After washing in PBS (3 × 10 min), incubation with a secondary antibody labeled with fluorescein isothiocyanate (FITC)-conjugated swine anti-mouse or swine anti-rabbit immunoglobulins (SwAM-FITC, SwAR-FITC; Sevac, Prague, Czech Republic, and Anti-Mouse IgG (Fc specific) – FITC Conjugate, Sigma, Prague, Czech Republic) took 60 min under the same conditions as for the primary antibody. Thereafter, slides were washed again in PBS (3 × 10 min), blocked in PBS-blocking solution containing per 100 ml: 5 g skimmed milk powder, 5 ml bovine serum, 0.5 ml Tween, and mounted in Vecta-Shield mounting medium (Scandic, Prague, Czech Republic) either with or without propidium iodide. Slides were examined under a fluorescence microscope Olympus BX-40 (Olympus Prague, Czech Republic), equipped with a 8-bit B&W CCD/camera (Sony SPT-M320CE), and photographs were taken by a PM-20 camera (Olympus, Tokyo, Japan) on a Kodak Ektapress Gold II Professional ISO 400 film (Eastman Kodak Corp., Rochester, NY), Kodak T-MAX professional ISO 400 black-and-white film (TMY 135) and confocal microscopy Leica TCS-SP (Micro, Prague, Czech Republic).