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STUDIES ON THE MECHANISM OF CAPACITATION: CHANGES IN PLASMA MEMBRANE PROTEINS OF RAT SPERMATOZOA DURING INCUBATION IN VITRO[1]

B. K. Davis and A. F. Gergely

Long Island Research Institute, and Department of Obstetrics and Gynecology, Health Sciences Center 10T, SUNY, Stony Brook, N.Y. 11794

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SUMMARY: Plasma membrane from cauda epididymal rat spermatozoa underwent extensive polypeptide hydrolysis during incubation in Krebs Ringer bicarbonate medium. Hydrolysis was markedly increased by addition of 4 mg bovine serum albumin/ml of medium. Consistent with this result, expression of trypsin-like enzyme activity, attributed to release of acrosome contents, was higher in sperm cells from BSA containing medium.

Fusion between plasma membrane and outer acrosomal membrane in mammalian sperm cells is now widely recognized as a precondition for fertilization in this class of animals. The acrosome reaction involves the exocytotic-type release of hydrolytic enzymes that facilitate sperm passage through the corona cell layer and zona pellucida and, finally, penetration of an ovum (1,2). In recent years, several groups of investigators (3-8) have achieved sperm capacitation and fertilization under chemically defined conditions in the presence of serum albumin.

An endeavor has been made in this laboratory to elucidate the significance of serum albumin in sperm capacitation. These studies have concerned occurrence of lipid exchange between the protein and plasma membrane of rat sperm and resulting alterations in membrane cholesterol/phospholipid ratio (9,10). The changes observed are considered to alter microviscosity within the sperm plasma membrane and promote the acrosome reaction. Experiments described in this communication were performed to extend our characterization of molecular

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changes in the rat sperm plasma membrane that occur during incubation in a chemically simple medium, which has the ability to promote capacitation. Specifically, we aimed to establish if there is (a) significant change in sperm plasma membrane polypeptide during incubation *in vitro* and (b) an association between bovine serum albumin (BSA) and this membrane.

MATERIALS AND METHODS These experiments were conducted with mature Sprague Dowley rats purchased from Gofmoor Farms (Massachusetts). The animals were housed in constant light (700 to 1900) and temperature (21± 1°C) and fed Purina Chow and water *ad lib*. Spermatozoa were obtained by sectioning tubules of the cauda epididymis immediately after autopsy and suspended in 50 ml Krebs Ringer bicarbonate (KRB) medium containing, when present, 4 mg/ml bovine serum albumin (BSA). The incubation was conducted in a humidified atmosphere of 5 per cent CO_2 in air at 37°C while the sperm suspension (around 2 x 10^6 spermatozoa/ml) was immersed under a layer of sterile mineral oil. After incubation, the oil layer was removed in a separatory funnel and the spermatozoa sedimented, usually at 3000g for 30 min. at 4°C. The cells were resuspended in 5 ml 0.01 M Tris, pH 7.4, and, after 1 hr., frozen at -68°C. The suspension was thawed, ethylenediaminetetracetic acid added until 0.075 M, and then irradiated for 15 sec. at 20 kHz using a Braunsonic ultrasonicator attached to a microprobe (0.4 cm diameter). Following centrifugation at 3000 g for 30 min., the opalescent supernatant was then sedimented on a discontinuous density gradient with 60, 40, and 20 per cent sucrose zones in an SW27 rotor with an ultracentrifuge (Beckman). The sucrose gradient preparation was fractionated and each fraction (1 ml) assessed for absorbance at 280 nm. Membrane protein (150 mg) was placed on 7.5 (w/v) per cent polyacrylamide gel columns and electrophoresis performed with 0.188 M glycine buffer, 0.188 M Tris, pH 8.9, containing 0.26 (w/v) per cent sodium dodecyl sulfate according to the method of Laemmli (11). The gels were stained with 0.5 (w/v) per cent Coomassie Brilliant Blue in 7.0 (v/v) per cent acetic acid. A gel scanner (Helena), operated at a gain of 4.5, traced the polypeptide profile. Molecular weights were estimated by comparison with the mobility of standard proteins; BSA (67,000), ovalbumin (44,000), and cytochrome c (13,000).

Expression of trypsin-like enzyme activity was determined by the rate of hydrolysis of tosyl-arginine-methyl-ester (TAME). The assays were performed with spermatozoa (6 x 10^6 sperm cells/ml), which had been incubated for various intervals in KRB medium, in the presence of 0.04 per cent (w/v) TAME, 0.1 M Tris, pH 8.11, 0.01 M Ca Cl_2, and they required 45 min. at 27°C. Absorbance at 247 nm was then assessed in the supernatants obtained, after sedimenting the sperm cells for 5 min. at 1000 g.

RESULTS Figure 1 shows that the method employed resulted in a single major peak at the 20/40 per cent sucrose interface. This peak accounted for over ninety per cent of the sedimented protein, judging by absorbance at 280 nm. The peak shows electron microscopic, enzymatic, physical

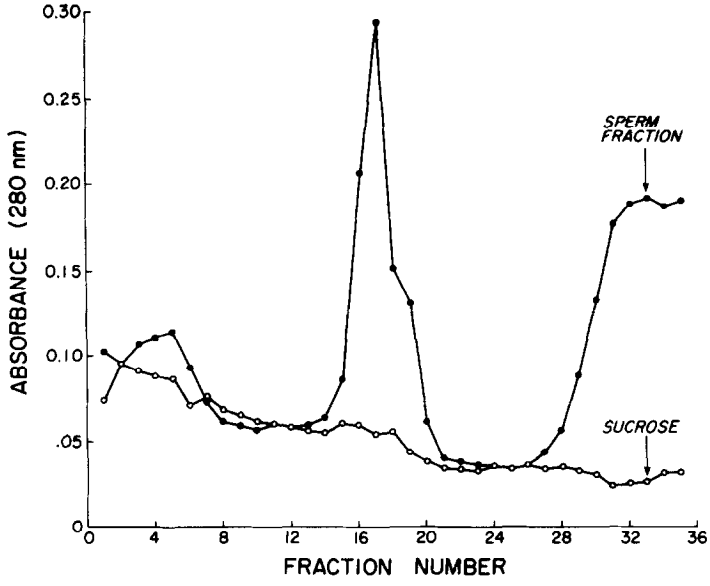

Fig. 1 - Optical density profile (280 nm) following sedimentation on a discontinuous denisty gradient of a plasma membrane preparation from epididymal rat spermatozoa, which had been incubated in KRB medium containing albumin. The membranes were isolated after brief sonication of spermatozoa and sedimentation of 130,000 g for 1.5 hr. on a gradient comprising zones of 60, 40 and 20 per cent sucrose dissolved in 0.01 M Tris, pH 7.4. A large peak corresponding to plasma membrane can be seen at the 20/40 per cent sucrose interface. Background absorbance by sucrose was determined in a density gradient containing no sperm fraction.

and chemical characteristics of plasma membrane (paper submitted for publication).

Membrane polypeptide profiles shown in Fig. 2 indicate there was extensive hydrolysis of these proteins during incubation in vitro. This hydrolysis was conspicuously elevated in the presence of 4 mg BSA/ml. Membranes from spermatozoa incubated for 2.5 hrs. with BSA displayed no polypeptides over 70,000 daltons. By contrast, three major polypeptide peaks with molecular weights above 150,000 are visible in the profile for native plasma membrane from epididymal rat sperm cells. However, even in the absence of BSA, there was virtually complete hydrolysis of these polypeptides after 2.5 hrs. of incubation at 37°C. This was associated with the appearance of lower molecular weight peaks (around

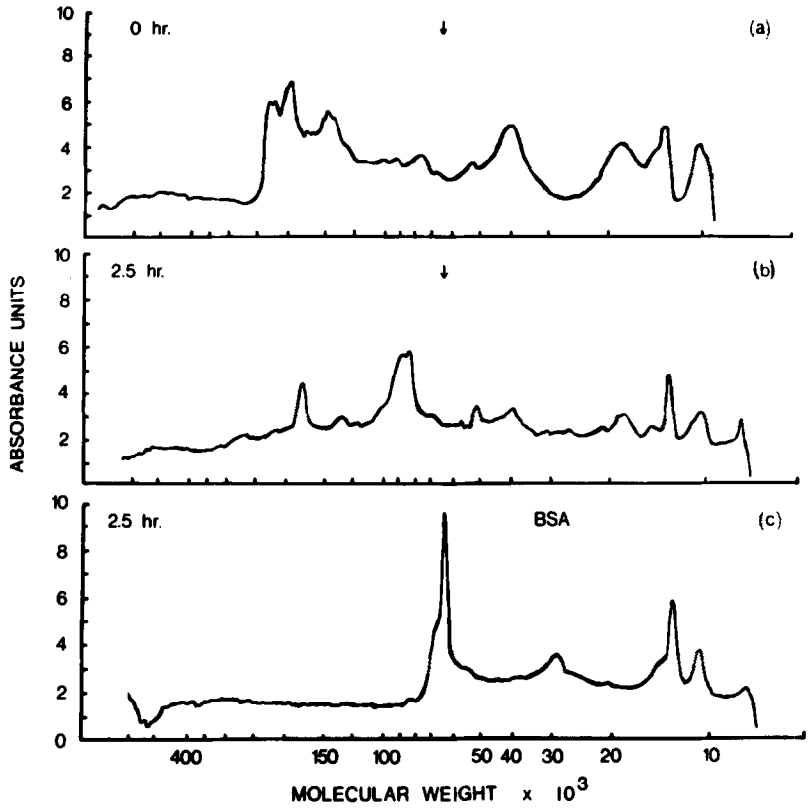

Fig. 2. - Polypeptide profiles obtained on SDS-gel electrophoresis of plasma membranes from epididymal rat spermatozoa that were incubated in KRB medium for indicated intervals. (a) Unincubated, (b) incubated for 2.5 hrs., and (c) incubated in medium containing 4 mg BSA/ml for 2.5 hrs. An arrow gives location of the albumin zone on a standard gel. Stain, Coomassie Brilliant Blue.

85,000 daltons) in this membrane preparation. It is apparent that smaller polypeptides in the native plasma membrane have also been hydrolyzed, such as those forming the peaks at 50,000 and 22,000 daltons. Another conspicuous feature of the results presented by Fig. 2 is the prominent BSA peak in plasma membrane from spermatozoa incubated in KRB medium containing the protein. Association of this protein with cauda epididymal rat spermatozoa was also observed when these cells were incubated with [^{125}I]BSA.

Trypsin-like enzyme activity rapidly increased among spermatozoa incubated in BSA containing medium to reach a level of 1.67 x 10^{-9} moles

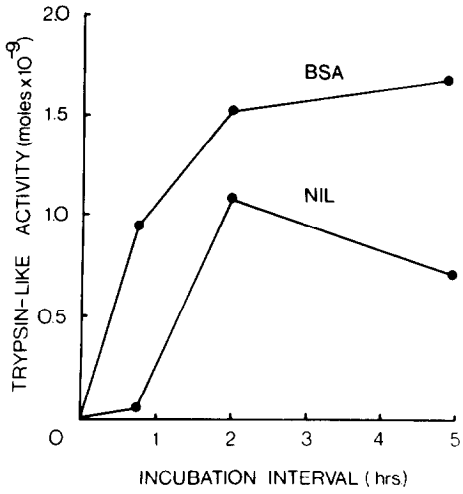

Fig. 3. - Effect of BSA on expression of trypsin-like activity by rat spermatozoa during incubation in KRB medium. Enzyme activity was established from the rate of TAME hydrolysis after various intervals of sperm incubation. Duplicate determinations were performed.

after 5 hrs. in a suspension with 6 x 10^6 sperm cells (Fig. 3). Activity in the absence of BSA showed a lag for about 1 hr. Lower rates of hydrolysis of the synthetic trypsin substrate, TAME, were also evident at longer intervals; at 5 hrs., it was 0.72 x 10^{-9} moles/6 x 10^6 sperm cells. As may have been anticipated, elevations in proteolytic enzyme activity shown in Fig. 3 precede the time required for expression of fertilizing ability by epididymal rat sperm cells *in vitro*.

DISCUSSION These results clearly demonstrate extensive hydrolysis of plasma membrane proteins during incubation of rat spermatozoa in Krebs Ringer bicarbonate medium. This is associated evidently with release of the acrosomal contents from these sperm during incubation. The presence of BSA in the medium caused a conspicuous increase in the extent of protein digestion, and, significantly, this was associated with elevated levels of trypsin-like enzyme activity, which can be attributed to acrosin (E.C. 3.4.21.10) (12). This activity among spermatozoa in medium lacking BSA probably resulted from spontaneous release of acrosomal enzymes, which can accompany sperm degeneration, rather than by induction of the acrosome

reaction. The marked changes observed in the sperm plasma membrane polypeptide profile are considered to be effects, rather than causes,of either true or false acrosome reactions. In a recent study, Hirao and Yanagimachi (13) examined the effect of proteolytic digestion, by a variety of enzymes, on hamster gamete function. They reported that proteolysis did not impair the fertilizing ability of hamster ova; interestingly, fertilization was inhibited by digestion of the eggs with phospholipase C. The interaction of BSA with the plasma membrane of rat spermatozoa, as noted in this study, is consistent with its involvement in lipid exchange. Formation of comparatively stable associations with this lipid-solubilizing protein may also contribute to destabilization of the sperm plasma membrane during capacitation and seems to merit further investigation.

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