Protein involvement in the fusion between the equatorial segment of acrosome-reacted human spermatozoa and liposomes

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Artificial membranes (liposomes) can interact with the equatorial segment (ES) of human spermatozoa, provided that the acrosome reaction (AR) has occurred [Arts, Kuiken, Jager and Hoekstra (1993) Eur. J. Biochem. **217**, 1001–1009]. Using fluorescently labelled liposomes, this interaction can be seen as either punctate fluorescence in the ES (lip-ESp), reflecting only bound liposomes, or as diffuse fluorescence in this region (lip-ESd), indicating that the liposomes have fused with the ES membrane. Only equatorial segments that still contain constituents of the acrosomal matrix have the capacity to bind liposomes and eventually to fuse with them. Since the exposure of such intact equatorial segments is the exclusive result of induction of the AR under physiological conditions, these results imply that liposomes can be used for the rapid detection of acrosome-reacted spermatozoa. The lip-ESp and lip-ESd patterns were shown to be reflections of two distinct properties of the ES. Proteolytic treatment after AR completely inhibited the formation of a lip-ESd pattern, whereas formation of the lip-ESp pattern was only marginally inhibited by the proteolytic treatment. The same results were obtained using anti-sperm antibodies, which did not react with acrosome-intact spermatozoa. Proteolytic treatment of spermatozoa before AR induction had no effect on the fusion capacity of the ES after subsequent AR, which implies that the putative fusion protein is not accessible before AR. Thus fusion of liposomes with the ES of human spermatozoa is mediated by a sperm protein(s), whereas the lip-ESp pattern is not likely to represent the liposome-binding stage that precedes the fusion step.

INTRODUCTION

The molecular basis of the fusion between the mammalian spermatozoon and oocyte is still largely unresolved. Much more is known about the preceding events occurring in the sperm head that are required to gain fusion competence.

After binding to the zona pellucida, the egg's extracellular glycoprotein coat, the acrosome reaction (AR) is induced in the spermatozoa. During AR, multiple focal point fusion events between the outer acrosomal membrane and the overlying plasma membrane cause a vesiculation, resulting in a release of the lytic enzyme contents of the acrosome, which probably facilitates the penetration of the zona pellucida [1–3].

During AR the equatorial segment (ES) of the acrosome is left behind, and the plasma membrane of this domain is joined to the remaining part of the outer acrosomal membrane, forming a continuous membrane with a hairpin-like structure [4]. In this structure remnants of the acrosomal contents are left behind, such as binding factors for *Pisum sativum* agglutinin (PSA) and soya-bean trypsin inhibitor (SBTI) [5]. In human sperm the ES encircles the equator of the head. After penetrating the zona pellucida, the spermatozoon binds to the oocyte plasma membrane (oolemma), and subsequently fusion between the two gametes may occur.

In electron microscopic studies, the ES has been reported to be the region where fusion is initiated [3,6–8]. Since only acrosome-reacted sperm can fuse with the oolemma, the ES apparently acquires its fusion competence during AR. Recently, we showed that, after AR induction, the ES is indeed the only domain involved in the interaction with artificial membranes (liposomes) [9]. Additionally, we showed that liposomal lipids are incorporated in the ES membrane by fusion and remain within the margins of this domain by diffusion barriers [10].

Not much is known about the acquisition of fusion competence of the ES during AR. The involvement of a protein that was activated by a metalloendoprotease (MEP) during AR was reported for the interaction of human spermatozoa and zona-free hamster oocytes [11,12]. Inhibition of MEP activity by phosphoramidon resulted in partial inhibition of fusion activity.

Current knowledge concerning biological membrane fusion has, in particular, been derived from studies on virus–host cell fusion [13,14]. Binding of the virus envelope to the target membrane and the subsequent merging of the two bilayers require the participation of unique viral proteins. A similar protein-dependent mechanism may also apply to sperm–egg fusion. Indeed, the involvement of specific proteins in the fusion of mammalian spermatozoa has been proposed [15–18]. However, at present no direct evidence is available that these proteins have fusion-inducing capacity. Recently, a sperm membrane protein from guinea pig, termed PH30 or fertilin, has been proposed to be the inducer of sperm–egg fusion [19]. The protein is a heterodimer composed of α and β subunits. A disintegrin domain in the β subunit is most probably involved in the initial sperm–egg binding [20]. The α subunit has been shown to contain an amino acid sequence with features similar to those of a viral fusion peptide [21]. A synthetic analogue of the putative fusion domain was able to induce fusion of liposomal bilayers, suggesting that fertilin-α might mediate sperm–egg fusion [22].

Abbreviations used: AR, acrosome reaction; ASA, anti-sperm antibodies; ES, equatorial segment; lip-ESd, diffuse fluorescence in the ES; lip-ESp, punctate fluorescence in the ES; MEP, metalloendoprotease; NHS-LC-biotin, sulphosuccinimidyl 6-(biotinamido)hexanoate; PS, phosphatidylserine, PSA, *Pisum sativum* agglutinin; PSA–FITC, fluorescein isothiocyanate-labelled PSA; SBTI, soya-bean trypsin inhibitor; *N*-Rh-PE, *N*-(lissamine Rhodamine B-sulphonyl)phosphatidylethanolamine.

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Identification of sperm factors responsible for the fusion capacity of the ES is complicated by the presence of egg factors in systems that contain both gametes. Although egg factors are definitely involved in binding of spermatozoa [21,23–25] and may be of importance for the species specificity of the process, previous work revealed that sperm factors suffice to accomplish fusion with artificial membranes [9,10]. In the present work, the involvement of sperm factors in the interaction between ES and liposomes is further defined. The results demonstrate the existence of two distinct appearances of the ES, which may have clinical significance in discriminating between fusion-active and -inactive spermatozoa. It could be established that the presence of residual acrosomal constituents in the ES region after AR reflects or possibly mediates the ability of this membrane domain to interact with liposomes. Furthermore, the present work indicates the involvement of proteins in sperm–liposome interaction. The functional properties of this (these) protein(s), i.e. fusion activity, become exposed only after AR.

MATERIALS AND METHODS

Chemicals

PSA, fluorescein isothiocyanate-conjugated PSA (PSA–FITC), SBTI (crude fraction), calcium ionophore A23187, phosphoramidon, pronase (*Streptomyces griseus* protease XIII) and anti-biotin antibodies were obtained from Sigma (St. Louis, MO, U.S.A.). Phosphatidylserine (PS; bovine brain) and *N*-(lissamine Rhodamine B-sulphonyl)phospatidylethanolamine (*N*-Rh-PE) were purchased form Avanti Polar Lipids (Birmingham, AL, U.S.A.). Sulphosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin), avidin, streptavidin and avidin–Texas Red were from Pierce (Rockford, IL, U.S.A.).

Preparation of spermatozoa

Human ejaculated spermatozoa were obtained from normal donors or patients who were normospermic. Spermatozoa were harvested by using a 70 % Percoll cushion as described before [9]. The spermatozoa were centrifuged at 600 ***g*** for 30 min, washed once with sperm buffer and suspended in the same buffer to the desired concentration.

Sperm buffer consisted of 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM NaH_2PO_4, 10 mM Hepes, 10 mM $NaHCO_3$, 115 mM NaCl and 0.1 % (w/v) glucose; the pH was adjusted to 7.4 and the osmolarity to 285 mOsm. Calcium was omitted to avoid ion-induced fusion of liposomes with spermatozoa and liposome–liposome fusion.

Induction of AR in suspension

Spermatozoa (10^8 cells/ml of sperm buffer) were preincubated with 5 mM $CaCl_2$ for 10 min at 37 °C. AR was initiated by addition of calcium ionophore A23187 from a 2 mM stock solution in DMSO to a final concentration of 10 μM. The AR was allowed to proceed for 60 min at 37 °C. The incubation was stopped by centrifugation for 3 min at 600 ***g*** followed by a wash with sperm buffer.

This treatment causes a loss of vitality, and more than 85 % of the cells become stained when incubated with ethidium bromide [9]. However, the ES retains its specific properties seen for vital acrosome-reacted spermatozoa, including its fusogenic activity, as shown previously [9].

Determination of AR

Acrosome-reacted spermatozoa in suspension were detected either with PSA–FITC or SBTI–biotin.

PSA–FITC

This fluorescent lectin conjugate detects acrosomal contents in methanol-permeabilized cells. Incubation was performed as described by Cross et al. [26], with modifications as described previously [9]. At least 100 spermatozoa were scored for FITC fluorescence.

The labelling patterns were classified according to Cross et al. [26]: PSA-A_1, fluorescence located in the acrosomal region; PSA-A_2, fluorescence located in a clearly damaged acrosome; PSA-ES, fluorescence confined to ES; PSA-0, absence of detectable fluorescence.

Since the cells are permeabilized by methanol while the acrosomal contents precipitate, type PSA-A_1 is indicative of sperm with intact acrosomes. Pattern PSA-ES probably reflects acrosome-reacted spermatozoa after mild AR, whereas pattern PSA-0 might represent a degenerate form of AR [5,26].

SBTI–biotin

Biotinylated SBTI was prepared as described previously [5]. Untreated spermatozoa were incubated with 2 mg/ml biotinylated SBTI for 30 min at room temperature in sperm buffer. After washing once, the sperm were incubated for 15 min with 0.25 mg/ml avidin–Texas Red in sperm buffer. The suspension was washed and was examined by fluorescence microscopy.

The labelling patterns of the head were classified as follows [5]: SBTI-H, fluorescence distributed all over the head; SBTI-A, labelling confined to the apical part of the head; SBTI-ES, fluorescence confined to the ES; SBTI-0, no fluorescence detected on the head.

In the experiments described below, at least 100 spermatozoa were counted to score the presence of these labelling patterns.

Preparation of liposomes

Large unilamellar vesicles consisting of PS and containing 0.6 mole % *N*-Rh-PE were prepared by reverse-phase evaporation [27,28]. The vesicles, made in 5 mM sodium acetate/5 mM Hepes/140 mM NaCl, pH 7.4, were sized by extrusion through polycarbonate Unipore membranes (pore size 0.1 μm; Nucleopore, Pleasanton, CA, U.S.A.).

Interaction of spermatozoa with liposomes

Samples were prepared by mixing 10 μl of a liposome suspension (20 nmol of phospholipid) and 100 μl of a spermatozoa preparation [(100×10^6)/ml] at room temperature. Loosely associated liposomes were immediately removed by centrifugation for 3 min at 600 ***g***, and an additional wash step with 1 ml of sperm buffer. The sperm pellet was resuspended in 100 μl of sperm buffer. Fusion was examined by fluorescence microscopy. At least 100 spermatozoa were counted.

The labelling patterns observed with human sperm were placed in the following categories [10]: lip-H, diffuse fluorescence throughout the head, often including midpiece and (parts of) the tail; lip-ESd, a diffuse fluorescent band located at the middle of the head and considered to be the ES; lip-ESp, punctate fluorescence in the ES region, reflecting attached liposomes, without a significant occurrence of fusion, as indicated by a lack of ES-incorporated *N*-Rh-PE; lip-0, absence of sperm-incorporated fluorescence. Spermatozoa with associated liposomes

that could not be specifically defined as liposomal attachment to the ES region were also included in this category.

Zonae pellucidae

Zonae pellucidae were obtained from fertilized and cleaved human oocytes that were not transferred in the *in vitro* fertilization program. These embryo-derived zonae pellucidae still harboured sperm-binding and AR-inducing properties [5]. The embryos were washed through several drops of PBS and were frozen at −60 °C without any cryoprotectant. After thawing, the embryos had completely collapsed, whereas the zonae remained virtually intact. The remaining zonae were washed three times by pipetting in BWW medium supplemented with 10 % pooled serum from pregnant women as described previously [9].

Previous results have shown that zonae derived from fertilized human oocytes usually retain the ability to bind spermatozoa and induce AR [5,9]. This would suggest that, in contrast with some other mammalian systems, the process of zona hardening by oocyte factors apparently is not effectively expressed on the outer surface of the human zona pellucida (cf. [5]).

Induction of AR by zonae pellucidae

Spermatozoa were centrifuged over 70 % Percoll, washed twice with BWW/serum and suspended at a concentration of $(5–10)\times10^6$ motile sperm/ml. To induce capacitation, the suspensions were incubated for 18 h at 37 °C in test tubes. The capacitated spermatozoa (50 μl) in BWW/serum were incubated with 5–10 zonae in a plastic dish under mineral oil at 37 °C. After 6 h the zonae were washed in sperm buffer containing 1 % BSA to remove calcium ions, serum and non-bound spermatozoa. The zonae were finally resuspended in 50 μl of sperm buffer containing 1 % BSA. To this mixture 5 μl of liposomes (final concentration 0.25 mM phospholipid) were added, and fusion was allowed to proceed for 15 min at room temperature. The zonae were washed once with sperm buffer containing 1 % BSA and 5 μg/ml ethidium bromide, followed by two wash steps with sperm buffer/1 % BSA. The bound sperm were scored for labelling patterns as described above. Spermatozoa that had taken up ethidium bromide were considered as permeable and thus non-vital.

Surface labelling

Spermatozoa were surface-labelled with NHS-LC-biotin. Before use, the NHS-LC-biotin was freshly dissolved to 6 mg/ml in DMSO and added to 100×10^6 spermatozoa/ml of sperm buffer at a final concentration of 0.15 mg/ml. Labelling was carried out for 30 min at room temperature. The spermatozoa were pelleted at 600 ***g*** for 5 min, and unreacted NHS-LC-biotin was removed by two wash steps with sperm buffer. The labelled spermatozoa were resuspended in sperm buffer to the desired concentration.

Determination of labelling efficiency

In preliminary experiments, the extent of biotinylation was assessed by SDS/PAGE. Spermatozoa were suspended in 10 mM glycine, pH 6.0/1 % SDS/1 mM PMSF to a concentration of 100×10^6/ml and extracted for 1 h at room temperature. Non-solubilized material was removed by centrifugation at 15000 ***g*** for 5 min. The resulting supernatant, containing approx. 2 mg of extracted protein/ml, was brought to 2 % SDS/10 mM Tris, pH 6.8/0.01 % (w/v) Bromophenol Blue/2 mM dithioerythritol to reduce disulphide bonds. Samples were boiled for 5 min and electophoresed on 12.5 % discontinuous polyacrylamide gels. Approx. 100 μg of protein (the equivalent of 5×10^6 sperm) was loaded per slot. After electrophoresis, part of the gel was silver-stained, and the separated proteins in the corresponding part were transferred to nitrocellulose. Protein blots were incubated with PBS/1 % gelatin for 1 h at room temperature to block free nitrocellulose groups. The blots were incubated with avidin–alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) at a final concentration of 1 μg/ml in PBS/1 % (w/v) gelatin for 1 h. Then, the blots were washed with PBS/0.05 % (v/v) Tween-20, and colour was developed with Nitro Blue Tetrazolium (0.15 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (75 μg/ml) in 100 mM Tris, pH 9.5/100 mM NaCl/5 mM $MgCl_2$. The procedure was found to label reproducibly 20–30 proteins (results not shown).

In subsequent experiments, the efficiency of biotinylation was determined by monitoring the ability of motile spermatozoa to bind avidin-coated latex beads. A volume of a carboxylated latex-bead suspension (Polybead Carboxylate microspheres, #18327, diameter 2 μm; Polysciences, Inc., Warrington, PA, U.S.A.) was washed twice with 0.9 % (w/v) NaCl at 6000 ***g*** for 5 min. The sediment was incubated with the same volume of avidin (1 mg/ml) for 60 min at room temperature. Non-bound avidin was removed by washing, and the beads were suspended in the same volume of 0.9 % NaCl. The beads were stored at 4 °C until use. Droplets of the avidin-coated beads/10 % BSA in sperm buffer/sperm suspension were mixed on slides and examined by microscopy. After labelling, all motile spermatozoa were completely (head and tail) covered with beads.

Proteolytic treatment of spermatozoa

To test the involvement of proteins in the interaction with liposomes, spermatozoa were suspended to 100×10^6 cells/ml of sperm buffer and were incubated with 100 μg/ml pronase E for the desired time at room temperature. Sperm-directed proteolysis was then inhibited by addition of 1 % BSA. The suspension was immediately centrifuged, washed once with sperm buffer containing 1 % BSA and resuspended in the desired buffer.

Adsorption of a serum containing anti-sperm antibodies (ASA) with intact spermatozoa

In the fusion-inhibition studies a patient's serum was used that contained a high titre (>8192) of sperm-agglutinating ASA. To remove antibodies against sperm surface antigens, part of the serum was adsorbed with intact spermatozoa as follows. Serum (1 ml) was incubated for 1 h with spermatozoa. The cells were removed by two centrifugation steps, successively 3 min at 600 ***g*** and 10 min at 10000 ***g***. After each adsorption cycle, the presence of surface-directed antibodies was assessed by the tray agglutination test and a mixed anti-globulin reaction test for IgG, both performed as described previously [29]. After five adsorption cycles (involving a total of 1.5×10^9 spermatozoa) the tray agglutination test agglutination titre was decreased from >8192 to <4 and the percentage of motile cells with IgG was reduced from approx. 100 % to 0 %, indicating that virtually all surface-directed antibodies had been removed.

RESULTS

ESs after AR induction can be revealed by using PS liposomes

After induction of the AR, ESs may display PSA-binding sites. As shown in Figure 1(a), the fraction of sperm cells displaying PSA-binding capacity correlated very well with that showing lip-ESd and lip-ESp patterns, as obtained after incubating acrosome-

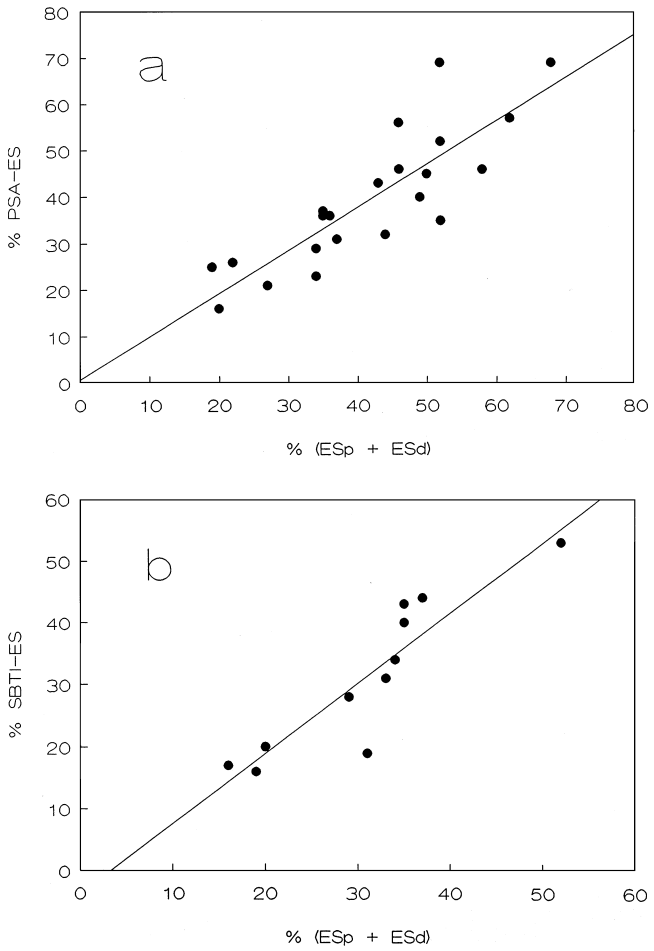

Figure 1 Detection of ESs with liposomes: correlation with the PSA–FITC and SBTI–biotin methods

Spermatozoa (10^8/ml of sperm buffer) were incubated with 5 mM Ca^{2+}/10 μM ionophore A23187 for 60 min at 37 °C. The reaction was terminated by centrifugation, and the cells were resuspended in sperm buffer. A 50 μl aliquot was fixed in methanol for PSA–FITC staining; 100 μl aliquots (non-fixed sperm) were incubated with PS liposomes or SBTI–biotin/avidin–Texas Red. All preparations were examined by fluorescence microscopy, and the labelling patterns were categorized. Only the results for ES patterns are depicted (PSA-ES, SBTI-ES and lip-ESd + lip-ESp). (**a**) Correlation between the proportions of spermatozoa interacting with liposomes (lip-ESp + lip-ESd) and sperm with residual PSA-binding factor in the ES (PSA-ES). Data of least-squares analysis: $y = 0.929x + 0.65$, $r = 0.848$, $P \ll 0.001$, $n = 22$. (**b**) Correlation between the proportions of spermatozoa interacting with liposomes (lip-ESp + lip-ESd) and sperm with residual SBTI-binding factor in the ES (SBTI-ES). Data of least-squares analysis: $y = 1.13x - 3.72$, $r = 0.908$, $P = 0.001$, $n = 11$.

reacted spermatozoa with PS liposomes. By least-squares analysis, a slope of 0.93 was obtained with an intercept of 0.65 ($r = 0.85$, $P \ll 0.001$). It should be noted that the preparations used here often contained high fractions of sperm that had lost all PSA-binding factor (pattern PSA-0; up to 40% of the suspension). The proportions of these PSA-0 fractions were of no influence on the relationship between the liposome- and PSA-binding qualities. Thus ESs that have lost PSA-binding factor also appeared to have lost the ability to interact with liposomes.

As shown in Figure 1(b), a rather good correlation was also observed between the proportion of sperm interacting with liposomes and sperm containing SBTI-binding factor in the ES region (pattern SBTI-ES). A slope of 1.13 was determined with an intercept of -3.72 ($r = 0.91$, $P = 0.001$).

Table 1 Surface proteins are not involved in the interaction between the ES and liposomes

(A) Sperm surface proteins were biotinylated, and subsequently the AR was induced with ionophore A23187. After termination of the reaction, the cells were washed and suspended in sperm buffer/0.3% BSA. Buffer (control), anti-biotin IgGs (0.1 mg/ml) or streptavidin (0.1 mg/ml) was added, and the solutions were incubated for 1 h. After washing with sperm buffer, PS liposomes were added and further treated as described in the Materials and methods section. (B) Spermatozoa (10^8/ml of sperm buffer/0.3% BSA) were preincubated with buffer (control) or 10× diluted serum containing ASA for 1 h. After washing with sperm buffer, the AR was induced as described above. After AR, liposomes were added. (C) Spermatozoa (10^8/ml of sperm buffer) were incubated with 2 mg/ml pronase at 37 °C. After 15 min, BSA was added (final concentration 1%), and the suspension was immediately centrifuged and washed once with sperm buffer. AR induction and subsequent incubation with PS liposomes were as described above. Results are the means of eight experiments.

	Labelling patterns [% of total (S.D.)]			
	Lip-H	Lip-ESd	Lip-ESp	Lip-0
(A) Shielding of biotinylated surface proteins				
Control	11.0 (3.0)	23.0 (2.0)	14.0 (4.0)	52.0 (5.2)
+ Anti-biotin	6.7 (0.6)	21.7 (2.1)	11.7 (2.5)	60.0 (4.4)
+ Streptavidin	9.3 (0.6)	20.7 (4.2)	12.7 (5.0)	57.3 (9.1)
(B) Preincubation with ASA before AR				
Control	6.3 (6.0)	25.7 (5.8)	23.3 (6.1)	44.7 (6.5)
+ ASA	5.0 (4.4)	25.3 (2.9)	22.7 (8.1)	47.0 (9.2)
(C) Proteolysis before AR				
Control	7.0 (7.6)	19.3 (3.7)	17.8 (3.2)	55.8 (12.2)
+ Pronase	5.5 (4.8)	18.8 (6.5)	18.0 (5.7)	57.8 (15.5)

Since there was a strong correlation between the presence of acrosome-specific compounds in the ES region and its capacity to interact with liposomes, it was of interest to determine whether both acrosomal binding factors were directly involved in the interaction with the phospholipid vesicles. Incubation of acrosome-reacted spermatozoa with 200 μg/ml (unconjugated) PSA for 1 h before liposome addition resulted in a very prominent agglutination of the vesicles, mainly in the ES region. Unfortunately, this phenomenon masked the underlying surface, thus making it impossible to establish whether fusion still occurred. Preincubation of the spermatozoa with 2 mg/ml SBTI–biotin for 1 h had virtually no effect on the subsequent interaction with liposomes, as reflected by unchanged proportions of lip-ESp and lip-ESd profiles in control versus treated cells (results not shown).

Surface proteins of acrosome-intact spermatozoa exposed before AR are not involved in fusion

To determine the localization of proteins involved in fusion, acrosome-intact spermatozoa were treated with pronase for 15 min. The treatment effectively removed surface proteins, as determined with biotinylated spermatozoa: after proteolytic treatment only three protein bands could be observed, whereas in non-treated cells more than 30 protein bands were apparent on silver-stained gels. Protease treatment of biotinylated spermatozoa did not affect motility, but eliminated the ability to bind avidin-coated beads. After subsequent AR induction followed by an incubation with PS liposomes, the spermatozoa showed no difference in liposome binding and fusogenic properties, when compared with non-pronase-treated spermatozoa (Table 1).

In another approach, the spermatozoal surface proteins were labelled with biotin before AR induction. After AR induction, the cells were incubated with either anti-biotin IgGs or

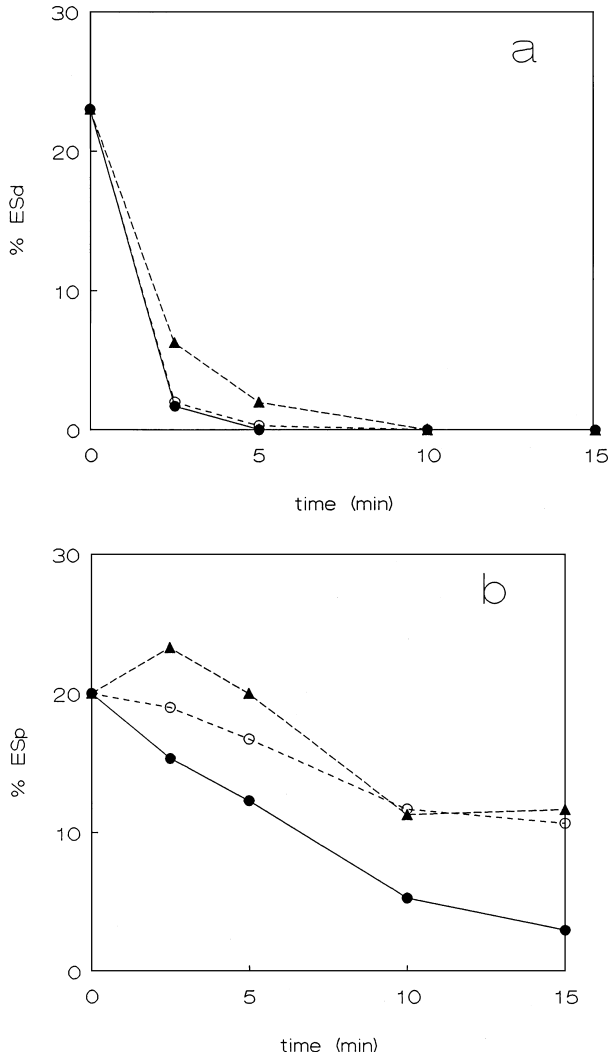

Figure 2 Effect of proteolytic treatment on the capacity of acrosome-reacted sperm to interact with PS liposomes

Spermatozoa (10^8/ml) were acrosome reacted with A23187 as described in the legend to Figure 1, washed once and resuspended to the initial concentration in sperm buffer. The suspension was divided in portions of 1 ml and received 20 (●), 8 (○) and 4 (▲) μg of pronase respectively. Proteolysis was carried out at 37 °C. At the indicated times 100 μl samples were removed, immediately supplied with 1% BSA and centrifuged for 3 min. The sperm pellet was washed once with sperm buffer/1% BSA. Subsequently, the cells were resuspended in sperm buffer containing PS liposomes (0.25 mM phospholipid) and immediately centrifuged for 3 min. After resuspension in sperm buffer, at least 100 spermatozoa were scored for fluorescence labelling patterns. Only the results (means of four separate experiments) for patterns lip-ESd (**a**) and lip-ESp (**b**) are shown.

streptavidin. Fusion and binding of liposomes were only marginally reduced, suggesting that shielding of the ES surface in this manner did not interfere with liposome–sperm interaction (Table 1). Incubation of the acrosome-reacted, biotinylated cells with avidin, instead of streptavidin, resulted in strong aggregation of liposomes over the entire surface of the spermatozoa, including the ES, thereby preventing an accurate scoring of the fluorescence pattern of the underlying ES (results not shown).

Finally, spermatozoa were also incubated with a serum containing a high concentration of antibodies, as judged by IgG–mixed anti-globulin reaction. The preincubation with ASA did not hinder AR induction by ionophore A23187, and no difference in liposome interaction patterns could be observed between ASA-loaded and control spermatozoa (Table 1).

In summary, these experiments demonstrate that surface proteins of intact spermatozoa, i.e. non-acrosome-reacted spermatozoa, are not involved in the binding or fusion of the cells with PS liposomes.

Table 2 Effect of ASA on the interaction between liposomes and the ES of acrosome-reacted spermatozoa

Spermatozoa (10^7/100 μl of sperm buffer) were acrosome reacted by addition of ionophore A23187 as described in the Materials and methods section. After washing, the cells were sedimented, and the pellet was incubated for 30 min with 50 μl of control serum (without ASA), the serum of a patient with ASA against total sperm, or the latter serum but adsorbed with intact sperm. After washing with sperm buffer, the spermatozoa were incubated with liposomes as described in the Materials and methods section. Results are the means of eight experiments, except for the percentages of control, which are the means of the individual percentages.

Serum added after AR	Labelling patterns [% of total ± S.D. (% of control)]			
	Lip-H	Lip-ESd	Lip-ESp	Lip-0
Control	7.1 ± 7.6	23.4 ± 3.3 (100)	25.1 ± 4.6 (100)	44.3 ± 7.1
Total ASA	5.4 ± 7.4	6.1 ± 1.6 (26.0 ± 5.0)	19.6 ± 7.6 (79.4 ± 28.7)	68.9 ± 11.0
Adsorbed ASA	5.0 ± 5.3	6.7 ± 3.0 (29.1 ± 12.7)	24.4 ± 7.5 (98.7 ± 26.7)	64.6 ± 9.8

Protease treatment of acrosome-reacted spermatozoa inhibits fusion

To establish whether binding and/or fusion of liposomes with the ES is protein dependent, the AR was induced by A23187 treatment, and the cells were subsequently treated with different concentrations of pronase before addition of liposomes (Figure 2).

Before pronase treatment, approx. 25% of the spermatozoa displayed a lip-ESd pattern and 20% displayed a lip-ESp pattern. Treatment with different concentrations of pronase rapidly eliminated fusogenic activity (Figure 2a), whereas the capacity of binding liposomes only gradually diminished (Figure 2b). Complete abolition of the binding capacity was only achieved after treatment with high concentrations of pronase (1 mg/ml for 15 min; results not shown).

Non-agglutinating ASA inhibit liposome–ES fusion

To obtain additional evidence for the involvement of specific sperm proteins, we investigated whether sera containing antibodies against sperm-specific antigens (ASA) could inhibit interaction of the ES with PS liposomes.

Incubation of acrosome-reacted spermatozoa with serum containing a high agglutination titre of ASA resulted in a strong inhibition of fusion (type lip-ESd; Table 2), whereas a negative control serum had no inhibiting activity. By contrast, the proportion of sperm showing pattern lip-ESp was only slightly diminished. ASA-containing sera from other patients gave comparable results (not shown).

To test whether ASA against surface proteins were involved in the inhibition, we adsorbed the serum to acrosome-intact spermatozoa (i.e. sperm surface antigens) until surface-directed ASA were undetectable. The adsorbed serum was equally potent in the inhibition of fusion of acrosome-reacted spermatozoa with liposomes. However, the adsorbed serum fraction had no effect on the proportions of spermatozoa displaying the lip-ESp pattern (Table 2).

MEP-activated protein is not involved in the fusion of the ES with liposomes

It has been reported that the acquisition of fusion competence during AR is mediated by an MEP [12]. To examine whether such an activation step would also affect the fusion of spermatozoa with liposomes, the AR was induced by incubation of sperm with calcium ionophore A23187 in the presence and absence of the MEP inhibitor phosphoramidon. No effect of phosphoramidon on subsequent liposome fusion or binding properties of the ES could be observed [pattern lip-ESd: 22.5 ± 3.1 (S.D.)% in the absence and 21.8 ± 6.2% in the presence of phosphoramidon; pattern lip-ESp: 19.3 ± 15.4% in the absence and 21.0 ± 11.5% in the presence of phosphoramidon; results of five independent experiments].

Initiation of AR by A23187 under the given conditions leads to a significant degree of ruptured membranes and loss of vitality [9]. To exclude the possibility that concomitant release of (aspecific) proteases could have accounted for activation of a potential fusion protein, the following control experiment was carried out. AR was induced on the zona pellucida surface, which produces vital acrosome-reacted spermatozoa [5]. The vital sperm bound to the zona showed either a lack of interaction with the liposomes (pattern lip-0) or the fusion-related pattern lip-ESd. A punctate fluorescence (pattern lip-ESp) was conspicuously absent. The presence of phosphoramidon throughout the co-incubation procedure of zonae pellucidae and spermatozoa had no inhibitory effect on the percentage of bound sperm displaying fusion (pattern lip-ESd). On the contrary, the presence of the MEP inhibitor slightly elevated the number of sperm bound per zona, as well as the percentage of sperm with pattern lip-ESd (results not shown).

DISCUSSION

Insight into the mechanism of biological membrane fusion has been derived, in particular, from studies involving the fusion of enveloped viruses with a host cell membrane. It has been shown that both the binding and the actual fusion step are mediated by specific viral proteins. Both activities may be located on one or two different proteins [13,14]. In fertilization, fusion between sperm and oocyte membranes also appears to involve proteins. Some invertebrate sperm contain proteins with putative fusion domains [30]. Isolated proteins of such species were shown to be capable of inducing fusion of liposomes [31–33]. Recently, a potential fusion protein was identified in guinea pig sperm [21]. This protein seems to form dimers with an integrin-like protein, which may be involved in the initial binding of the sperm to the oocyte membrane. The observation that fusion is inhibited by monoclonal antibodies against sperm proteins further supports the involvement of mammalian sperm proteins in sperm–egg fusion [15,17]. However, at present no direct evidence is available that would indicate that these proteins function as fusion peptides; inhibition of fusion by antibodies may be due to shielding (see also [34]), or may be attributed to other, non-specific, effects on the interaction with an egg factor. In this regard, the monoclonal produced by Okabe et al. [17] most probably inhibits sperm–egg binding, rather than fusion [35].

In a previous paper, we demonstrated that the ES of spermatozoa is the only membrane domain capable of interaction with liposomes, provided that the cells have undergone AR [9]. With pure phospholipid vesicles as target membranes for spermatozoa, the potential involvement of egg factors can be ruled out. Hence, this approach allows us to characterize further and define the role of human sperm protein(s), potentially involved in sperm–egg fusion.

The experiments revealed that fractions of sperm suspensions with patterns lip-ESd and lip-ESp correlated very well with the fractions containing PSA- and SBTI-binding factor in the ES region (Figure 1). When AR was induced with the calcium ionophore A23187, increased fractions in the sperm preparations were observed that did not contain any PSA-binding factor (pattern PSA-0). As discussed previously [5], the pattern PSA-0 may reflect a damaged ES as a result of harsh AR induction conditions, in which case also no ES-localized SBTI-binding factor is observed. As demonstrated here, such ESs do not interact with liposomes. Thus the presence of remaining acrosomal factors in the ES appears to be a prerequisite for the ability of this membrane domain to interact with liposomes. Although a direct involvement of the SBTI- and PSA-binding factors in this interaction could be, at least in part, excluded, it may well be that the remaining acrosomal matrix after AR is indispensible for the maintenance of a specific ES structure, providing a scaffold for interaction with liposomes.

It should be mentioned that, in contrast with ionophore-induced AR, all spermatozoa that were acrosome reacted on zonae pellucidae contain both SBTI- and PSA-binding factors that remain associated to the ES [5]. Also, these acrosome-reacted cells are readily detected with liposomes. Given its simplicity, this method is, therefore, very convenient for the detection of acrosome-reacted spermatozoa on zonae pellucidae.

Evidence that sperm proteins are directly involved in the fusion of (liposomal) membranes with the ES was derived from experiments with acrosome-reacted human sperm treated with protease. Such treatment abolished all fusion activity in the ES within 5 min (Figure 2). It would thus appear that sperm–liposome fusion resembles virus–liposome fusion [13], requiring a fusion peptide to induce membrane merging. As for virus-induced fusion, sperm–liposome fusion also seems Ca^{2+} independent, as the addition of 2 mM Ca^{2+} (final concentration) did not affect overall fusion (results not shown). Interestingly, protease treatment allowed a differentiation between binding and fusion functions of the human ES (reflected by patterns lip-ESp and lip-ESd respectively). As shown previously [10], within 3 min after addition of liposomes to acrosome-reacted spermatozoa, those cells capable of interaction have either undergone fusion with the vesicles (pattern lip-ESd) or only bound them (lip-ESp). Both fractions remain constant during prolonged incubation for up to 1 h [10]. Apparently, spermatozoa displaying pattern lip-ESp do not proceed towards the fusion-reflecting pattern lip-ESd. The present results further support the suggestion that pattern lip-ESp is unlikely to represent the binding step preceding fusion. Within 5 min after commencing proteolysis, fusion activity was almost completely abolished (Figure 2). In contrast, the fraction of cells displaying bound liposomes (lip-ESp) was not significantly changed over the same time interval. The bound fraction only gradually decreased during prolonged incubation. Evidently, before fusion, liposomes first have to bind to the ES. If this occurred via the mechanism that also leads to pattern lip-ESp, approximately a doubling in the fraction of spermatozoa displaying lip-ESp would have been expected after a short period of proteolytic treatment, given the high rate of loss of fusion activity and the relative insensitivity of pattern lip-ESp (Figure 2). Instead, the fraction of sperm with lip-ESp pattern appeared to be rather stable, whereas the proportion of ESs showing pattern lip-ESd seemed to lose both fusion and binding capacity upon proteolysis. Furthermore, ASA only inhibited the fusion of liposomes with the ES, but did not cause an enhancement of the cell fraction displaying a lip-ESp pattern (Table 2).

Thus our results suggest that the lip-ESp pattern does not represent a dynamic, transient binding stage that precedes the

fusion step. The existence of different binding mechanisms between mammalian sperm and egg, one leading to fusion, the other to mere attachment, has also been suggested by Myles [19]. It should be noted that the lip-ESp pattern was only rarely found on zona pellucida-bound spermatozoa, but was prominently detectable after more harsh, *in vitro* AR induction treatments. Thus arguing, the lip-ESp pattern could reflect an aberrant ES, i.e. different from a damaged ES that has lost acrosomal matrix compounds, since a binding pattern is virtually absent in that case.

The changes in the ES during AR that eventually result in the acquisition of fusion competence are largely unknown. The formation of a continuous membrane by junction of the plasma membrane with the outer acrosomal membrane in the ES might allow a redistribution of proteins and/or lipids. In this paper, we have shown that a protein(s) is (are) involved. Hence, the alteration in ES properties might, among other possibilities, be due to activation of proteins or to exposure of previously hidden/shielded peptides derived from the inner acrosomal membrane or plasma membrane. Pronase treatment of spermatozoa before AR induction (shown to remove surface proteins) had no effect on the fusion capacity of the ES after subsequent AR. Nor did shielding of biotinylated surface proteins by streptavidin and anti-biotin IgG after AR exert any effect (Table 1). Moreover, ASA that had been adsorbed with non-acrosome-reacted sperm, and which no longer contained agglutination capacity, had not lost inhibitory capacity (Table 2). The experiments described here provide evidence that the proteins involved in fusion are not accessible and/or fusion active before AR induction.

A possible mechanism by which activation during/after AR may occur could involve selective proteolysis by an MEP, as proposed for sea urchin sperm [36] and human sperm [12]. Furthermore, the guinea pig fertilin-β subunit contains an MEP domain with a potentially active site [37]. Addition of specific MEP inhibitors partially inhibited fusion of human sperm with zona-free hamster eggs [12]. However, inhibition of MEP activity had no effect on the capacity of acrosome-reacted human sperm to fuse with PS liposomes. It might be possible that the protein activated by an MEP is involved in the interaction with an oocytal factor, whereas in our model system only sperm factors are examined.

An alternative mechanism for activation of a fusion protein could be the modulation of its conformation by the lipid environment. The Semliki Forest virus has been shown to depend on sphingolipid to express its activity [38]. In boar sperm the ES becomes enriched in sperm-specific sulphogalactosylglycerolipid during capacitation and is retained in this region after AR [39]. This lipid is able to induce the hexagonal II phase, which was suggested to enhance the fusogeneity of the ES region. Whether sulphogalactosylglycerolipid influences the activity of sperm fusion protein(s) and/or the fluidity of the ES membrane remains to be investigated.

The apparent inaccessibility of the putative fusion protein in human sperm before AR could be explained by assuming an exclusive location of this activity in the acrosomal membrane. During AR the acrosomal membrane is joined to the plasma membrane in the ES, and the protein(s) might then be able to diffuse to the surface-located part of this domain. The protein would be retained in the ES by barriers to lateral diffusion [10], thereby achieving a locally enriched fusion-protein gradient.

Our results contrast with the putative fusion protein fertilin of guinea pig sperm. This protein has been initially identified by monoclonal antibodies raised against surface proteins, exposed on the plasma membrane [40]. Furthermore, fertilin appears to mature by selective proteolysis in the epididymis (before ejaculation), and no further activation seems to be necessary [41]. Finally, guinea pig fertilin is (surface) located in the post-acrosomal plasma membrane of both intact and acrosome-reacted spermatozoa [16]. Such a localization does not agree with the proposed mechanism that the ES of mammalian sperm is the initial membrane domain involved in fusion [6], a mechanism supported by our observations with liposomes [9,10]. Although a synthesized peptide, corresponding to the proposed fusion peptide of fertilin-α, could induce fusion of liposomes [22], reconstitution of its fusogenic activity in its natural environment (fertilin is a transmembrane protein) has yet to be carried out. At present, one more fusion-inhibiting antibody has been described that is only reactive with the ES of acrosome-reacted spermatozoa [15]. The putative fusion proteins of marine invertebrates, bindin and lysin, are aqueous soluble acrosomal proteins, rather than integral membrane proteins [30–33]. The proteins are associated with the acrosomal process after AR. However, it is unclear whether this protruding acrosomal membrane domain can be compared with the inner acrosomal membrane or, better, to the ES. The presence of the ES is restricted to the spermatozoa of eutherian mammals [6]. Compared with invertebrate sperm, the environment of this membrane domain on the fusion protein might be quite different.

In conclusion, we have shown that liposomes can be applied as a convenient tool to detect ESs after AR that contain remnants of the acrosomal matrix. Since such ESs are thought to represent the endstage of the physiological AR, the method offers a rapid, and reliable, means of detecting acrosome-reacted spermatozoa, induced in suspension or when bound to zonae pellucidae. Furthermore, liposomes reveal that these apparently intact ESs are present in two distinct forms. One form primarily binds liposomes, whereas the other leads to the actual merging with artificial membranes. Since zona pellucida-induced AR results predominantly in the fusogenic ES-form (as reflected by an almost exclusive presence of pattern lip-ESd), this appearance may represent spermatozoa ultimately involved in the fusion with the oocyte membrane. The pattern lip-ESp seems to reflect an appearance that does not represent the preceding step in fusion but rather a pattern revealed after artificial (ionophore-induced) AR. This artificial association of liposomes is further emphasized by the fact that protease treatment does not cause an enhanced or otherwise altered binding pattern, in spite of the fact that the fusogenic form is no longer apparent after proteolytic treatment. Hence, a frustrated fusion event does not bring about a binding feature seen at conditions where 'binding' (lip-ESp) is not yet affected (compare Figure 2a with 2b). It is tempting to speculate that this finding could imply that the 'prefusion'-binding and fusion functions are expressed in one and the same protein.

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