Evidence for the existence of lipid-diffusion barriers in the equatorial segment of human spermatozoa

Eugène G. J. M. ARTS,* Siemen JAGER*† and Dick HOEKSTRA†

Departments of *Obstetrics & Gynaecology and †Physiological Chemistry, State University of Groningen, Groningen, The Netherlands

Liposomes consisting of negatively charged phospholipids interact almost exclusively with the equatorial segment (ES) of human spermatozoa provided the cells have undergone the acrosome reaction (AR) [Arts, Kuiken, Jager and Hoekstra (1993) *Eur. J. Biochem.* **217**, 1001–1009]. Using fluorescently tagged liposomes, this interaction can be observed by fluorescence microscopy, showing either a diffuse fluorescence in the ES region (pattern ESd, presumably reflecting membrane-incorporated lipids as a result of fusion) or a punctate fluorescence (pattern ESp, representing adhering liposomes). These distribution patterns remain unchanged during prolonged incubation, up to 40 min. Not only do these observations suggest the existence of fairly specific liposomal binding sites, associated with the ES region, but also that a barrier to lipid lateral diffusion seems to

exist in the ES membrane. Using liposomes that contain fluorescent lipid analogues in either both leaflets or in the inner leaflet only, we demonstrate that this putative barrier entails both membrane leaflets. Treatment with EDTA caused fluorescence to spread from the ES towards other membrane domains. Since only spermatozoa displaying pattern ESd were affected by the chelator, the randomization was not caused by EDTA-induced fusion activity. Therefore, this observation provides further evidence that in spermatozoa displaying pattern ESd the fluorescent lipid analogues were incorporated in the ES membrane as a result of fusion. Furthermore, these experiments support the view of the existence of a transmembranous block to lipid lateral diffusion in the ES, the stability of which may be governed by bivalent cations.

INTRODUCTION

The mammalian spermatozoon is a highly polarized cell type with respect to both morphology and molecular structure. Morphologically, two main features may be distinguished: the head and the flagellum. The polarized nature is also expressed at the molecular level. Within the head of human sperm, the anterior part of the nucleus is covered by the acrosome. The plasma membrane (PM) overlying most of the acrosome is separated from the postacrosomal PM by the equatorial segment (ES), which constitutes the equator of the head. The PM consists of domains that harbour specific proteins [for a review, see Eddy (1988)], while the existence of non-diffusible lipid fractions has also been suggested (Wolf et al., 1986).

The regional differentiation of the sperm head becomes even more apparent after the acrosome reaction (AR). The AR is an exocytotic process and involves a vesiculation, caused by focal-point fusion events between the apical part of the PM and the underlying outer part of the acrosomal membrane. Most probably, the AR is induced by the zona pellucida, the glycoprotein matrix surrounding the oocyte and as a result the (former) inner acrosomal membrane becomes exposed to the environment (Yanagimachi, 1988). The ES is left behind as a rigid structure formed by remnants of the inner and outer acrosomal membranes and PM. Only after onset of the AR can the spermatozoon penetrate the zona pellucida and acquire the competence to fuse with the oocytal membrane.

Electronmicroscopic evidence suggests that fusion with the oolemma starts at the ES of acrosome-reacted spermatozoa

(Bedford et al., 1979; Talbot and Chacon, 1982; Yanagimachi, 1988; Clark and Koehler, 1990). Recently, we have shown that the ES of acrosome-reacted human spermatozoa is the sole membrane domain of the head that is involved in sperm-liposome fusion (Arts et al., 1993). Indeed, fluorescence microscopy indicated that fluorescent phospholipid analogues were specifically incorporated in the ES by membrane fusion. In the present paper we corroborate and extend our previous observations and demonstrate that lateral diffusion of these analogues, subsequent to the fusion event, is restricted to the area bounded by the ES. The diffusion barriers of the ES display properties similar to those of tight junctions of epithelial cells, and where the diffusion block was relieved by calcium depletion (van Meer and Simons, 1986). Consequently lipid derivatives diffused throughout all sperm surface membranes. However, in contrast with tight junctions, the diffusion barrier existing in the ES encompasses both the outer and the inner monolayer.

MATERIALS AND METHODS

Chemicals

Phosphatidylserine (PS, bovine brain), *N*-(lissamine Rhodamine B sulphonyl)phosphatidylethanolamine (N-Rh-PE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine (N-NBD-PE) and 1-acyl-2-{6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino}hexanoyl}phosphatidylcholine (*C*₆-NBD-PC) were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A. Hepes was from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Calcium ionophore A23187 and fluorescein isothiocyanate-

Abbreviations used: AR, acrosome reaction; *C*₆-NBD-PC, 1-acyl-2-{6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino}hexanoyl}phosphatidylcholine; DMSO, dimethyl sulphoxide; ES, equatorial segment; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B-sulphonyl)phosphatidylethanolamine; PM, plasma membrane; PS, phosphatidylserine; PSA-FITC, fluorescein isothiocyanate-labelled *Pisum sativum* (pea) agglutinin; RET, resonance energy transfer.

† To whom correspondence should be sent.

labelled *Pisum sativum* (pea) agglutinin (PSA-FITC) were from Sigma, St. Louis, MO, U.S.A. Bisbenzimidazole Hoechst 33258 was from Calbiochem, San Diego, CA, U.S.A. Percoll was purchased from Pharmacia-LKB Biotechnology, Uppsala, Sweden, and $10\times$ -concentrated Earle's balanced salt solution was from Serva, Heidelberg, Germany.

Preparation of spermatozoa

Human ejaculated spermatozoa were obtained from men who were normospermic according to WHO criteria (World Health Organization, 1987). Spermatozoa were harvested by using a 70% (v/v) Percoll cushion as described before (Arts et al., 1993). 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 2.8 mM KCl, 0.5 mM MgCl₂, 0.7 mM NaH₂PO₄, 10 mM Hepes, 10 mM NaHCO₃, 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 CaCl₂ for 10 min at 37 °C. AR was initiated by addition of 10 μ M calcium ionophore A23187 from a stock solution in dimethyl sulphoxide (DMSO) [final DMSO concn. 0.5% (v/v)]. 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.

Occasionally, vitality was determined by addition of Hoechst 33258 to a final concentration of 50 μ g/ml. Spermatozoa that showed fluorescent nuclei were considered non-vital.

Determination of AR

Acrosome-reacted spermatozoa in suspension were detected with PSA-FITC, which binds to acrosomal contents in methanol-permeabilized cells as described by Cross et al. (1986), and modified as described previously (Arts et al., 1993).

Preparation of liposomes

Large unilamellar vesicles (liposomes) consisting of PS and containing small amounts of fluorescent lipid analogues were prepared by reverse-phase evaporation (Szoka and Papahadjopoulos, 1978; Hoekstra and Düzgüneş, 1986). 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 μ m; Bio-Rad).

For fusion measurements, liposomes were used that contained 0.8 mol % each of the lipid analogues N-Rh-PE and N-NBD-PE.

Symmetrically labelled liposomes for fluorescence microscopy contained either 0.6 mol % N-Rh-PE or N-NBD-PE. Asymmetrically labelled liposomes were prepared as described by McIntyre and Sleight (1991). Briefly, symmetrically labelled vesicles containing N-NBD-PE (1 ml of 2.5 mM phospholipid) were incubated with 30 mM of the NBD quencher sodium

dithionite for 15 min at room temperature. To remove the sodium dithionite, the vesicles were subsequently dialysed for 16 h at 4 °C against 100 ml of sperm buffer with two changes of buffer. Dithionite treatment reduces the fluorescence by 50% (for large unilamellar vesicles) or 70% (for small unilamellar vesicles), indicating a specific abolishment of NBD fluorescence in the outer leaflet and leaving that in the inner leaflet intact (Hoekstra et al., 1993).

Fusion measurements

Non-treated or acrosome-reacted spermatozoa were incubated with the liposomes and fusion was measured by monitoring lipid mixing with an assay based on resonance energy transfer (RET) or was analysed by fluorescence microscopy.

RET assay

With this assay, fusion is followed by monitoring lipid mixing (Struck et al., 1981). The liposomes contain 0.8 mol % each of the fluorescent phospholipid analogues N-Rh-PE and N-NBD-PE. At these probe concentrations photons emitted by the excited N-NBD-PE will be transferred to N-Rh-PE and NBD-fluorescence will be almost negligible. Upon fusion with a non-labelled membrane the probes will be diluted, resulting in a decrease in energy transfer efficiency and hence an increase in NBD fluorescence.

NBD fluorescence was measured with a Perkin-Elmer MPF 43 spectrofluorimeter at excitation and emission wavelengths of 465 nm and 530 nm respectively. The temperature was maintained at 37 °C with a thermostatically controlled cuvette holder equipped with a magnetic stirring device. The final incubation volume was 2 ml.

For lipid-dilution measurements in the absence of free liposomes, 50×10^8 spermatozoa in sperm buffer were mixed with the liposomes (100 nmol of lipid), immediately layered on top of 35% Percoll and centrifuged for 3 min at 600 *g*. After being washed once, the cells were incubated in sperm buffer and the fluorescence scale was set to 0%. Lipid dilution was initiated by addition of EDTA. At the end of the reaction, 1% (v/v) Triton X-100 was added to determine 100% fluorescence (infinite dilution). Corrections were made for sample dilution and for effects of the detergent on the quantum yield of NBD. The resulting value was taken to represent the fraction liposomes associated with the spermatozoa. To determine the maximal fluorescence level of the initially added liposomes, the vesicles (100 nmol of phospholipid) were disrupted in 1% (v/v) Triton X-100 in a volume of 2 ml and corrected as described above.

Fluorescence microscopy

Samples were prepared by mixing 10 μ l of a liposome suspension (20 nmol of phospholipid and labelled with 0.6 mol % N-Rh-PE) and 100 μ l of a spermatozoa preparation (10^8 /ml) at room temperature. Loosely associated liposomes were immediately removed by centrifugation for 3 min at 600 *g*. Fusion was studied by fluorescence microscopy in a Leitz Dialux microscope using incident illumination. At least 100 spermatozoa were counted.

The labelling patterns observed with human sperm were placed into the following categories: type H (Figure 1, panel a), diffuse fluorescence throughout the head, often including midpiece and (parts of) the tail; type ESd (Figure 1, panel b), a continuous fluorescent band located at the middle of the head and considered to be the ES; type ESp (Figure 1, panel c), punctate fluorescence in the ES region, reflecting attached liposomes, without a

Figure 1. Labelling patterns observed after incubation of spermatozoa with liposomes

Spermatozoa were acrosome-reacted with A23187 and incubated for 3 min with PS liposomes (0.25 mM phospholipid). After removal of free liposomes the cells were studied by fluorescence microscopy. Panel a, type H: fluorescence distributed throughout head and tail. Panel b, type ESd: diffuse fluorescence in the ES (large arrow); and type 0: no fluorescence incorporated in the sperm head (large arrowheads). The picture was obtained using combined bright-field and incident (rhodamine-channel) illumination. Panel c, type ESP: punctate fluorescence in the ES only (small arrowhead); in spermatozoa viewed from the side the pattern may appear patchy due to accumulation of fluorescence from different focal planes (small arrow). The bar represents 5 μm .

significant occurrence of fusion as indicated by a lack of ES-incorporated N-Rh-PE; this clearly punctate pattern can be readily distinguished from the continuous, diffuse ESd-pattern if examined at magnifications higher than $400\times$; type 0 (Figure 1, panel b), absence of sperm-incorporated fluorescence. Spermatozoa with associated liposomes that could not be specifically defined as liposomal attachment to the ES region were also counted 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. The embryos were washed through several drops of PBS and 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 thrice by pipetting in Biggers, Whitten and Whittingham (BWW) medium (Biggers et al., 1971), supplemented with 10% pooled serum from pregnant women (Zeilmaker and Rijkmans, 1986). Previous results (Arts et al., 1993; 1994) have shown that zonae derived from fertilized human oocytes usually retain the ability to bind spermatozoa and induce AR. This would suggest that, in contrast with some other mammalian systems (Dietl and Rauth, 1989), the process of zona hardening by oocyte factors apparently is not effectively expressed on the outer surface of the human zona pellucida (cf. Arts et al., 1994).

Induction of AR by zonae pellucidae

Spermatozoa were centrifuged over 70% Percoll, washed twice via centrifugation with BWW/serum and were suspended at a

concentration of $(5-10) \times 10^6$ motile sperm/ml. To induce capacitation, the suspensions were incubated for 18 h at 37°C in test tubes. Capacitated spermatozoa in BWW/serum (50 μl) were coincubated 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 Ca^{2+} , serum and non-bound spermatozoa. The zonae were finally resuspended in 50 μl of sperm buffer containing 1% BSA. To this mixture 5 μl liposomes (final concentration 0.25 mM phospholipid) were added and fusion was allowed to proceed for 15 min at room temperature. After washing three times with sperm buffer containing 1% BSA, the zonae were incubated with 30 mM EDTA (prepared by diluting a 0.1 M stock solution in distilled water, and set to 300 mosM, with sperm buffer) or an equal volume of sperm buffer (control) for 20 min at room temperature. The zonae were washed twice with sperm buffer containing 1% BSA, followed by a final wash step with the same buffer containing 50 $\mu\text{g/ml}$ Hoechst 33258. The bound sperm were scored for labelling patterns and supravitality, as described above.

Estimation of the human sperm surface area

For quantification of the lipid mixing, occurring after fusion of liposomes with the ES and subsequent lateral diffusion throughout the cell, we determined the total sperm PM surface. Since the human sperm head is oval when flat-lying, but chisel-shaped when viewed from the side, it is difficult to set up an accurate equation for the calculation of the surface. Therefore we only made a rough estimation of the total surface of the head by doubling the surface area, determined for the projected area (van Duijn, 1975). Thus a value of $21 \mu\text{m}^2$ was calculated. The midpiece was assumed to be a cylinder with a length of 4.5 μm and a diameter of 1 μm (van Duijn, 1975; Cummins and Woodall, 1985), giving a surface area of $14 \mu\text{m}^2$. From the known volumes of the principal piece and endpiece of the tail (van Duijn, 1975) and the length of both domains (45 and 5 μm , respectively; Fléchon and Hafez, 1976), a total area of approx. $100 \mu\text{m}^2$ was estimated, assuming both to be cylinders. The total membrane surface of the human spermatozoon was therefore estimated to be approx. $135 \mu\text{m}^2$.

The ES was taken as an ellipsoid encompassing the head at its broadest and thickest part (width 3.2 μm , diameter 2 μm ; van Duijn, 1975) and 1 μm height (as estimated from electron micrographs). With these estimates a surface of approx. $8 \mu\text{m}^2$ for the PM-derived part of the ES can be calculated. Taking into account the hairpin structure of the ES after AR, this value may be doubled or tripled, when the outer and inner acrosomal membrane-derived parts, respectively, are included.

Statistical analysis

Data were analysed by the Wilcoxon (1945) matched-pairs signed-rank test.

RESULTS

Fluorescent lipid analogues fail to diffuse beyond the boundaries of the ES

Liposomes consisting of the negatively charged phospholipid PS and containing 0.6 mol % of the fluorescent analogue N-Rh-PE interact with the ES of spermatozoa only after AR (Arts et al., 1993). To study the ultimate fate of membrane-incorporated N-Rh-PE, we induced the AR with calcium ionophore A23187 and co-incubated spermatozoa and liposomes for a prolonged period (Table 1). Within 4 min after addition of the liposomes, 10–15%

Table 1 Prolonged co-incubation of spermatozoa and PS liposomes has no effect on fluorescence localization

Acrosome-reacted spermatozoa (with A23187) were incubated with PS liposomes (0.25 mM lipid) at room temperature. At the indicated times non-associated liposomes were removed by washing and the patterns were scored as described in Figure 1. At least 100 spermatozoa were counted. The results are means (S.D.) for four separate experiments. Paired data for the different sampling times within each labelling pattern were analysed with the Wilcoxon matched-pairs signed rank test and found not to be significantly different ($P > 0.05$).

Fusion time (min)	Type ...	Proportion of spermatozoa of each type (%)			
		H	ESd	ESp	0
3		7.2 (4.2)	36.0 (7.7)	16.8 (5.1)	40.0 (6.5)
15		16.8 (10.4)	33.4 (5.0)	14.4 (4.0)	35.4 (10.2)
30		12.2 (6.5)	32.0 (12.2)	16.0 (3.7)	39.8 (6.4)
45		12.8 (5.4)	37.2 (5.4)	15.4 (4.9)	34.6 (6.4)

of the sperm displayed fluorescence throughout the head (type H; see Figure 1, panel a). Approx. 50% of the spermatozoa interacted with the liposomes at the ES (types ESd and ESp), which corresponds fairly well with the number of acrosome-reacted cells containing PSA-binding factor in the ES region as determined with the AR assay. On the basis of diffuse fluorescence in the ES region, more than half of this fraction had fused with the liposomes (type ESd; Table 1). No change in the distribution of these patterns occurred when the incubation was extended to 45 min. Assuming that N-Rh-PE was indeed incorporated into the ES membrane, the lipid analogue was apparently restricted in its mobility within the boundaries of this membrane domain. The same observations were made when N-Rh-PE was substituted for a lipid analogue which has an NBD-group attached to the ethanolamine head group (N-NBD-PE) (results not shown).

A transbilayer lipid-diffusion barrier is present in the ES

Provided that the lipid(s) are properly intercalated in the lipid phase of the sperm acceptor membrane, the above results suggest the existence of junctions preventing lateral diffusion to membrane domains other than the ES. Moreover, since the fluorescent lipid analogues are symmetrically distributed over the liposomal membranes (i.e. present in both inner and outer membrane leaflet), and when it is assumed that the topology is maintained upon fusion, the barrier must exist in both outer and inner leaflet. To show that a barrier was indeed present in the inner leaflet, we

fused the acrosome-reacted spermatozoa with PS liposomes, asymmetrically labelled with N-NBD-PE. The latter vesicles were prepared by treating symmetrically labelled vesicles with sodium dithionite, which irreversibly destroys NBD fluorescence (see the Materials and methods section). As with symmetrically labelled liposomes, fusion with asymmetrically labelled vesicles resulted in fluorescence that was restricted to the ES (cf. Fig. 1, panel b).

Unfortunately, no lipid dilution could be detected using the RET assay, perhaps because of excessive crowding (see below) of molecules within the ES domain, giving rise to inefficient relief of RET, or due to other, as yet unknown, causes. These considerations prompted us, therefore, to examine the ability to eliminate the possible lipid-diffusion barriers, arguing that free lateral diffusion would indicate both the existence of these barriers and the proper incorporation of N-Rh-PE, as reflected by lateral mobility.

Relieving the block to free lateral diffusion

Initial efforts were aimed at removing the lipid-diffusion barriers by proteinase treatment. After AR induction with calcium ionophore A23187 and fusion with liposomes, subsequent incubation of the cells with Pronase for 1 h had no effect on N-Rh-PE localization (Table 2). However, addition of 10 mM EDTA resulted in a 2–3-fold enhancement of the proportion of cells, showing fluorescence spread over the entire head (type H). The same results were obtained when asymmetrically labelled liposomes were used, containing N-NBD-PE in the inner leaflet (results not shown).

The distinct shift in fluorescence distribution from ESd to an H-type (Table 2) suggested that the fluorescence emanated from the ES and was, after treatment of the spermatozoon with EDTA, free to diffuse towards all other membrane domains. The more random redistribution of fluorescence was not the result of an induction of fusogenic activity by the chelator, since the proportion of spermatozoa containing only associated liposomes (type ESp) remained unchanged. Furthermore, non-acrosome-reacted spermatozoa did not fuse with PS liposomes, nor was this the case when these cells were simultaneously incubated with liposomes and EDTA (Table 2).

Pretreatment of acrosome-reacted spermatozoa with EDTA for 10 min before addition of liposomes inhibited the fusogenic activity of the ES. However, some characteristics of the ES structure were maintained, since preferential association of liposomes to the ES region still occurred (Table 2). Simultaneous incubation of the cells with PS liposomes and EDTA had no

Table 2 Effect of EDTA on fluorescence localization of spermatozoa in suspension

Spermatozoa were either ^aacrosome-reacted with calcium ionophore A23187 or ^bincubated with 0.5% DMSO (v/v) for 1 h. After washing, the spermatozoa were incubated with ^cPS liposomes (0.25 mM lipid) for 3 min, ^dPS liposomes followed by a wash step and incubation in 10 mM EDTA for 10 min, ^esimultaneously PS liposomes and 10 mM EDTA for 3 min or ^f10 mM EDTA for 10 min, followed by a wash step and incubation with PS liposomes. Before determination of the labelling patterns, a final wash step was performed. Results are means (S.D.) for five different experiments.

Spermatozoa	Incubation	Type ...	Proportion of spermatozoa of each type (%)			
			H	ESd	ESp	0
Acrosome-reacted ^a	PS ^c		15.6 (6.2)	29.7 (12.7)	17.6 (4.2)	37.0 (11.0)
	PS, wash, EDTA ^d		45.2 (17.4)	5.6 (4.4)	15.6 (9.1)	35.6 (11.2)
	PS + EDTA ^e		31.7 (11.6)	15.0 (3.3)	19.7 (14.9)	33.6 (15.3)
	EDTA, wash, PS ^f		8.5 (5.8)	10.5 (5.3)	34.2 (8.3)	46.8 (11.2)
Non-treated ^b	PS ^c		16.7 (6.5)	0 (0)	0 (0)	83.3 (6.5)
	PS + EDTA ^e		20.3 (11.5)	0 (0)	0 (0)	79.7 (11.5)

Figure 2. Redistribution of N-Rh-PE after EDTA treatment of zona-pellucida-induced acrosome-reacted spermatozoa

Induction of AR by zona pellucidae was carried out as described in Table 3. Panel a, sperm bound to a zona pellucida only interact with liposomes at the ES after AR (pattern ESd). Note the typical bar-like structure. The bar represents 25 μm . Panel b, fluorescence is seen in other membrane domains of part of the spermatozoa (arrowheads) after incubation with EDTA. Panel c, as panel b, but at higher magnification. Acrosome-reacted spermatozoa that are not affected by the EDTA treatment are indicated by arrows; in the spermatozoa indicated by arrowheads, fluorescence is localized throughout heads and tails, but most extensive in the ES. The bar represents 10 μm .

effect on the proportion of spermatozoa with incorporated N-Rh-PE (types H + ESd), indicating that the fusion event is rapid, as already reflected by the time course (Table 1), and faster than the inhibition of fusogenic activity by the chelator.

The above observations were done on spermatozoa after AR induction with ionophore in suspension. Although these sperm are non-vital, the ES retains its specific properties seen for vital acrosome-reacted sperm, including its fusogenic activity, as

shown previously (Arts et al., 1993). To determine the site of action of EDTA, we also examined the effect of EDTA treatment on the ES boundaries of vital (and non-permeable) acrosome-reacted spermatozoa, produced on the surface of human zona pellucidae. With such cells, and before EDTA addition, it was observed that the liposomes only interacted with the ES (Figure 2, panel a; Table 3). Subsequent treatment with EDTA resulted in migration of the fluorescent lipid analogues towards all

Table 3 Effect of EDTA on fluorescence localization using zona-pellucida-induced acrosome-reacted spermatozoa

Capacitated spermatozoa were co-incubated with human zonae pellucidae for 6 h. After removing non-bound sperm by washing, the zonae (with firmly bound sperm) were incubated for 15 min with PS liposomes (0.25 mM). After washing the zonae were incubated 20 min with buffer (control) or 30 mM EDTA. The mean results for four independent experiments are presented (S.D. values are omitted).

Expt.	EDTA	No. of zonae	Spermatozoa/ zona	AR/zona (%)	Proportion of acrosome-reacted spermatozoa of each type (%)		
					Type...H	ESd	ESp
1	—	7	79.0	28.6	0	100	0
	+	8	28.4	55.6	20.0	80.0	0
2	—	6	74.5	31.0	0	100	0
	+	8	41.6	52.9	15.8	84.3	0
3	—	7	22.9	13.3	0	85.7	14.3
	+	10	16.5	24.2	23.3	14.4	62.2
4	—	16	32.8	27.6	0	47.1	52.9
	+	8	28.1	23.3	10.2	8.8	81.0

Figure 3. Lipid mixing is induced by addition of EDTA

Acrosome-reacted spermatozoa ($50 \times 10^6/2$ ml sperm buffer) were incubated with PS liposomes containing 0.8 mol-% of both N-Rh-PE and N-NBD-PE (the amount of phospholipid added was 100 nmol). Immediately after addition of the liposomes, the cells were centrifuged over a Percoll gradient, washed once, and suspended in 1.9 ml of sperm buffer in a cuvette. After injection of EDTA (100 μ l of a 0.1 M isotonic solution; arrow) into the mixture an increase in NBD fluorescence was observed. Total cell-bound fluorescence was determined by addition of 1% (v/v) Triton X-100 after termination of the lipid mixing event. Maximum fluorescence of the initially added liposomes was determined by disruption of the vesicles (100 nmol/2 ml) with Triton X-100.

membrane domains, head as well as tail (Figure 2, panel b; Table 3). The efficiency of the EDTA treatment was limited, but, commonly, random distribution of the N-Rh-PE was seen in 10–25% of the acrosome-reacted sperm (defined as those sperm showing interaction with the liposomes; Table 3).

In contrast with the experiments in suspension, EDTA did not exert an immediate effect after zona pellucida triggered AR. Co-incubation of cells and chelator for at least 20 min was necessary. Furthermore, a final concentration of 30 mM EDTA or higher was needed (osmolarity was kept at 290 mosM) to achieve an effect. All spermatozoa showing randomization of the N-Rh-PE were also sensitive towards the nuclear stain Hoechst 33258, which is indicative of permeable membranes. The dye was excluded from spermatozoa showing ES-restricted fusion and which did not respond to EDTA treatment.

Quantification of lipid mixing after EDTA treatment

The lateral diffusion of lipids after EDTA treatment allowed a determination of lipid mixing using an assay based on RET (Figure 3). As noted above, addition of acrosome-reacted spermatozoa to PS liposomes did not result in a measurable relief of energy transfer. After removal of free liposomes by washing, 10 mM EDTA was added, which resulted in an increase in NBD fluorescence of 10%. By comparing fluorescence levels of liposomes and liposome-cell complexes after addition of Triton X-100, it can be estimated that 15% of the initially added liposomes associated with the spermatozoa.

Thus, assuming a total relief of RET, in this approach about 1.5% of the initially present liposomes ($= 1.4$ nmol of lipid) were involved in lateral diffusion. As determined by fluorescence microscopy, the number of spermatozoa involved is about 40% ($= 20 \times 10^6$ cells). Per spermatozoon, 4.5×10^7 lipid molecules are then eventually introduced via fusion, representing a total area of $26 \mu\text{m}^2$, when assuming that one lipid molecule covers a surface area of approx. 0.6 nm^2 (Schieren et al., 1978). Therefore, the fluorescent lipid probes dilute over a surface area of $135 \mu\text{m}^2$ (total sperm surface), which is about a 7-fold dilution of the labelled area. Therefore, a maximal relief of RET of $100 \times 6/7 = 84\%$ may be obtained, which agrees fairly well with the initial assumption of total relief of energy transfer per fusion event. Thus it may be estimated that approx. $30 \mu\text{m}^2$ of liposomal membrane will fuse with the ES of one spermatozoon (see the Discussion).

DISCUSSION

In the present work we have provided evidence for the existence of lipid diffusion barriers in ES membranes of acrosome-reacted human spermatozoa.

PS liposomes, containing fluorescent phospholipid derivatives, largely attach to, and probably fuse almost exclusively with, the ES of spermatozoa, provided that the cells have undergone the AR (Arts et al., 1993). Here we have shown that the fluorescent lipids are maintained within the boundaries of this membrane domain for at least 45 min (Table 1). The restricted lateral diffusion was not an artefact due to an artificially triggered AR, since vital sperm also, acrosome-reacted by the zona pellucida, displayed an ES-restricted association of fluorescence.

Addition of the bivalent-cation chelator EDTA caused randomization of the fluorescent lipid analogues throughout the head and tail membranes, which could also be measured by a lipid mixing assay. The results in Table 2 indicate that all redistributed fluorescence originated from the spermatozoa displaying diffuse fluorescence in the ES (type ESd) before EDTA addition. Previously, we have provided evidence that the ESd labelling pattern reflected those acrosome-reacted spermatozoa that had actually fused with the liposomes (Arts et al., 1993). The redistribution of fluorescence, initially restricted to the ES, throughout the cells was not due to initiation of ensuing fusion events by EDTA, since the fluorescence appearance of sperm containing only attached liposomes (type ESp) was not affected, and non-acrosome-reacted sperm never fused with liposomes in the presence of the chelator. Given the fact that the lipid diffusion after EDTA treatment could be measured with an assay based on RET, the redistribution of the fluorescent lipid analogues cannot be explained by free lateral diffusion of ES proteins containing attached (but unfused) liposomes. Thus the fluorescent lipid analogues must have been introduced in the ES membrane by fusion, prior to treatment with EDTA. These incorporated lipids are limited in their mobility by diffusion blocks at the borders of the ES, which can be relieved by the

action of EDTA. Previously, we have shown that random distribution of fluorescent lipid analogues also occurred when frozen-thawed spermatozoa were incubated with liposomes (Arts et al., 1993), which might be the result of non-specific fusion events with perturbed membranes. However, the outcome of this experiment can also be explained by lipid redistribution from the ES, whose diffusion blocks are eliminated by the freeze-thawing treatment.

The extensive lipid redistribution occurring after disrupting the ES barrier was also reflected in a relief of energy transfer between the fluorescent lipid probes N-NBD-PE and N-Rh-PE. However, as noted before (Arts et al., 1993), no relief of RET can be measured when acrosome-reacted spermatozoa are incubated with liposomes and, hence, when the lipid distribution is limited to the ES region. As shown here, this inability probably stems from the fact that, in conjunction with the extent of fusion, the ES area available for dilution of the probes in the initial fusion event is too small to provide sufficient NBD fluorescent signal, arising upon dilution. For the initial fusion event it was calculated that $30 \mu\text{m}^2$ of liposomal surface would fuse with $8 \mu\text{m}^2$ of ES surface, or, when taking into account the hairpin-like structure of this domain, an area of about $16 \mu\text{m}^2$. Without an adequate compensation mechanism, incorporation of such an amount of exogenous lipid would likely result in severe membrane deformation. Occasionally, a swollen appearance of the ES domain was seen. However, by fluorescence microscopy such deformations were not generally apparent, suggesting that other causes for quenching are possibly involved. Thus liposomes that are bound to the ES might participate in additional quenching of fluorescence of incorporated N-NBD-PE. However, neither can the possibility be ruled out that as-yet-unknown quenchers in the ES domain artificially diminish NBD fluorescence.

The data in Table 2 indicate that, to some extent, EDTA affects the fusion capacity of the ES by disrupting the lipid-diffusion barriers. This suggests an involvement of lipids, arranged in a specific concentration or configuration, in the fusion activity of the ES. The effect of EDTA on the fusion capacity appears to be a slower process than the initial fusion events and subsequent diffusion of the lipids. The attachment of liposomes to the ES is not affected by the presence of EDTA, suggesting that different sperm factors may be involved in the fusion and attachment function of the ES. Alternatively, both functions may be harboured by the same protein, but in distinct domains or subunits. In this respect, a putative fusion peptide and an integrin-binding domain have been reported to be located on different subunits of the guinea pig sperm protein PH-30 (Blobel et al., 1992).

Barriers to lipid lateral diffusion have been reported for other polarized cell types. In epithelial cells the basolateral and apical membrane domains are separated by tight junctions, which have been shown to prevent lipid diffusion in the exoplasmic, but not the cytoplasmic, leaflet of the plasma membrane (van Meer and Simons, 1986). Using asymmetrically labelled liposomes, fluorescent lipid derivatives can be incorporated into the exoplasmic leaflet of the apical membrane domain. Redistribution of these probes to the basolateral domain occurs when the tight junctions are opened by incubation in a Ca^{2+} -free medium. This is very reminiscent of the present observation that incorporated N-Rh-PE (and other fluorescent analogues) randomized through the spermatozoal membranes after depletion of bivalent cations by EDTA. A very prominent and interesting distinction is that, in the spermatozoal system, the lipid-diffusion barrier seems to be located in both membrane leaflets, since using symmetrically labelled liposomes N-Rh-PE is expected to be introduced in both leaflets of the ES membrane. Indeed, cells incubated with

liposomes containing N-NBD-PE in the inner leaflet only, i.e. in which the fluorescence of the outer leaflet had been quenched by dithionite, showed the same fluorescence appearance as cells incubated with symmetrically labelled liposomes.

A bilayer spanning diffusion barrier has been reported to exist in polarized neurons (Kobayashi et al., 1992). Lipids introduced in the axon failed to diffuse to the cell body. In these cells the necessity for a physical barrier has been doubted by arguing that the lipid-diffusion coefficients would be insufficient to cover the large distances between axon and cell body within the time of observation (Futerman et al., 1993). However, we have shown here that, in principle, such barriers may exist, since relieving the diffusion blocks with EDTA resulted in a redistribution of the fluorescent lipids, even to the endpiece of the tail, i.e. over a distance of some $50 \mu\text{m}$.

As yet, the nature of these efficient diffusion barriers in the ES structure is unclear and several mechanisms may be proposed for their maintenance.

EDTA may affect the cell surface, possibly by perturbing surface proteins such as lipid-binding proteins (Lingwood, 1986; Jones and Hall, 1991) or fibronectin (Glander et al., 1987). Furthermore, stable bridging structures, which are possibly membrane-embedded, are observed in the extracellular space between the inner and outer acrosomal parts of the ES of hamster and boar sperm (Bedford et al., 1979; Russell et al., 1980; Huang and Yanagimachi, 1985). Yet human spermatozoa which had been acrosome-reacted on zonae pellucidae had to be incubated for a prolonged period with EDTA to achieve the redistribution of fluorescence. At the same time, those cells that were affected by the chelator were also permeable to the nuclear stain Hoechst 33258. Hence it is possible that the action of EDTA is not solely restricted to the cell surface.

The existence of transmembrane protein barriers cannot be excluded. By electron microscopy it is seen that, in several mammalian species, a serrated band of oblique 'rods', visualized as alternating elevations and depressions, is encircling the sperm head at the posterior margin of the ES [see, for references, Eddy (1988)]. It should be noted that the post-acrosomal and inner acrosomal membranes immediately overlie the perinuclear material, from which the ES hairpin structure is excluded. On the other hand, freeze-fracture studies on guinea-pig and boar sperm reveal a clearance of intramembranous particles from the ES after AR (Friend et al., 1977; Aguas and Pinto da Silva, 1989).

An explanation for the structure of lipid-diffusion barriers that has gained more interest recently is the occurrence of lateral phase separation of lipids. It has been noted that, in ram sperm PMs, non-diffusing lipid fractions exist that are preserved on lipid extraction (Wolf et al., 1988). Therefore it was proposed that these domains might be maintained by lipid factors such as plasmalogens or sperm-specific glycolipids, like the sulphatoylgalactosylglycerolipid (seminolipid). In this respect, an antibody against seminolipid revealed a specific localization in the ES of acrosome-reacted boar spermatozoa (Gadella, 1994). The seminolipid may promote the formation of the hexagonal II phase of the phospholipid membrane (Gadella et al., 1993). Whether this phase separation can affect the free lateral mobility of membrane components remains to be established.

It is finally of interest to note that annexins, which may be involved in the membrane attachment of cytoskeletal elements [see for a review, Creutz (1992)], have been reported to be specifically present in the ES of human spermatozoa (Berruti, 1988; Feinberg et al., 1991). Since this class of proteins is well known to require Ca^{2+} for their functioning (Creutz, 1992), its participation in the maintenance of a fusogenic ES is worth investigating.

REFERENCES

- Aguas, A. P. and Pinto da Silva, P. (1989) *J. Cell Sci.* **93**, 467–479
- Arts, E. G. J. M., Kuiken, J., Jager, S. and Hoekstra, D. (1993) *Eur. J. Biochem.* **217**, 1001–1009
- Arts, E. G. J. M., Kuiken, J. and Jager, S. (1994) *Fertil. Steril.*, in the press
- Bedford, J. M., Moore, H. D. M. and Franklin, L. E. (1979) *Exp. Cell Res.* **119**, 119–126
- Berruti, G. (1988) *Exp. Cell Res.* **179**, 374–384
- Biggers, J. D., Whitten, W. K. and Whittingham, D. G. (1971) in *Methods in Mammalian Embryology* (Daniel, J. C., Jr., ed.), pp. 86–116, Freeman, San Francisco
- Blobel, C. P., Wolfsberg, T. G., Turck, C. W., Myles, D. G., Primakoff, P. and White, J. M. (1992) *Nature (London)* **356**, 248–252
- Clark, J. M. and Koehler, J. K. (1990) *Mol. Reprod. Dev.* **27**, 351–365
- Creutz, C. E. (1992) *Science* **258**, 924–931
- Cross, N. L., Morales, P., Overstreet, J. W. and Hanson, F. W. (1986) *Gamete Res.* **15**, 213–226
- Cummins, J. M. and Woodall, P. F. (1985) *J. Reprod. Fertil.* **75**, 153–175
- Dietl, J. A. and Rauth, G. (1989) *Human Reprod.* **4**, 869–875
- Eddy, E. M. (1988) in *The Physiology of Reproduction* (Knobil, E. and Neill, J., eds.), pp. 27–68, Raven Press, New York
- Feinberg, J. M., Rainteau, D. P., Kaetzel, M. A., Dacheux, J.-L., Dedman, J. R. and Weinman, S. J. (1991) *J. Histochem. Cytochem.* **39**, 955–963
- Fléchon, J.-E. and Hafez, E. S. E. (1976) in *Human Semen and Fertility Regulation in Men* (Hafez, E. S. E., ed.), pp. 76–82, the C. V. Mosby Company, St. Louis
- Friend, D. S., Orci, L., Perrelet, A. and Yanagimachi, R. (1977) *J. Cell Biol.* **74**, 561–577
- Futerman, A. H., Khanin, R. and Segel, L. A. (1993) *Nature (London)* **362**, 119
- Gadella, B. M. (1994) Ph. D. Thesis, University of Utrecht
- Gadella, B. M., Colenbrander, B., van Golde, L. M. G. and Lopes-Cardozo, M. (1993) *Biol. Reprod.* **48**, 483–489
- Glander, H.-J., Herrmann, K. and Haustein, U.-F. (1987) *Andrologia* **19**, 456–459
- Hoekstra, D. and Düzgünes, N. (1986) *Biochemistry* **25**, 1321–1330
- Hoekstra, D., Buist-Arkema, R., Klappe, K. and Reutelingsperger, C. P. M. (1993) *Biochemistry* **32**, 14194–14202
- Huang, T. T. F. and Yanagimachi, R. (1985) *Am. J. Anat.* **174**, 249–268
- Jones, R. and Hall, L. (1991) *Biochim. Biophys. Acta* **1080**, 78–82
- Kobayashi, T., Storrie, B., Simons, K. and Dotti, C. G. (1992) *Nature (London)* **359**, 647–650
- Lingwood, C. A. (1986) *Biochem. Cell Biol.* **64**, 984–992
- McIntyre, J. C. and Sleight, R. G. (1991) *Biochemistry* **30**, 11819–11827
- Russell, L., Peterson, R. N. and Freund, M. (1980) *Anat. Rec.* **198**, 449–459
- Schieren, H., Rudolph, S., Finkelstein, M., Coleman, P. and Weissmann, G. (1978) *Biochim. Biophys. Acta* **542**, 137–153
- Struck, D. K., Hoekstra, D. and Pagano, R. E. (1981) *Biochemistry* **20**, 4093–4099
- Szoka, F. C. and Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4194–4198
- Talbot, P. and Chacon, R. S. (1982) *Fertil. Steril.* **37**, 240–248
- van Duijn, C. (1975) *Bibl. Reprod.* **25**, 121–248
- van Meer, G. and Simons, K. (1986) *EMBO J.* **5**, 1455–1464
- Wilcoxon, F. (1945) *Biometrics Bull.* **1**, 80–83
- Wolf, D. E., Hagopian, S. S., Lewis, R. G., Voglmayr, J. K. and Fairbanks, G. (1986) *J. Cell Biol.* **102**, 1826–1831
- Wolf, D. E., Lipscomb, A. C. and Maynard, V. M. (1988) *Biochemistry* **27**, 860–865
- World Health Organization (1987) *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*, 2nd edn., pp. 55–58, The Press Syndicate of the University of Cambridge, Cambridge
- Yanagimachi, R. (1988) in *The Physiology of Reproduction* (Knobil, E. and Neill, J., eds.), pp. 135–185, Raven Press, New York
- Zeilmaker, G. H. and Rijkman, C. M. P. M. (1986) *Int. J. Androl. (Suppl.)* **6**, 123–130