

## Visualization and quantification of glycolipid polarity dynamics in the plasma membrane of the mammalian spermatozoon

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### SUMMARY

Seminolipid (sulphogalactosylalkylacylglycerol), the glycolipid that is specific for mammalian germ cells, is located exclusively in the outer leaflet of the sperm plasma membrane. In this study the lateral distribution of seminolipid on sperm heads has been investigated by indirect immunofluorescence labelling and detection with digital imaging fluorescence microscopy. In freshly ejaculated sperm cells this glycolipid was present primarily at the apical ridge subdomain of the plasma membrane of the sperm head. After binding the sperm cells to zona-coated coverslips seminolipid migrated, in 40 minutes, from the apical ridge to the equatorial subdomain of the plasma membrane. A similar redistribution of seminolipid was observed during capacitation of sperm cells *in vitro* induced by Ca<sup>2+</sup> or bovine serum albumin. Comparable migration of seminolipid was also found after prolonged storage of ejaculated sperm cells, albeit at a much slower

rate. Addition of arylsulphatase A, an enzyme present in seminal plasma that desulphates seminolipid, significantly enhanced the migration of seminolipid during storage of sperm cells. Its breakdown product desulphoseminolipid (galactosylalkylacylglycerol) appeared highly specifically at the equatorial segment. The measured fluorescence intensity over the sperm head surface correlated linearly with the spatial probe distribution as was checked by fluorescence lifetime imaging microscopy. This paper demonstrates and quantifies for the first time the polarity of seminolipid on the surface of the sperm cell and the dynamic alterations that occur in this polarity during post-ejaculatory events.

Key words: seminolipid, sperm, plasma membrane, lipid polarity, fluorescence lifetime imaging microscopy, immunolocalization, capacitation

### INTRODUCTION

The sperm cell is a highly differentiated and polarized cell (Holt, 1984; Peterson and Russell, 1985). It is divided into three compartments: the head, the mid-piece and the tail. The plasma membrane of these compartments is separated into three corresponding domains by two specialized tight-junctional structures; namely, the nuclear and annular rings (Friend, 1982; Holt, 1984; Cowan and Myles, 1993). These rings prevent the lateral diffusion of proteins and lipids from one domain of the plasma membrane to another (Holt, 1984).

Evidence has accumulated that membrane-glycoconjugates (Cross and Overstreet, 1987; Srivastava et al., 1988; Bearer and Friend, 1990; Focarelli et al., 1990; Nikolajczyk and O'Rand, 1992), integral membrane proteins (Myles et al., 1984; Saxena et al., 1986b; Peterson et al., 1987; Cowan and Myles, 1993) and lipids (Bearer and Friend, 1982; Holt and North, 1984, 1986; Wolf et al., 1988, 1990) are further organized into distinct subdomains of each of the three domains of the sperm plasma membrane. Initially, lectin and antibody binding studies revealed that integral membrane proteins and glycoconjugates of the sperm plasma membrane

were organized in subdomains (for references see Peterson and Russell, 1985). Additional support came from freeze-fracture replicas of the sperm plasma membrane, demonstrating the subdomain polarity of intramembranous particles representing integral membrane proteins (Fléchon et al., 1986; Aguas and Pinto da Silva, 1989). Interestingly, most of these proteins showed lateral diffusion, indicating that they were not anchored to a protein matrix (Myles et al., 1984). On addition of extracellular Ca<sup>2+</sup> to washed sperm cells the distribution of the integral membrane proteins changed into different subdomain patterns (Fléchon et al., 1986; Aguas and Pinto da Silva, 1989). Subdomain reorganizations at the sperm head plasma membrane were also demonstrated by lectin and antibody binding studies (Saxena et al., 1986a,b; Cowan et al., 1991). Remarkably, there were no ultrastructural barriers (such as the ring structures between the three domains) to prevent lateral diffusion of these molecules between the subdomains (Fléchon et al., 1986; Kan and Pinto da Silva, 1987; Aguas and Pinto da Silva, 1989). The lateral distribution of integral membrane proteins also changed upon altering the environmental temperature (Holt and North, 1984, 1986; De Leeuw et al., 1990). Subdomain-specific redistribution of integral

membrane proteins was also observed during physiological changes of the sperm cells during: (i) maturation along the epididymal tract (Saxena et al., 1986a; Toyama and Nagano, 1988); (ii) capacitation in the female genital tract (Saxena et al., 1986b, Cross and Overstreet, 1987); and (iii) binding to the zona pellucida (O'Rand and Fisher, 1987; Richardson et al., 1991; Nikolajczyk and O'Rand, 1992). During these processes the spermatozoa encounter a series of changes in extracellular environment that probably induce the migration of transmembrane and peripheral membrane proteins from one subdomain of the sperm plasma membrane to another.

Evidence for the organization of lipids in subdomains of the sperm plasma membrane has been reported. This lipid polarity was observed with fluidity markers (Holt and North, 1984, 1986; Wolf and Voglmayer, 1984; Wolf et al., 1986, 1988) or lipid-binding probes (Bearer and Friend, 1982, 1990; Friend, 1982) and by differential scanning calorimetry (Wolf et al., 1990). The reorganization of lipids during capacitation *in vitro* (Bearer and Friend, 1982, 1990; Wolf et al., 1986), epididymal maturation (Wolf and Voglmayer, 1984) and cooling (Holt and North, 1984, 1986; De Leeuw et al., 1990) appeared to be comparable with that of membrane proteins.

Although plasma membrane polarity and organization into subdomains is now well established for mammalian sperm, little is known about the molecular mechanisms underlying these phenomena. It has been suggested that the limited metabolic properties of the sperm cell and the absence of membrane flow via vesicle-mediated transport contribute to the abnormal polar organization of the sperm cell (Holt, 1984). In addition, the unusual lipid composition of the sperm plasma membrane may be a factor contributing to the unique polarity of this membrane. (i) The cholesterol content is unusually low (~15%; Parks and Hammerstedt, 1985; Nikolopoulou et al., 1986). (ii) The phosphoglycerides contain alk-1-enyl- (55%; almost exclusively hexadecenyl) and alkyl residues (25%; almost exclusively hexadecyl) rather than acyl chains (20%; almost exclusively hexadecanoyl) at the 1-position of glycerol (Selivonchick et al., 1980). The 2-position is esterified predominantly with long-chain polyunsaturated (C22:6 or C22:5) fatty acids (Parks and Hammerstedt, 1985; Nikolopoulou et al., 1986; Graham and Foote, 1987). (iii) The glycolipid content is high (~8%) and consists exclusively of seminolipid (1-*O*-alkyl-2-*O*-acyl-3- $\beta$ -(3'-sulphogalactosyl)-glycerol, referred to as SGaAAG) in mammalian spermatozoa (Kornblatt et al., 1972; Gadella et al., 1993a). This unusual lipid composition undoubtedly contributes to the high immobile fraction of lipids (up to 50%) in the sperm plasma membrane (Wolf et al., 1988). As a consequence, the thermotropic phase behaviour of this lipid mixture may contribute to the organization of the sperm plasma membrane into subdomains (Holt and North, 1984, 1986; Wolf et al., 1988, 1990).

As the transition temperature of SGaAAG ( $T_m=36.4^\circ\text{C}$ ) is close to the physiological temperature, this glycolipid has been suggested to play a key role in the establishment of subdomains on spermatozoa (Parks and Lynch, 1992). In the present study we investigated the lateral topology of SGaAAG in the sperm plasma membrane. We were able to demonstrate and quantify lateral SGaAAG polarity in the boar sperm plasma membrane and to visualize for the first time the dynamic redistribution of this lipid during various post-ejaculatory events. Use was made of the fact that SGaAAG is an antigenic lipid (Goujet-Zalc et al., 1986; Lingwood, 1986; Bansal et al., 1989; Tsuji et al.,

1992) and is exclusively localized in the outer-leaflet of the sperm plasma membrane (Gadella et al., 1993a), which allowed its immunolocalization without the need to permeabilize the sperm cell. The distribution of immunolabelled SGaAAG over the sperm head surface was visualized and quantified by digital imaging fluorescence microscopy. With the use of the newly developed technique of fluorescence lifetime imaging microscopy (FLIM, Gadella et al., 1993b), we were able to rule out the possibility that the detected spatial differences in immunofluorescence were due to differences in dynamic quenching processes caused by microheterogeneity of the fluorophore's molecular environment. Hence fluorescence truly reflects the spatial distribution of the probe.

## MATERIALS AND METHODS

### Materials

Four anti-galactolipid monoclonal antibodies were used: O4 (Mouse IgM anti-sulphogalactolipids) and O1 (Mouse IgM anti-desulphogalactolipid), R (Mouse IgG3 anti-galactolipid) (Bansal et al., 1989), and Z (Mouse IgM anti-SGaAAG) (Goujet-Zalc et al., 1986). The O4 and O1 antibodies were kindly provided by Dr J. Trotter (Heidelberg, FRG), the R antibody by Dr M. Noble (Ludwig Institute for Cancer Research, London, UK) and the Z antibody was purchased from Immunotech. (cat. no. 224-58, Nouzilly, France). Goat anti-mouse anti-IgM antibody conjugated with fluorescein isothiocyanate (GaM-FITC), goat anti-mouse anti-IgG3 antibody conjugated with tetramethyl rhodamine B isothiocyanate (GaM-TRITC) and goat anti-mouse anti-Ig conjugated with horseradish peroxidase (GaM-HRP) were purchased from Nordic (Tilburg, the Netherlands). Mowiol® was from Aldrich (Steinheim, FRG). High-performance TLC silicagel-60 plates and polyisobutylmetacrylate were obtained from Merck (Darmstadt, FRG), plastic-backed TLC plates (Bakerflex, silicagel 1B2) were from J. T. Baker (Phillipsburg, NJ). The peroxidase substrate 3,3'-diaminobenzimidine tetrahydrochloride (DAB) was obtained from Sigma (St Louis, MO). Orcinol was purchased from the British Drughouse Ltd (London, UK).

### Isolation and hydrolysis of glycolipids

SGaAAG was purified from sperm lipid extracts as described previously (Gadella et al., 1992). To check the purity of SGaAAG, 50 nmol of the isolated glycolipid fraction was spotted on high performance TLC plates and developed with chloroform/methanol/ammonia, 25% (65:24:4, by vol.). The glycolipids were visualized with the orcinol spray reagent (Kundu, 1981). Purified SGaAAG (1  $\mu\text{mol}$ ) was desulphated enzymically as previously described (Gadella et al., 1992) or via mild acid hydrolysis with 1 ml methanol containing 50 mM HCl during 2 hours at 37°C. The reaction mixture was cooled to room temperature after which 1.8 ml distilled water and 1 ml chloroform were added for two-layer partition. The aqueous layer was washed five times with 3 ml chloroform/methanol (85:15, v/v) and the pooled organic layers were dried under nitrogen and loaded on a silica column (activated for 2 hours at 130°C and equilibrated with distilled chloroform), and galactosylalkylacylglycerol (GaAAG) was purified according to Kornblatt et al. (1972). The isolated SGaAAG and GaAAG were also deacylated via mild alkaline hydrolysis in 1 ml methanol containing 0.1 M KOH for 1 hour at 37°C. The two-layer partition and further purification of the deacylated galactolipids were performed as described above. The purity of GaAAG and the deacylated galactolipids were checked on high-performance TLC as described above. *N*-acyl and *N*-[2-hydroxy]acyl standards of sulphogalactoceramide and galactoceramide were purchased from Sigma (St Louis, MO, USA). Galactodiacylglycerols isolated from plant chloroplasts were kindly provided by Dr R. van't Hof (Van't Hof et al., 1993).

### Immunostaining of glycolipids on TLC plates

Immunological detection of glycolipids was performed, with some modifications, by the method of Kniep and Mühlradt (1990). Briefly: 5 nmol samples of each purified glycolipid were spotted on plastic-baked silicagel TLC plates. The plate was developed with chloroform/methanol/ammonia (25%) 65/25/4 (by vol.) and dried under a hair dryer (30 minutes). A stock solution of 2.5% (w/v) poly(isobutyl)metacrylate in distilled chloroform (Van Genderen et al., 1991) was diluted with distilled *n*-hexane to a 0.1% (w/v) solution. The TLC plate was soaked for 90 seconds in this solution, dried for 15 minutes and blocked for 2 hours with a buffer containing 1% (w/v) gelatin in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4 (GTS buffer, Bhat et al., 1991). Subsequently, GTS buffer containing the primary antibody (dilution: 1:100 for O1 and O4; 1:300 for R and Z) was carefully applied to the silica surface (60 µl/cm<sup>2</sup>). The TLC plate was subsequently placed in a glass Petri dish after placing a slightly smaller piece of parafilm on top of the plate and it was gently rocked for 2 hours in a humid atmosphere. Unbound antibodies were removed from the plate by washing it with GTS buffer (5 times during 5 minutes). Thereafter, the plate was incubated with secondary antibody (1:1000 dilution of GaM-HRP in GTS buffer), washed four times with GTS buffer and twice with 50 mM Tris-HCl, pH 7.4. The peroxidase reaction was started with 50 mM Tris-HCl containing 0.2% (v/v) H<sub>2</sub>O<sub>2</sub> and 0.1% (w/v) of the colour substrate DAB. When enough colour formation was visible (3–10 min) the reaction was stopped by washing the plate three times with distilled water. All steps were carried out at room temperature. Control experiments were carried out in which the primary monoclonal antibody incubation step was omitted and no colour formation was detectable.

### Sperm storage

Ejaculates from healthy and fertile boars were collected in fractions and only sperm-rich fractions were used (Gadella et al., 1991). The following sperm preparations were made for long storage experiments: (i) undiluted semen; (ii) diluted semen (1:10 with Beltsville Thawing Solution BTS; Johnson et al., 1988); (iii) BTS-washed semen (Gadella et al., 1991); and (iv) BTS-washed semen with addition of a physiological concentration of an AS-A preparation isolated from seminal plasma (Gadella et al., 1993a). These samples were stored in sealed packages of 100 ml (with a minimum of air above the buffer to prevent degassing of the buffer) in a 17°C incubator (Johnson et al., 1988).

### Capacitation in vitro

Capacitation experiments in vitro were carried out in a 37°C incubator with a 5% CO<sub>2</sub> humidified atmosphere. The sperm cells were first washed in Ca<sup>2+</sup>-free Hanks' buffered salt solution (HBSS: 5.4 mM KCl, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 4.2 mM NaHCO<sub>3</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.6 mM D-glucose, 0.6 g/l penicillin G Na and 1.0 g/l dihydrostreptomycin at pH 7.2). HBSS-washed sperm samples were capacitated in vitro with 2 mM CaCl<sub>2</sub> (Aguas and Pinto da Silva, 1989) or with 4% (w/v) BSA fraction V (Langlais and Roberts, 1985).

### Preparation of zona-coated coverslips

Pig ovaries were obtained from a slaughter house (Soest, the Netherlands), transported on ice and used as a source of oocytes. The ovaries were submerged in a buffer (130 mM NaCl solution containing 10 mM sodium phosphate, 2 mM EGTA and 11 mM sodium citrate, pH 7.0) and the follicles were disrupted according to the method of Dunbar et al. (1980). The oocytes were selected from this preparation with a series of nylon gauzes (respectively, 1500 µm, 1000 µm, 500 µm, 350 µm, 200 µm, 150 µm, 75 µm; Nylon Monofil, Nybolt, Zürich, Switzerland). Smaller cells such as erythrocytes, and single follicle cells and other debris, were removed from the oocytes with several extra washing steps of the oocyte preparation over the 150 and 75 µm screens. Follicle cell-free oocyte preparations (average oocyte diameter ~100 µm) were homogenized in a Pierce Reactivar tube with a glass Dounce pestle. Egg cells were disrupted by this treatment while the zonae remained largely intact. The cell free zonae were collected

on a 50 µm nylon screen. This preparation was washed five times to remove remaining cell debris and other soluble contaminants. Purity was checked by: (i) microscopical evaluation of the zona ghosts; (ii) assays for glucosaminidase (McGuire et al., 1972) and naphthylamidase (Suszkiw and Brecher, 1970) to monitor the complete removal of cellular remnants; and (iii) assays for alkaline phosphatase as a plasma membrane marker (Lindhart and Walter, 1963; Buhr et al., 1989). The zona preparations containing residual enzyme activities that were less than 3% of the original oocyte homogenate were stored at -20°C. Solubilization of zonae was carried out in a sodium carbonate buffer (2 hours, pH 10, 70°C) and insoluble material was pelleted (O'Rand and Fisher, 1987). A smear was made with 2 µl of a zona solution (0.5 mg protein per ml) on a coverslip (15 mm × 15 mm) (Richardson et al., 1991). This coverslip was allowed to dry in air at 37°C and washed thoroughly with HBSS prior to the sperm binding experiments.

### Binding of sperm cells to zona-coated coverslips

The zona-coated coverslip was carefully placed on a 100 µl drop of a HBSS-washed sperm solution (10<sup>7</sup> sperm cells per ml) with forceps (in a 37°C incubator with a 5% CO<sub>2</sub> humidified atmosphere). After 10 minutes the unbound sperm cells were removed with a gentle stream of HBSS. The flushed coverslips with bound spermatozoa were replaced in the incubator.

### Immunolabelling

#### Washing procedure

Sperm cells in suspension were centrifuged in a swing-out centrifuge (500 g, 5 min, at 23°C). The sperm pellet was gently resuspended to the original volume with medium. Sperm cells bound to zona-coated coverslips were carefully removed from the drop of medium and gently washed in a beaker of medium with forceps. Coverslips with a minimum of fluid were carefully placed on a new drop of medium. The coverslips were allowed to float on these drops with the zona-coated face down and the attached spermatozoa in the medium. Air-bubbles under the coverslip were carefully removed with a Pasteur pipette. The integrity of sperm cells was checked after washing as described previously (Gadella et al., 1991).

#### Immunoincubations

The sperm samples were washed in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and fixed with PBS containing 2% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde at 4°C for 1 hour. After fixation the sperm samples were quenched by three incubation steps with PBS containing 50 mM glycine (10 minutes at 23°C). The sperm samples were washed three times in PBS containing 1% (w/v) gelatin (PBS-gelatin) and incubated with a 1:40 dilution of O1, O4 or a 1:100 dilution of R or Z in PBS-gelatin. The unbound primary antibodies were removed with six PBS-gelatin washing steps (5 minutes at 23°C). Thereafter, the sperm cells were incubated with secondary antibody (1:100 dilution in PBS-gelatin of GaM-FITC (for Z, O1, O4) or GaM-TRITC (for R)). The unbound secondary antibody was removed with six PBS-gelatin washing steps. Subsequently, the sperm samples were washed three times in PBS and mounted in Mowiol® containing 1,4-diazo-bicyclo-(2,2,2)-octane to inhibit photobleaching. Total immunofluorescence measurements of sperm samples were performed with a Luminescence Spectrometer LS50 from Perkin Ellmer (Beaconsfield, UK). The excitation and emission wavelength were 550 and 580 nm for TRITC-labelled samples, and 470 and 500 nm for FITC-labelled samples, respectively (slit-width 10 nm). In control experiments sperm cells were incubated as above but not labelled with the secondary fluorescent-conjugated antibody. On these samples no fluorescent signal was detectable, showing that there was no autofluorescence. In another control experiment the incubation step with the monoclonal antibody dilution was omitted. In these samples no immunofluorescence was detectable, indicating that there was no aspecific binding of the fluorescent-conjugated antibodies.

### Digital imaging fluorescence microscopy

A Zeiss Axioplan microscope equipped with epi-illumination (100 W mercury lamp), fluorescence/phosphorescence detection optics and a Zeiss plan-neofluar  $\times 100$  NA 1.3 oil immersion objective was used. Fluorescein fluorescence was selected with a Zeiss 450-490 nm excitation filter, an Omega FT 500 dichroic mirror (Omega Optical Inc. Brattleboro, VT) and an Omega 525 DF 30 emission filter. For rhodamine-stained preparations we used a Zeiss BP 546 excitation filter, a Zeiss FT 580 dichroic mirror and a Zeiss LP 590 emission filter. A slow-scan Series 200 (CH220) CCD-camera (Photometrics Inc., Tucson AZ) was placed on top of the microscope.

### Fluorescence lifetime imaging microscopy

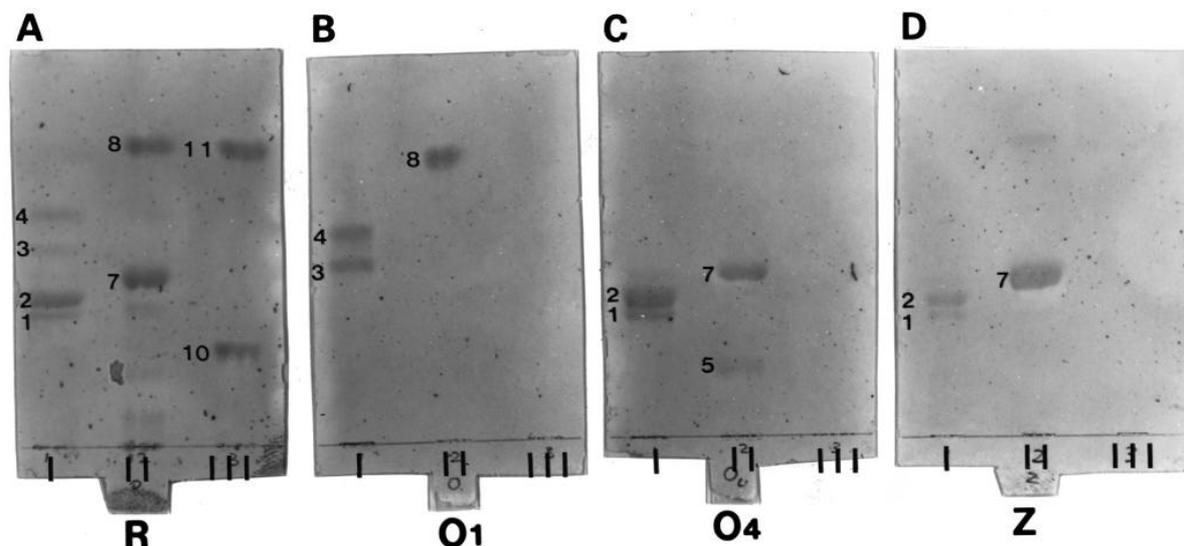
The equipment and set up of the frequency-domain-fluorescence-lifetime imaging microscope has been described in detail elsewhere (Gadella et al., 1993b). Briefly, the microscope consists of an epi-illumination Zeiss Universal Microscope (Oberkochen, FRG), coupled to an image-intensifier-CCD-camera detection system. An argon-ion laser was used as light source (488 nm for fluorescein-stained samples or 514 nm for rhodamine-stained samples). To specifically collect the fluorescein fluorescence, we used a Zeiss FT 505 nm dichroic mirror and a Zeiss 515-565 nm emission filter. For rhodamine-stained prepa-

rations, the filters were a Zeiss FT 580 nm dichroic mirror and a Zeiss LP 590 nm long-pass filter. For other technical details see Gadella et al. (1993b) and the legend to Fig. 7 of that article in particular.

## RESULTS

### Immuno-TLC of galactolipids

In order to immunolocalize SGaAAG and GaAAG on the surface of the sperm cell we used four different monoclonal anti-galactolipid antibodies: O4 and O1 (Sommer and Schachner, 1981; Bansal et al., 1989), Z (Goujet-Zalc et al., 1986) and R (Ranscht et al., 1982). These antibodies showed high binding specificity towards various (sulpho)galactolipids but did not bind to a range of other glycolipids, phospholipids and neutral lipids (e.g. see Goujet-Zalc et al., 1986; Bansal et al., 1989). O4 and R antibodies were originally reported to recognize (sulpho)galactoceramides but do bind also to SGaAAG (Bansal et al., 1989); Fig. 1A,C. The Z antibody was reported to specifically bind to SGaAAG and sulphogalactoceramides (Goujet-Zalc et al., 1986). Additionally, we tested



**Fig. 1.** Immunoreactivity of monoclonal antibodies against various galactolipids. (Sulpho)galactolipids were isolated from various sources and 5 nmol of each was spotted on plates A-D which were developed as described in materials and methods: lane I, four different (sulpho)galactosylceramides (I.1-4); lane II, four different (sulpho)galactosyletherglycerides (II.5-8); lane III, three different (sulpho)galactosyl-diglycerides (III.9-11). Plates: (A) the R antibody, (B) the O1 antibody, (C) the O4 antibody, (D) the Z antibody. In all plates GaM-HRP was allowed to bind to the monoclonal antibodies adsorbed to the TLC plates. The binding was visualized with the DAB peroxidase staining method (Kniep and Mühlrad, 1990). Reference lipids were run on parallel lanes and stained with orcinol; the numbers (1-11) correspond with the determined  $R_F$ -values.

Location on TLC	Reference lipids	Immunoreactivity with antibodies			
		R	O1	O4	Z
I.1	Galactose (3-sulphate) $\beta$ 1-1'(N-[2-hydroxy]-acyl)-sphingosine)	++	-	++	+
I.2	Galactose (3-sulphate) $\beta$ 1-1'(N-acyl)-sphingosine)	++	-	++	+
I.3	Galactose $\beta$ 1-1'(N-[2-hydroxy]-acyl)-sphingosine)	+	++	-	-
I.4	Galactose $\beta$ 1-1'(N-acyl)-sphingosine)	+	++	-	-
II.5	1-O-alkyl-3- $\beta$ -(3'sulphogalactosyl)-glycerol	-	-	+	-
II.6	1-O-alkyl-3- $\beta$ -galactosyl-glycerol	-	-	-	-
II.7	1-O-alkyl-2-O-acyl-3- $\beta$ -(3'sulphogalactosyl)-glycerol*	++	-	++	++
II.8	1-O-alkyl-2-O-acyl-3- $\beta$ -galactosyl-glycerol†	++	++	-	-
III.9	Sulphoquinovosyldiacylglycerol	-	-	-	-
III.10	Digalactosyldiacylglycerol	+	-	-	-
III.11	Galactosyldiacylglycerol	++	-	-	-

-, no antibody binding; +, weak antibody binding; ++, strong antibody binding; \*, SGaAAG; †, GaAAG.

for the first time the binding characteristics of the four antibodies to various galactoglycerolipids (and derivatives) by immuno-TLC (the data are summarized in Fig. 1). For the immunolocalization experiments described below it is important to note that both the O4 and Z antibodies bind strongly to SGaAAG (Fig. 1C,D) and not to GaAAG or other desulphogalactolipids. The R antibody binds to SGaAAG and GaAAG (Fig. 1A) whereas the O1 antibody recognizes GaAAG but not SGaAAG (Fig. 1B). The reactivities of the four antibodies to galactocerebrosides and sulphogalactocerebrosides are in agreement with data from previous studies (Goujet-Zalc et al., 1986; Bansal et al., 1989). There is a slight difference in specificity between the Z and O4 antibodies: (i) the Z antibody binds more strongly to SGaAAG than to sulphogalactocerebrosides whereas the O4 antibody has a higher affinity for sulphogalactocerebrosides. (ii) The O4 antibody binds weakly to deacylated SGaAAG whereas the Z antibody appears not to recognize this lipid. None of the antibodies binds exclusively to hydroxy or nonhydroxy forms of sulphogalactoceramide or galactoceramide. Galactodiacylglycerol and digalactodiacylglycerol were only recognized by the R antibody; indicative of the relatively lower specificity of the R antibody that probably recognizes all lipids with a terminal 3- $\beta$ -galactosyl moiety independently of the sulphate group and the structure of the lipid backbone. However, the lyso-ethergalactolipids were not recognized by the R antibody (Fig. 1A). None of the antibodies showed binding to sulphoquinovosyldiacylglycerol (Fig. 1).

### Immunolocalization of SGaAAG and GaAAG

#### Freshly ejaculated sperm

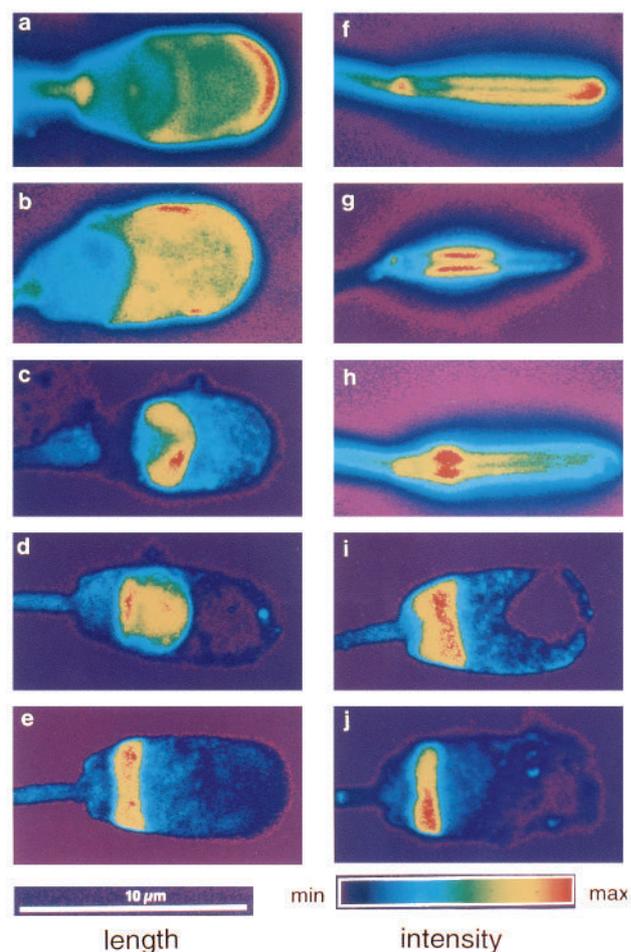
Indirect immunolabelling of SGaAAG was performed to detect the lateral distribution of the lipid on the surface of boar spermatozoa. In Fig. 2 the surface distribution of SGaAAG is visualized with the Z antibody that is most specific for SGaAAG (see Fig. 1). The Z label was found to be inhomogeneously distributed over the sperm surface of freshly ejaculated spermatozoa (Fig. 2A). The apical ridge subdomain of the sperm plasma membrane was highly labelled with this antibody. Similar distribution patterns were obtained with R and O4 antibodies (not shown).

On the other hand, no fluorescent labelling was detectable with the O1 antibody, corroborating the finding that GaAAG is absent from freshly ejaculated sperm cells (Gadella et al., 1993a).

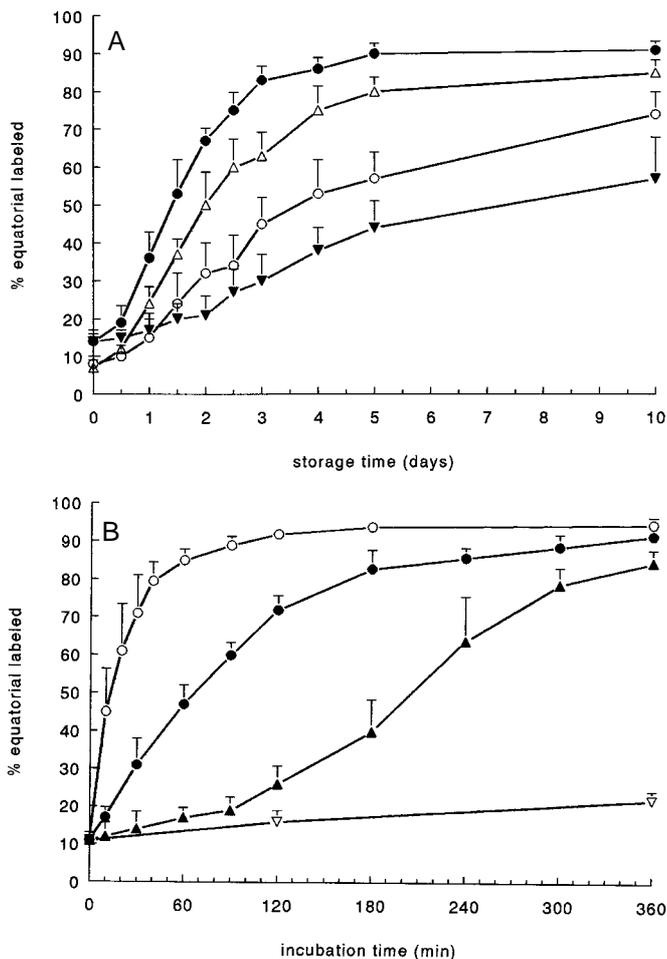
#### Arylsulphatase A incubations

Previously, we described the presence of high concentrations of an extracellular form of arylsulphatase A (AS-A; EC 3.1.6.8) in seminal plasma. This enzyme can desulphate SGaAAG on intact spermatozoa (Gadella et al., 1991, 1992, 1993a). We investigated whether incubation with AS-A alters the SGaAAG label pattern on spermatozoa with the Z antibody. Because AS-A converts SGaAAG into GaAAG the appearance of O1 labelling of the sperm surface was also followed. Fig. 2A-E shows that during AS-A incubation the SGaAAG labelling pattern shifted from the apical ridge to the equatorial subdomain of the sperm plasma membrane. Characteristically, boar spermatozoa, like other eutherian sperm cells, have very flat sperm heads (Bedford, 1991; see also Fig. 2F-H, where the sperm head flat faces are parallel to the

bundle of the microscope). The distribution and shift of the Z label on these sperm heads was similar to that seen in micrographs of sperm heads oriented with their flat face perpendicular to the light bundle (compare Fig. 2F-H with Fig. 2A,C,E). Moreover, Fig. 2F-H demonstrates that the Z antibody labelled only the surface of the sperm cells. The kinetics of this AS-A-induced shift of Z labelling in sperm populations is depicted in Fig. 3A, which demonstrates that the addition of AS-A stim-



**Fig. 2.** Immunofluorescence labelling of SGaAAG and GaAAG on the sperm head of cells treated with AS-A. (a-h) Indirect immunolabelling of SGaAAG with the Z antibody and the GaM-FITC fluorescent immunoconjugate. (a-e) Sperm heads oriented with their flat face perpendicular to the light bundle: (a) A freshly ejaculated sperm cell with apical ridge labelling; (b) A sperm cell incubated for 12 hours with AS-A showing a more diffuse pre-equatorial labelling; (c and d) two sperm cells incubated for 24 hours with AS-A showing intermediate equatorial labelling; (e) a sperm cell that has been incubated with AS-A for 48 hours. (f-h) Sperm cells oriented in parallel with the light bundle: (f) a freshly ejaculated sperm cell; (g,h) sperm cells incubated with AS-A for 1 day (g), or 2 days (h). (i and j) Indirect immunolabelling of GaAAG with O1 antibody and GaM-FITC fluorescent immunoconjugate after incubation sperm cells with of AS-A for 1 day (i) or 2 days (j). The dimensions of the boar sperm head are  $8 \mu\text{m} \times 4 \mu\text{m} \times 0.6 \mu\text{m}$  (length  $\times$  width  $\times$  thickness). The intensity bar represents the linear scale of the digitized fluorescence intensities in the images. The maximum/minimum (background) intensity is  $>10$  for a-e, i and j and  $>25$  for f, g and h.



**Fig. 3.** Kinetics of migration of the SGaAAG label from apical to equatorial subdomain of the plasma membrane on sperm populations as visualized with the Z antibody and the GaM-FITC immun-conjugate. The relative amounts of sperm cells that were equatorially labelled are expressed as a function of time. The shift of the SGaAAG label was followed during different storage experiments (A) and during *in vitro* capacitation and zona binding experiments (B). Of each sample  $3 \times 400$  sperm cells were counted. Data that are mean values  $\pm$  s.d. of five independent experiments are expressed. (A) BTS-washed sperm cells (▼); commercially 10 $\times$  BTS-diluted boar semen (○); undiluted boar semen (△) and washed sperm cells, with an addition of AS-A isolated from seminal plasma (Gadella et al., 1993a; ●). (B) HBSS-washed sperm populations (▽), during *in vitro* capacitation of HBSS-washed sperm cells, with 2 mM CaCl<sub>2</sub> (●) or with 4% (w/v) BSA (▲), and after binding to coverslips coated with immobilized zona matrix (○).

ulated the shift of label from the apical towards the equatorial subdomain significantly. In the control (washed sperm cells stored for 48 hours) the percentage of equatorially labelled cells increased slightly from 15% to 20%, whereas in the presence of AS-A the shift was complete in almost 70% of the cells after this period. Although, O1 did not label freshly ejaculated spermatozoa, the cells became gradually O1 positive at the equatorial subdomain of the sperm plasma membrane upon incubation with AS-A (Fig. 2I,J). After prolonged AS-A incubations GaAAG tended to concentrate in the distal equatorial subdomain (Fig. 2J).

### Effect of storage

In order to determine whether the shift of Z label on sperm cells also occurs during long-term storage of sperm cells in seminal plasma (which contains AS-A; Gadella et al., 1991), we prepared boar sperm samples with different amounts of seminal plasma: (i) undiluted semen; (ii) diluted sperm samples (10 $\times$  in BTS; Johnson et al., 1988); and (iii) BTS-washed sperm samples with minimal amounts of seminal plasma (Gadella et al., 1991). The Z label pattern was followed for 10 days. During this period a redistribution of Z label on the sperm surface occurred similar to that occurring upon incubation of washed sperm with AS-A (e.g. Fig. 2A-H). However, this shift was slower in undiluted sperm samples compared with that in the washed sperm cells that were incubated with a physiological concentration of AS-A (see Fig. 3A). Dilution of the semen with BTS resulted in a decreased migration rate of the Z-label towards the equatorial subdomain of the sperm head. Furthermore, Fig. 3A shows that this rate was lowest in semen samples from which seminal plasma was removed completely by washing and resuspending the cells in BTS. However, the washing procedure causes some damage to a small population of spermatozoa (Gadella et al., 1991), which may explain the slightly enhanced number of equatorially labelled cells observed at zero time.

### Effect of *in vitro* capacitation on SGaAAG distribution

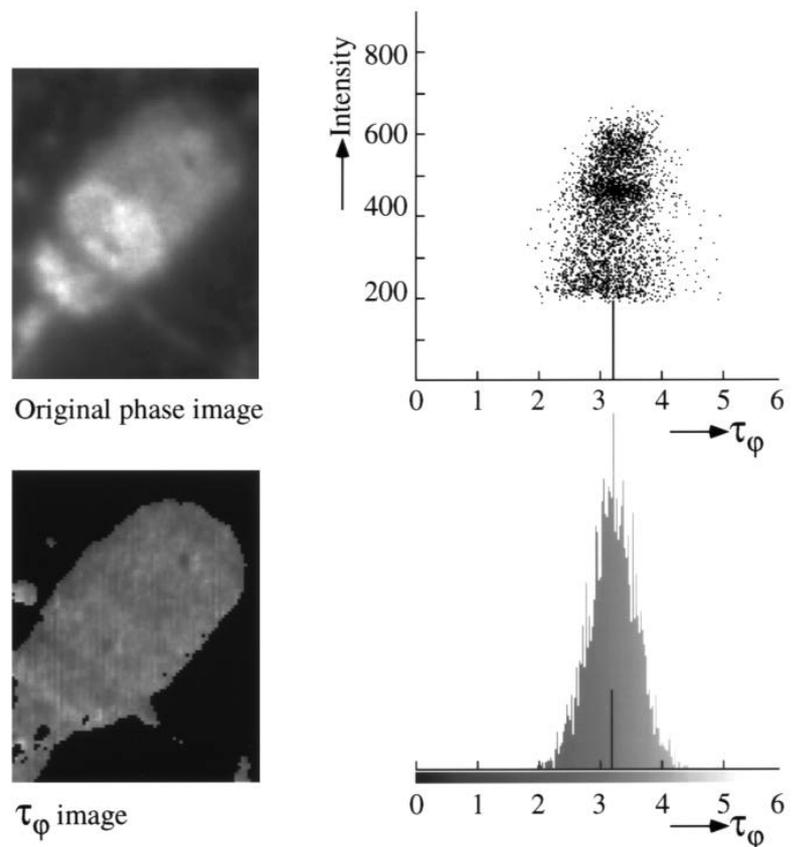
As mentioned in the Introduction, the surface organization of the sperm cell changes during their capacitation *in vitro*. For this reason we tested the effect of various inducers of capacitation (Ca<sup>2+</sup>, BSA) on the Z-labelling of spermatozoa. The BTS buffer is not appropriate for this purpose because it contains EDTA. Therefore, the sperm cells were washed with HBSS, which caused no deterioration of the sperm cells during the incubation period of 6 hours (results not shown, see also Aguas and Pinto da Silva, 1989), and the Z-label remained restricted to the apical subdomain during this period (Fig. 3B). When Ca<sup>2+</sup> or BSA was added to the HBSS, the Z-label moved to an equatorial position on the sperm cell head. This shift of Z label was complete within 2 hours after Ca<sup>2+</sup> addition, and within 5 hours after addition of BSA (see Fig. 3B), which is much faster than was observed during storage experiments (Fig. 3A).

### Zona binding induces SGaAAG movement

Binding to the zona pellucida triggers the sperm cell to undergo surface reorganizations leading to the physiological acrosome reaction (O'Rand and Fisher, 1987; Richardson et al., 1991). Hence, we investigated whether zona pellucida also affected the Z-labelling of sperm cells. To determine the distribution of Z-label on sperm cells that have bound to intact zonae is difficult. Therefore, sperm cells were allowed to bind to coverslips coated with components of the zona matrix (51) and the unbound sperm cells (approx. 75%) were removed after 10 minutes. Remarkably, already more than 40% of the bound sperm cells were Z-labelled at the equatorial subdomain at that moment. This percentage increased rapidly and approached 90% within 1 hour (Fig. 3B). The shift of Z labelling was similar to that depicted in Fig. 2A-E.

### Immunofluorescence lifetime imaging microscopy

The fluorescence lifetime of the bound immunofluorescent label was analysed with FLIM. In all cases the fluorescence



**Fig. 4.** Fluorescence lifetime analysis of immunolabelled boar sperm heads. A boar sperm cell was labelled indirectly with the Z and GaM-FITC antibodies. The upper left subimage shows the original fluorescence distribution on the sperm head. The lower left subimage depicts the in situ fluorescence lifetime distribution on the same sperm. The distribution of fluorescence intensity versus the distribution of fluorescence lifetime of each analysed pixel is expressed in the upper right histogram. The temporal distribution of the fluorescence lifetime ( $\tau_{\phi}$ ) of the analysed pixels is given in the lower right panel.

lifetime was independent of the location of the probe over the various subdomains of the sperm heads, although the fluorescent intensities varied widely among these regions. An illustrative and representative example of such an analysis is shown in Fig. 4 for FITC labelling (mean lifetime 3.1 ns). Comparable results were obtained after TRITC labelling (mean lifetime 2.3 ns). The invariance of fluorescence lifetime, despite the three- to fourfold variation in fluorescence intensity, provides strong evidence that the fluorescence intensity measured in situ is linearly related to the concentration of bound fluorescent probe.

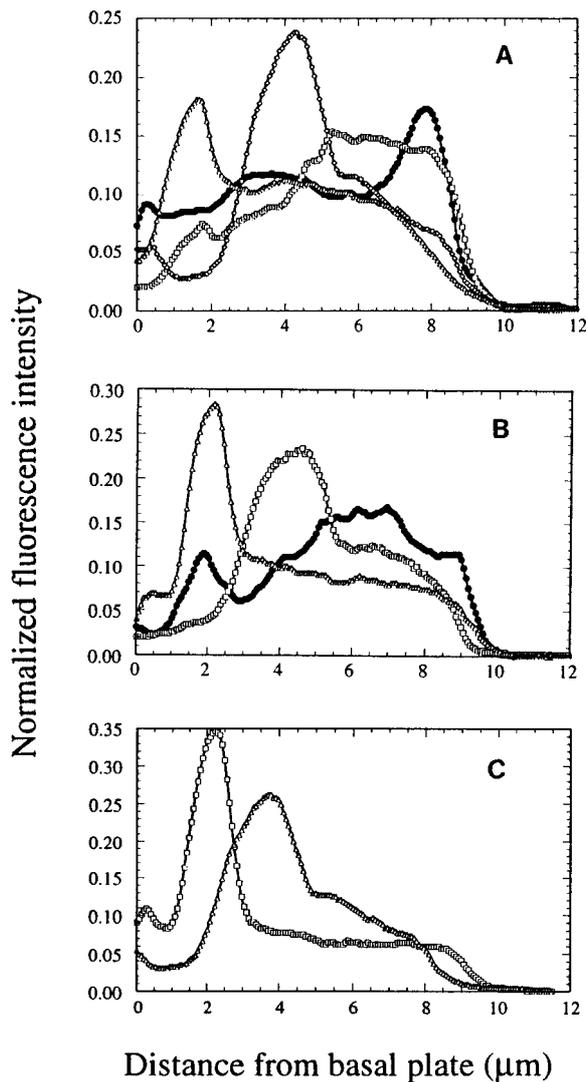
#### Quantitative analysis of fluorescence distribution

The digital images of fluorescence intensity distribution measured over the sperm head surface were processed to quantify the probe distribution. First, by image rotation the sperm cell was oriented with its tail to the left and its apical side to the right (for orientations see Fig. 2). Then the fluorescence intensities were integrated in the  $y$ -direction (top-bottom) and projected onto a single line ( $x$ -axis), and the resulting one-dimensional (axial) distribution was normalized for comparative reasons. The basal plate was used as a reference point (zero point on  $x$ -axes of Fig. 5A-C) as this is a morphological orientation point of the sperm head. At this site the nuclear ring separates the plasma membrane domains of the head and the mid-piece. The apical ridge of the head is less reliable as a reference point because the sperm acrosome swells and vesiculizes with the plasma membrane during the acrosome reaction (Gadella et al., 1991). The quantitative distribution of Z label is expressed as a function of distance from

the basal plate in Fig. 5A,B. The shift of Z label upon AS-A incubation is visualized in Fig. 5A. Clearly, the Z label was initially enriched at the apical site of the head, and then redistributed over the sperm head towards the equatorial segment. Similar redistribution of the Z label occurred during the other storage experiments as well as during the in vitro capacitation experiments with BSA or with  $\text{Ca}^{2+}$ . The rapid shift of Z label to the equatorial segment of sperm cells after binding to zona matrix-coated coverslips is depicted in Fig. 5B. Freshly ejaculated sperm cells are O1 negative but do show O1 labelling after AS-A treatment (Fig. 5C). In this figure the concentration of O1 label in the distal part of the equatorial segment is also visualized. The relative amount of fluorescent label bound per subdomain of the sperm head was calculated from the line plots and summarized in Table 1.

#### Comparison of R and O4 antibodies with the Z labelling

The antibodies R and O4, which also bind to SGaAAG (see Fig. 1), were also tested and gave very similar results to the Z antibody (see Table 1). The R antibody, which is less specific for SGaAAG than O4 or Z (see Fig. 1), did interact to some extent with the zona matrix on the coverslips. This aspecific binding was not observed with Z or O4. The more intense R labelling at the equatorial region of the sperm surface after incubating the cells with AS-A is probably caused by the accumulation of GaAAG, which is also an epitope for R but not for Z or O4 (Fig. 1). This is supported by the restricted equatorial subdomain appearance of GaAAG after AS-A incubation as visualized by O1. No significant differences in R compared with Z or O4 labelling were found during treatments



**Fig. 5.** The distribution of fluorescent probe over the sperm head expressed as a function of the distance from the basal plate. (A) Different Z labelling patterns on sperm cells incubated with AS-A; a freshly ejaculated sperm cell with a label pattern as in Fig. 2A (●); after incubation with AS-A for 12 hours (label pattern of Fig. 2B; □), 24 hours (label pattern as in Fig. 2C; ◇) and 48 hours (as in Fig. 2E; △). (B) Different Z labelling patterns at sperm cells that have adhered to zona matrix-coated coverslips. A sperm cell that was allowed to bind to the zona matrix for 10 minutes (●), and two sperm cells detected on the zona matrix after 30 minutes of binding; one with equatorial labelling (□) and one with Z label concentrated in a distal region of the equatorial subdomain (△). (C) Two sperm cells with O1 labelling after prolonged AS-A incubation (2 days): a sperm cell with equatorial labelling (△), and with a more distally concentrated labelling in the equatorial segment (□).

without AS-A (Table 1), which supports the notion that GalAAG is absent from freshly ejaculated sperm cells.

After immunolabelling, sperm suspensions were placed in a cuvette and the total fluorescence was measured in a fluorometer. Importantly, the amount of immunolabel bound to the sperm cells remained constant before and after migration. Similar observations were made with O4 and R antibodies

**Table 1. Labelling of boar spermatozoa after various treatments with four anti-galactolipid antibodies (Z, O4, R and O1) and quantitative analysis of their distribution over the sperm head**

Treatment	Antibody	Relative distribution of immunolabelling over the sperm head (% of total fluorescence per head)				
		Apical	Pre-equatorial	Equatorial	Distal equatorial	Post-equatorial
Control	Z	38	19	19	12	12
	O4	37	19	20	12	12
	R	36	18	22	12	12
Ca <sup>2+</sup>	Z	4	12	49	27	8
	O4	3	11	51	23	12
	R	4	10	50	30	6
BSA	Z	10	11	45	25	16
	O4	10	10	46	27	17
	R	10	12	43	24	20
ZP	Z	4	18	28	42	8
	O4	4	11	26	40	20
	R*					
AS-A	Z	5	16	31	39	9
	O4	7	16	30	41	6
	R	4	16	24	50	6
	O1†	4	4	10	72	10

Control, freshly ejaculated sperm cells with an apical labelling pattern (see Fig. 2A); Ca<sup>2+</sup>, sperm cells capacitated with 2 mM CaCl<sub>2</sub>; BSA, sperm cells capacitated with 4% (w/v) BSA; ZP, sperm cells bound to zona-coated coverslips; AS-A, washed sperm cells stored with AS-A (Fig. 2E); mean values are indicated (*n*=5); s.e.m. < 10%.

\*Data of R labelling on zona-bound spermatozoa were not reliable because of considerable background fluorescence.

†Labelling with O1 was not different from background fluorescence in the absence of AS-A.

during capacitation *in vitro*. On the other hand, 48 hours of AS-A incubation resulted in a 30% loss of Z and O4 immunolabel and a concomitant appearance of O1 label, whereas, the amount of R label remained constant during this procedure.

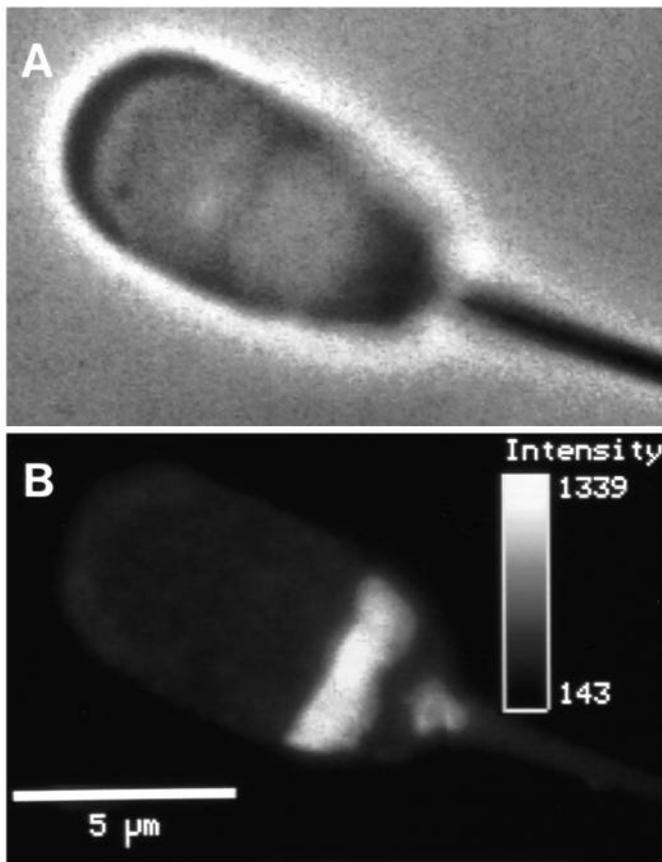
#### Surface reorganizations occur before the acrosome reaction

Sperm cells with intact acrosome morphology (Gadella et al., 1991) already showed equatorial labelling (see Fig. 6). Moreover, all acrosome-reacted sperm cells that we have observed showed an equatorial immunolabel pattern of (S)GalAAG (results not shown), indicating that the described surface reorganizations precede the acrosome reaction.

#### DISCUSSION

In this paper we demonstrate the polarity and lateral mobility of (S)GalAAG over the surface of the sperm head by immunolabelling with four monoclonal antibodies. On freshly ejaculated boar spermatozoa only the apical subdomain of the sperm head plasma membrane was found to be strongly labelled with antibodies against SGalAAG (Fig. 2A); an observation that is in agreement with the immunocytochemical localization of SGalAAG at the apical site of mouse and rat epididymal spermatozoa reported earlier by Lingwood (1986).

The most important observation of the present study is that the migration of the immunolabel from the apical towards an equatorial position is a general post-ejaculatory event



**Fig. 6.** Phase-contrast micrograph (A) and fluorescence micrograph (B) of a boar sperm cell exposed to 2 mM  $\text{CaCl}_2$  for 2 hours. This sperm cell is labelled at the distal end of the equatorial subdomain with the Z antibody (B), and its acrosome is still intact, since the normal apical ridge structure is still visible (A; see Gadella et al., 1991).

occurring before the acrosome reaction (Fig. 6). Furthermore, capacitating agents such as BSA, or  $\text{Ca}^{2+}$ , greatly accelerated this process (Fig. 3B). The lateral migration of SGalAAG immunolabel was even more facilitated in sperm cells that were allowed to bind to zona-matrix-coated coverslips. With regard to the latter observation we should note, however, that only one out of four sperm cells did bind to the coated coverslips and that only this bound subfraction has been analysed. Thus, we cannot exclude the possibility that the bound subfraction was a positive selection of cells prone to undergo the surface reorganization monitored by a shift of the SGalAAG immunolabel.

Another interesting aspect of the present study is that addition of an AS-A preparation (Gadella et al., 1993a; AS-A converts SGalAAG into GalAAG) induced the same shift of the SGalAAG-specific immunolabel (Fig. 2B-H). GalAAG was not found in freshly ejaculated boar sperm cells (Gadella et al., 1992) and, indeed, the GalAAG-specific antibody (O1) did not bind to freshly ejaculated sperm cells, but label gradually appeared at the equatorial segment of the sperm head plasma membrane during incubations with AS-A (Fig. 2I,J).

The fact that incubation of sperm cells with seminal plasma did also trigger the shift of SGalAAG-specific immunolabel

could be due to the presence of AS-A secreted by seminal vesicles in this extracellular fluid (Gadella et al., 1993a). In line with this are the observations that the migration was much faster in undiluted semen than in diluted and washed semen, as in these samples the extracellular AS-A content decreased in the same order (Gadella et al., 1992; Fig. 3A). GalAAG labelling was only detectable in the equatorial segment of the sperm plasma membrane, and this strongly suggests that, after desulphation by AS-A, the lipids immediately relocate to the equatorial segment. On the other hand, it is also possible that desulphation of SGalAAG by AS-A is only possible after its migration from the apical to the equatorial subdomain. Most probably, the removal of the hydrophilic sulphate group results in enhanced hydrophobic interactions of GalAAG (when compared with SGalAAG) with neighbouring membrane lipids. Massive desulphation also triggered SGalAAG to move to this segment. This indicates that a substantial loss of negative charge in the apical subdomain of the sperm head plasma membrane triggers the observed lipid redistribution. Interestingly,  $\text{Ca}^{2+}$  induced a similar lipid redistribution. We suggest that this effect is due to partial reduction of the lipid(sulphate) negative charge by complex formation with  $\text{Ca}^{2+}$ . Preliminary observations on artificial membranes demonstrated that SGalAAG and SGalCer can bind free  $\text{Ca}^{2+}$  and this binding causes an increase in the membrane fluidity that affects its lateral phase behaviour (Tupper et al., 1992).

#### Migration of (S)GalAAG: fact or artifact?

An important point of discussion remains as to whether the observed shift of immunolabel from the apical to the equatorial subdomain does indeed reflect the lateral migration of SGalAAG or is an artifact of the immunolabelling procedure. The following points were evaluated in order to provide an answer to this question.

(i) The specificities of the monoclonal antibodies to SGalAAG and derivatives were checked (see Fig. 1). It has been reported previously that the antibodies R, O4 bound (Bansal et al., 1989) and Z (Goujet-Zalc et al., 1986) recognize SGalAAG but the reactivity to its desulphated or deacylated analogues had not been determined previously. Although, SGalAAG is the exclusive glycolipid in mature spermatozoa, GalAAG and lyso(sulpho)galactoglycerides may emerge during the experiments as a result of degradation of SGalAAG by AS-A (Gadella et al., 1993) or by the action of acylhydrolase (Lingwood et al., 1990). Therefore, we additionally checked the specificities of the four monoclonal antibodies for these galactoglyceride derivatives (Fig. 1). From our results we can now conclude that: (a) the Z and O4 antibodies cannot cross-react with GalAAG; (b) the O1 antibody labels GalAAG but not SGalAAG, whereas the R antibody recognizes both lipids. The deacylated analogues are not recognized by R, Z and O1; only weak binding of O4 occurred with deacylated SGalAAG.

(ii) The labelling of SGalAAG and GalAAG appears to be restricted to the surface of the sperm cell (Fig. 2F-H), which is additional evidence for the exclusive location of SGalAAG in the outer leaflet of the plasma membrane (Gadella et al., 1993a). It is noteworthy that the observed redistribution of SGalAAG-specific immunolabel was very similar to that of AS-A over the surface of rabbit spermatozoa (Nikolajczyk and

O'Rand, 1992), probably because the enzyme colocalizes with its substrate.

(iii) In all labelling experiments a very narrow temporal lifetime distribution of the immunolabel was observed over the sperm head surface with FLIM (e.g. see Fig. 4). The important consequence of this invariance is that the differences in detected fluorescence intensities over sperm heads are not the result of subdomain-specific excited state kinetics of the immunofluorescent probe, but quantitatively reflect the spatial distribution of the fluorescent probe.

(iv) The accessibility of (S)GalAAG in the sperm membrane to antibodies appears to be proportional not only to the amount of antibody but also to the amount of (S)GalAAG per unit of the sperm plasma membrane because: (a) the total amount of SGalAAG-specific immunofluorescent labelling remained the same during the *in vitro* capacitation experiments although the sperm head distribution of the label altered. (b) During two-day incubations with AS-A the amount of Z and O4 label decreased with 30%, which corresponds with the relative amount of SGalAAG desulphated in this time period (Gadella et al., 1993a). In contrast, the amount of R label remained identical because it labels both SGalAAG and GalAAG, probably with similar affinity (Fig. 1). The increase in O1 immunofluorescence on sperm cells that were incubated with AS-A is in agreement with these observations.

(v) The label distribution of the four different antibodies was very similar under the various conditions (Table 1). The redistribution of SGalAAG followed a similar pattern during these experiments, albeit on a different time scale (Fig. 3).

Considering (i) to (v), we are confident that the digital image information about fluorescence intensity distribution does reflect the *in situ* concentration and migration of (S)GalAAG over the sperm head surface. Moreover, we have recently incorporated a fluorescent acylated sulphogalactolipid into the sperm plasma membrane and found it predominantly in the apical subdomain of freshly ejaculated sperm cells. Also the redistribution of this lipid upon *in vitro* capacitation was very similar to that observed by immunolabelling of SGalAAG. This strongly indicates that the immunolabelling patterns of SGalAAG described here do not result from subdomain polarities of the extracellular matrix and that a subdomain-specific steric hindrance to the binding of the antibodies to their epitope can be ruled out (Gadella et al., unpublished data).

### Models of subdomain polarity of the sperm cell plasma membrane

The limited capacity of the sperm cell for lipid metabolism as well as the absence of membrane flow via vesicle-mediated transport (endocytosis and exocytosis) certainly contribute to the polar appearance of its plasma membrane (Holt, 1984; Vos et al., 1994). However, at the molecular level fundamental understanding of how the sperm membrane subdomains are formed, maintained and reorganized is lacking.

Two models have been proposed to explain these phenomena (for review see Peterson and Russell, 1985). Briefly, one model states that the peripheral glycoconjugates of the sperm plasma membrane are ordered in subdomain-specific matrices and that, as a consequence, integral membrane proteins and lipids that interact with these matrices are also polarly ordered over the sperm surface (the matrix-model). According to the second model, the lateral polarity of

the sperm plasma membrane is induced by the thermotropic phase behaviour of the membrane lipids (the lipid-model). Above the phase transition temperature ( $T_m$ ) lipid molecules can diffuse rapidly within their leaflet of the membrane lipid bilayer (liquid-crystalline phase) whereas below the  $T_m$  they freeze into the gel phase, which prevents the lateral diffusion of lipid monomers. The formation of the gel phase results in an exclusion of fluid lipids and transmembrane proteins (lateral phase segregation); a phenomenon that has been observed during *in vitro* capacitation experiments (Aguas and Pinto da Silva, 1989).

Our results do not allow discrimination between the two models but they fit best into the lipid model because the matrix model fails to explain that after AS-A treatment not only GalAAG but also the remaining SGalAAG (supposedly bound to the matrix) moves to the equatorial subdomain. In our view, the matrix model would predict local and gradual changes in the lipid composition only in those regions where specific interactions between the plasma membrane lipids and the overlying extracellular matrix are disrupted or changed. In contrast, the changes in glycolipid distribution that we observed were axially symmetric changes (see Fig. 2), which is consistent with a lipid phase separation. Additional arguments favouring the lipid model are: (i) the number of SGalAAG molecules on the surface of a sperm cell ( $1.8 \times 10^8$  per sperm cell; Gadella et al., 1992) greatly exceeds the number of interaction sites on integral membrane proteins (1000 to 10,000 Aguas and Pinto da Silva, 1989) and on peripheral glycoconjugates (~2000 for the SGalAAG binding protein SLIP; calculated from data of Tanphaichitr et al., 1993); (ii) the unique lipid composition of the mammalian sperm cell probably plays an important role in the formation, establishment and reorganization of the sperm plasma membrane subdomains (see Introduction).

Parenthetically, the phase behaviour of SGalAAG might be of physiological interest because the thermotropic transition temperature of SGalAAG in boar spermatozoa is 36.4°C (Parks and Lynch, 1992) and the epididymal spermatozoa mature below that temperature, at 35°C, whereas an ejaculated spermatozoon will meet the egg cell at the higher oviductal temperature of about 40°C (Mann and Lutwak-Mann, 1981). This implies that a gel to liquid-crystalline phase transition of SGalAAG may occur during the progress of capacitation in the female genital tract. Such a phase transition influences the lateral organization of the sperm surface, and may enable the sperm cell to bind to the zona pellucida.

The amount of cytosol in the sperm cell is minimized during sperm maturation and consequently the plasma membrane is in very close contact with several intracellular organelle membranes (Mann and Lutwak-Mann, 1981; Vos et al., 1994). Hence, the shape of the subdomains in the sperm plasma membrane follows the polar organization of these underlying intracellular structures. Therefore, we propose that, as well as the lateral behaviour of the lipids, the inner environment of the sperm plasma membrane may also be important for its organization into these subdomains.

### Possible physiological functions of SGalAAG

The organization and dynamic changes of subdomains in the plasma membrane of a sperm cell are essential for its functioning, after ejaculation, in the female genital tract. We will

follow the sperm cell on this journey and focus on the roles that SGalAAG may play meanwhile. (i) During and after the ejaculation the acrosome must remain intact until the sperm cell binds to the zona pellucida. SGalAAG stabilizes the lamellar phase of lipid bilayers (Mannock et al., 1985; Curatolo, 1987; Mannock and McElhaney, 1991) and thus may block fusion reactions between membranes. The present results show that at this stage SGalAAG is concentrated in the apical ridge subdomain. There it prevents the acrosome reaction, which is a multiple fusion event between the plasma membrane and the outer acrosomal membrane. (ii) When the sperm cell approaches an oocyte it binds to the zona pellucida with its apical ridge subdomain, which contains specific adhesion molecules that can interact with zona proteins (Wassarman, 1987). A peripheral sperm protein called sulphogalactolipid immobilizing protein (SLIP) that is associated with the surface of mouse sperm has been shown to be involved in the binding of the sperm cell to mouse zonae (Tanphaichitr et al., 1992, 1993). (iii) After zona binding the zona pellucida triggers the acrosome reaction (O'Rand and Fisher, 1987). Importantly, the apical and pre-equatorial subdomains of the plasma membrane fuse with the underlying outer acrosomal membrane whereas the equatorial subdomain of the sperm head does not fuse with the underlying outer acrosomal membrane (Mann and Lutwak-Mann, 1981; Peterson and Russell, 1985). This can be explained by the observation that preceding the acrosome reaction (Fig. 6) SGalAAG migrates from the apical ridge (which destabilizes that segment of the plasma membrane) to the non-fusogenic equatorial subdomain (Fig. 2). The acrosome reaction results in the generation of mixed vesicles from the parts of the plasma membrane that have fused with the outer acrosomal membrane and in the release of the acrosomal fluid containing enzymes that will lyse the zona pellucida. Parenthetically, we may note that these vesicles did contain only marginal amounts of SGalAAG, indicating that the lipid was removed from that part of the surface area of the spermatozoon before the acrosome reaction took place (results not shown). After the acrosome reaction the sperm cell drills through the zona to its destination: the oocyte plasma membrane. (iv) The final fertilization of the oocyte is initiated by a specific single point fusion of its plasma membrane with the sperm cell plasma membrane at the equatorial subdomain (Wassarman, 1987). The interesting possibility that sperm SGalAAG can specifically interact with a peripheral oocyte membrane protein (SLIP) was recently reported (Law et al., 1988; Tanphaichitr et al., 1993). This interaction might serve to provide a close encounter between the two gametes and facilitate the fusion of their plasma membranes. The generation of GalAAG by AS-A (which is also restricted to the equatorial subdomain; Fig. 2I,J) may enhance the fusogenicity of this subdomain with the oocyte plasma membrane. It is known that GalAAG, like other monogalactolipids, can induce a transition of the lipid bilayer from the lamellar to the hexagonal II phase, which has been proposed as an intermediate in membrane fusion (Mannock et al., 1985; Curatolo, 1987; Mannock and McElhaney, 1991).

In conclusion, in this report we provide evidence that: (i) SGalAAG is concentrated over the apical surface of freshly ejaculated sperm cells; (ii) this sperm-specific sulphoglycolipid migrates to the equatorial subdomain of the sperm head after

capacitation in vitro with  $\text{Ca}^{2+}$  or BSA, or interaction with zona matrix components.

Our hypothesis with regard to biological function is that SGalAAG, because of its presence in the outer leaflet of the sperm head plasma membrane, can fulfil a double and paradoxical role in the sperm cell: (a) when present in the apical subdomain it prevents the plasma membrane fusing with the underlying outer acrosomal membrane; (b) after its migration to the equatorial subdomain of the sperm head it contributes to the final fusion event between the sperm and egg cell plasma membranes.

FLIM proved to be an efficient tool for analysing the subdomain polarity of SGalAAG. With this technique we could directly correlate the intensity of fluorescence to the local concentration of the probe (Fig. 4) and thus exclude the possibility that the observed differences in the fluorescence intensities were an artifact due to differences in fluorescence lifetime. Future studies should concentrate on the thermotropic phase behaviour of SGalAAG in relation to the observed polarity phenomena of the lipid in the sperm plasma membrane.

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