

# Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction

## Evidence for a primary capacitation event in boar spermatozoa

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### SUMMARY

In order to extend the static information of immunolabelling sulphogalactolipids in fixed boar spermatozoa, a fluorescent sulphogalactolipid analogue, galactose(3-sulphate)- $\beta$ 1-1'[(*N*-lissamine rhodaminy)-12-aminododecanoyl]-sphingosine, was incorporated into plasma membranes of living spermatozoa and its lateral distribution over the sperm head was studied. The fluorescent lipid was enriched in the apical ridge subdomain of freshly ejaculated sperm cells. After sperm binding to the zona pellucida the lipid redistributed to the equatorial segment of the sperm surface. A similar shift occurred during capacitation *in vitro* with 2 mM CaCl<sub>2</sub> or with 4% (w/v) bovine serum albumin. The desulphated derivative galactose- $\beta$ 1-1'[(*N*-lissamine rhodaminy)-12-aminododecanoyl]-sphingosine was also incorporated into the plasma membrane of freshly ejaculated sperm cells and clearly stained the apical ridge subdomain and the (pre)-equatorial

subdomains of the sperm heads. The desulphogalactolipid analogue showed a slightly faster migration to the equatorial segment of the sperm plasma membrane than did its sulphated counterpart. The measured fluorescence intensity distributions correlated linearly with the spatial probe distribution, which was checked by fluorescence lifetime imaging microscopy. The observed migration of the incorporated glycolipids precedes the acrosome reaction and is one of the underlying molecular events likely to be important in the process of sperm capacitation. The results of this study suggest that lipid phase segregation is an important driving force for the organization of the sperm head plasma membrane into subdomains.

Key words: sperm capacitation, fluorescent galactolipid, polarity dynamics, plasma membrane, fluorescence lifetime imaging microscopy, acrosome reaction

### INTRODUCTION

The biological function and molecular organization of (glyco)lipids in cellular membranes are important areas in current cell biology (Curatolo, 1987; Hoekstra and Kok, 1992; Marsh, 1992; Glaser, 1993; van Meer, 1993; Vaz and Almeida, 1993; Trotter and Voelker, 1994). Sperm cells and their plasma membranes offer an interesting model system, since they exhibit a distinct lipid composition with an unusual lateral polarity which is subjected to dynamic changes after ejaculation and probably plays an important role in fertilization (for reviews see Vos et al., 1994; Yanagimachi, 1994).

We have previously described the lateral distribution of the glycolipids seminolipid (SGalAAG) and desulphoseminolipid (GalAAG) in fixed boar sperm cells, as determined by immunocytochemistry (B. M. Gadella et al., 1994). Here, we describe the lateral polarity of (sulpho)galactolipids using a more direct approach: the incorporation of fluorescent (sulpho)galactolipids into the plasma membrane of living boar spermatozoa.

Two inherent disadvantages hamper interpretation of immunolocalization studies: (i) the sperm cells have to be fixed prior to the immunolabelling procedure as the anti-glycolipid antibodies alter the lipid topology in the membrane of unfixed cells (for Refs see Dyer, 1993); (ii) the antibodies bind to the hydrophilic headgroup of glycolipids (the epitope for the antibodies; B. M. Gadella et al., 1994) and this can only occur if the epitope is accessible to the antibody. The dynamic redistributions that we observed in post-ejaculatory spermatozoa may therefore reflect the accessibility of the glycolipid to the antibody rather than the real distribution of the glycolipids.

The approach used in this paper is to incorporate the two fluorescent tagged galactolipids: galactose(3-sulphate)- $\beta$ 1-1'[(*N*-lissamine rhodaminy)-12-aminododecanoyl]-sphingosine (SGalCer(C12-LRh)) and galactose- $\beta$ 1-1'[(*N*-lissamine rhodaminy)-12-aminododecanoyl]-sphingosine (GalCer(C12-LRh)) (Marchesini et al., 1990) into the lipid bilayer of sperm plasma membranes and follow their lateral distribution within the membrane. An obvious disadvantage of using fluorescent

lipids is that they differ from the natural glycolipids and this could introduce artifacts. The fluorescent lipid backbone (i.e. [(*N*-lissamine rhodaminy)-12-aminododecanoyl]-sphingosine or Cer(C12-LRh)) differs from the 1-*O*-alkyl-2-*O*-acyl glycerol of SGalAAG. However, the head group ( $\beta$ -galactose or  $\beta$ -galactose 3-sulphate) is identical and, moreover, the lissamine rhodamine moiety is hydrophobic and the effective hydrophobic length of Cer(C12-LRh) is comparable to that of 1-*O*-alkyl-2-*O*-acyl glycerol. We therefore think that structurally related artifacts are likely to be minimal. The important advantages of using these fluorescent probes over an immunocytochemical approach are: (i) after their incorporation the probes are subjected to the same restrictions in their lateral diffusion as are natural sperm plasma membrane lipids; (ii) the lateral distribution of the incorporated lipid probe can be monitored as a function of time on living sperm cells because sample fixation or post-incubation labelling procedures are not required.

Using this technique in combination with digital imaging fluorescence microscopy and fluorescence lifetime imaging microscopy (FLIM; T. W. J. Gadella et al., 1993), we found evidence for lateral glycolipid polarity and for glycolipid migration in the sperm head plasma membrane upon capacitation. This supports our previous immunocytochemical results (B. M. Gadella et al., 1994) and confirms the usefulness of fluorescent glycolipid probes in studying glycolipid polarity phenomena in sperm cells. In addition, we report novel information about the relationship between lateral glycolipid distribution on the sperm head plasma membrane and the initiation of the acrosome reaction.

## MATERIALS AND METHODS

### Materials

SGalCer(C12-LRh) and GalCer(C12-LRh) were prepared in the department of Biomedical Sciences and Biotechnology, University of Brescia (Marchesini et al., 1990) and kindly provided by Prof. Dr S. Marchesini. Sperm-rich ejaculate fractions were collected from healthy boars at the Cooperative Pig Artificial Insemination Centre

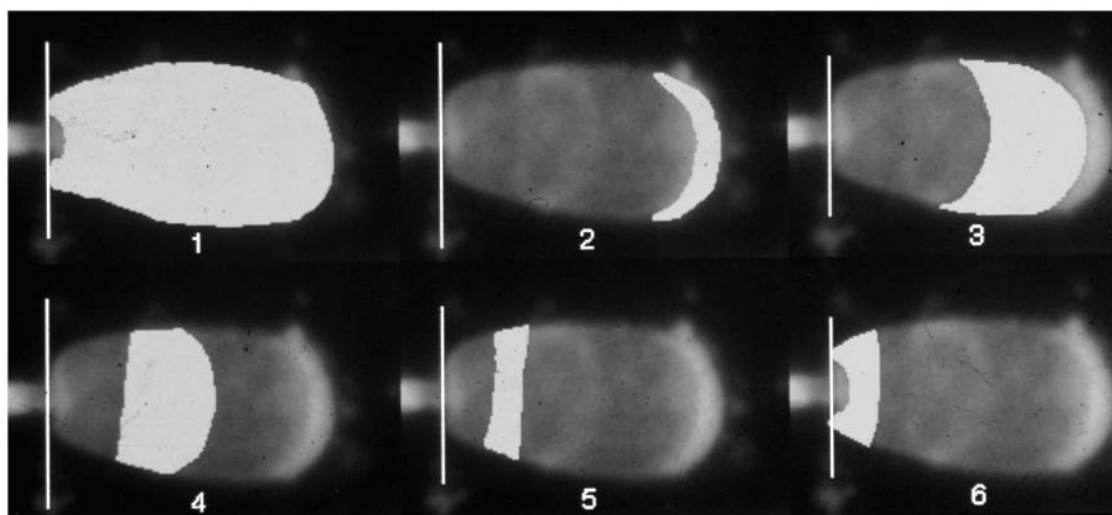
'Utrecht en de Hollanden' (Bunnik, the Netherlands).  $\text{Ca}^{2+}$  ionophore A23187 and egg phosphatidylcholine were purchased from Sigma (St Louis, MO).

### Incorporation of fluorescent glycolipids

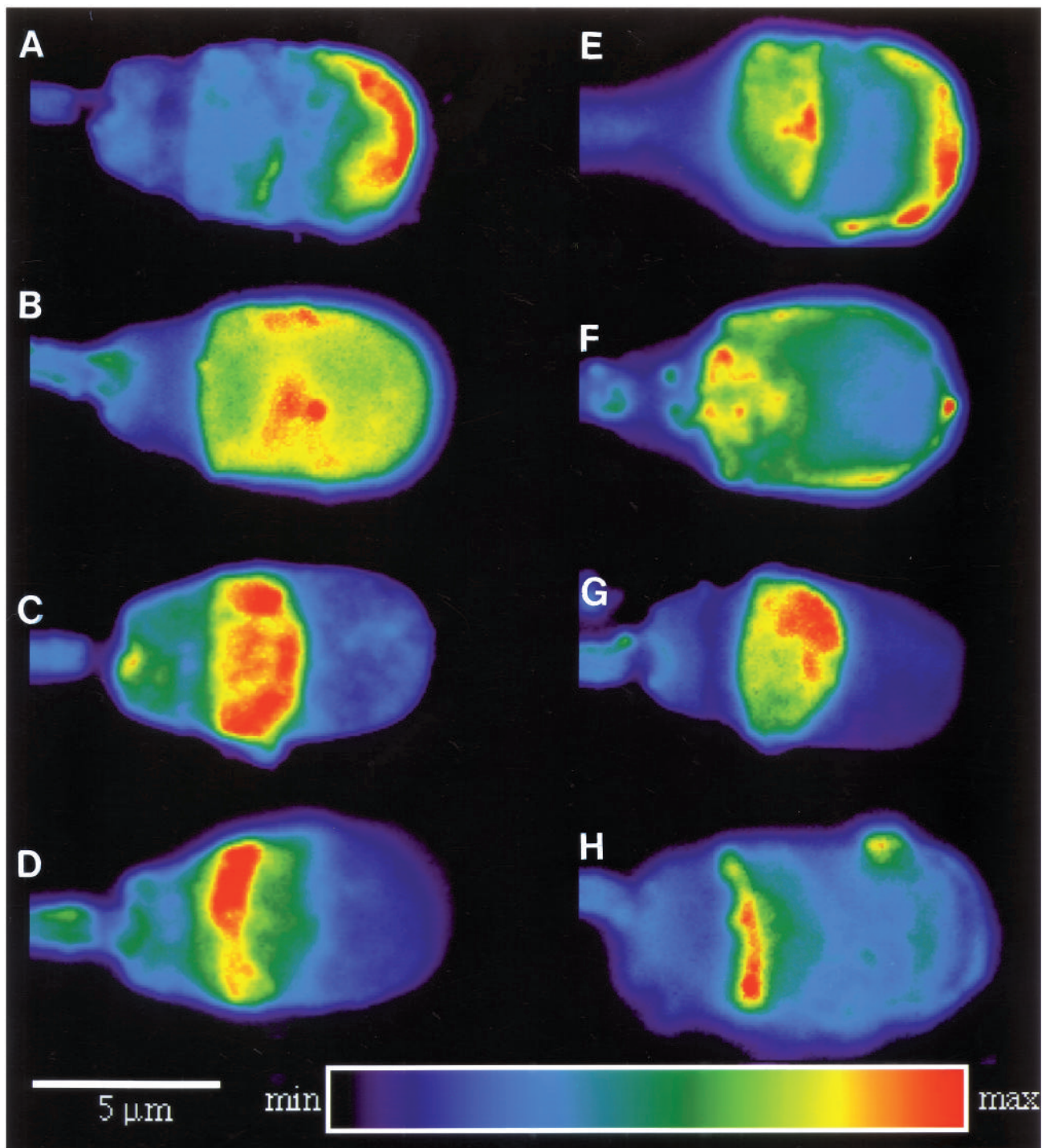
A lipid mixture of 85 mol% egg phosphatidylcholine and 15 mol% SGalCer(C12-LRh) or GalCer(C12-LRh) was dried under nitrogen and dissolved in ethanol to a concentration of 2.8 mM. Vesicles were prepared by injecting this ethanol solution into 13 volumes 10 mM Hepes-buffered Puck's saline cooled on ice (Vos et al., 1992). This solution was dialysed at 2°C overnight (Lipsky and Pagano, 1985) with Hepes-buffered Puck's saline and diluted with 18 mM Hepes-buffered MEM, pH 7.4, to a final lipid concentration of 20 nmol/ml (Lipsky and Pagano, 1983). The fluorescent vesicles were added to a sperm suspension (5 nmol total lipid/million cells) for 10 minutes at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . The remaining vesicles (i.e. free in suspension or loosely associated to the sperm surface) were removed from the sperm surface by washing the suspension three times through a 70% Percoll gradient (Arts et al., 1993). The removal of unfused vesicles, the integrity of the sperm samples (by scoring the acrosome morphology), the sperm motility and the integrity of the sperm plasma membrane (with Hoechst 33258 stain) were evaluated under a microscope (Gadella et al., 1991). The amount of incorporated lipid was ~0.2 mol% of total plasma membrane lipids as measured using a Luminescence Spectrometer LS50 from Perkin-Elmer (Beaconsfield, UK). During the experiments arylsulphatase conversion was minimal (<2.5% as checked according to Marchesini et al., 1990; see also B. M. Gadella et al., 1993, 1994).

### Other methods

Sperm washing, sperm capacitation *in vitro*, the preparation of zona-coated coverslips and the binding of sperm cells to these coverslips, as well as digital imaging microscopy and FLIM, are described in detail elsewhere (B. M. Gadella et al., 1994; T. W. J. Gadella, 1993, 1995). Digital image processing was done with TCL-image (TPD, Technical University of Delft; distributed by Multihouse, Amsterdam) and NIH-image (NIH, Bethesda, MD). Macros were written in TCL-image for contrast stretching, displaying distance bars and intensity scales (Figs 1-6). Image rotation and intensity profiles were generated using NIH-image (Figs 9, 10). These profiles were further analysed by Kaleidagraph (Abelbeck Software Inc.) to generate Table 1.



**Fig. 1.** Nomenclature and morphology of the subdomains of the plasma membrane on boar sperm heads. The sperm heads were oriented with their flat faces perpendicular to the optical axis of the microscope. The blank areas indicated in 1-6 refer to: (1) the complete sperm head; (2) the apical subdomain; (3) the pre-equatorial subdomain; (4) the equatorial subdomain; (5) the distal equatorial subdomain; (6) the post-acrosomal subdomain. The white line indicates the position of the basal plate.



**Fig. 2.** Lateral distribution of fluorescent glycolipids after their incorporation into the plasma membrane on sperm cells with intact acrosomes and plasma membranes. Lateral distribution of SGalCer(C12-LRh) (A-D) and GalCer(C12-LRh) (E-H) after their incorporation into the sperm plasma membrane. The sperm heads were oriented with their flat surface perpendicular to the optical axis of the microscope. (A) The distribution of SGalCer(C12-LRh) in the plasma membrane of a freshly ejaculated sperm cell. (B-D) Subsequent stages of migration of SGalCer(C12-LRh) during capacitation in vitro with HBSS containing 2 mM  $\text{CaCl}_2$ . (E) The distribution of GalCer(C12-LRh) in the sperm plasma membrane of a freshly ejaculated sperm cell. (F-H) Subsequent stages of redistribution of GalCer(C12-LRh) during capacitation (conditions as above). The micrographs were taken after a capacitation period of: (B,F) 30 minutes; (C,G) 90 minutes; (D,H) 4 hours. The fluorescence intensity bar represents a linear scale of digitized fluorescence intensities in the images. In all cases the maximum/minimum (background) fluorescence intensity ratio was approx. 25. The fluorescence intensities were scaled to match a common pseudocolour table.

## RESULTS

### Ultrastructural analysis of fluorescence patterns

Fig. 1 shows the positions of the different plasma membrane subdomains found in the boar sperm head. The surface distribution of SGalCer(C12-LRh) in the plasma membrane of freshly ejaculated sperm cells (Fig. 2A) shows a concentration in the apical subdomain. GalCer(C12-LRh) was found in both the apical and the equatorial subdomains (Fig. 2E).

After addition of  $\text{Ca}^{2+}$  and BSA (known to induce capacitation; Fléchon et al., 1986; Toyama and Nagano, 1988; Aguas and Pinto da Silva, 1989), the lateral distribution of incorporated fluorescent glycolipids changed. SGalCer(C12-LRh) gradually migrated to the distal end of the equatorial subdomain (see Fig. 2B-D), while GalCer(C12-LRh) also accumulated in the equatorial subdomain but tended to be concentrated in a smaller area than its sulphated counterpart (Fig. 2F-H). In order to demonstrate the surface phenomenon of intersubdomain glycolipid movement the characteristically very flat boar sperm heads were imaged in a parallel orientation to the optical axis of the microscope (Fig. 3). Note that the resolution of Fig. 3 is close to the diffraction limit due to the emission properties of lissamine rhodamine. A similar picture was obtained when data sperm cells were loaded with GalCer(C12-LRh) (data not shown).

Since binding to the zona pellucida of the egg cell triggers surface reorganizations on sperm cells leading to the acrosome reaction (O'Rand and Fisher, 1987), we looked at the effects of zonae pellucidae on glycolipid movements. Sperm cells, loaded with SGalCer(C12-LRh) or GalCer(C12-LRh) were allowed to bind to coverslips coated with solubilized zonae pellucidae (Nikolajczyk and O'Rand, 1992). We found that the redistribution of fluorescent glycolipids in sperm cells binding to the zona pellucida (~20% of the sperm population) was

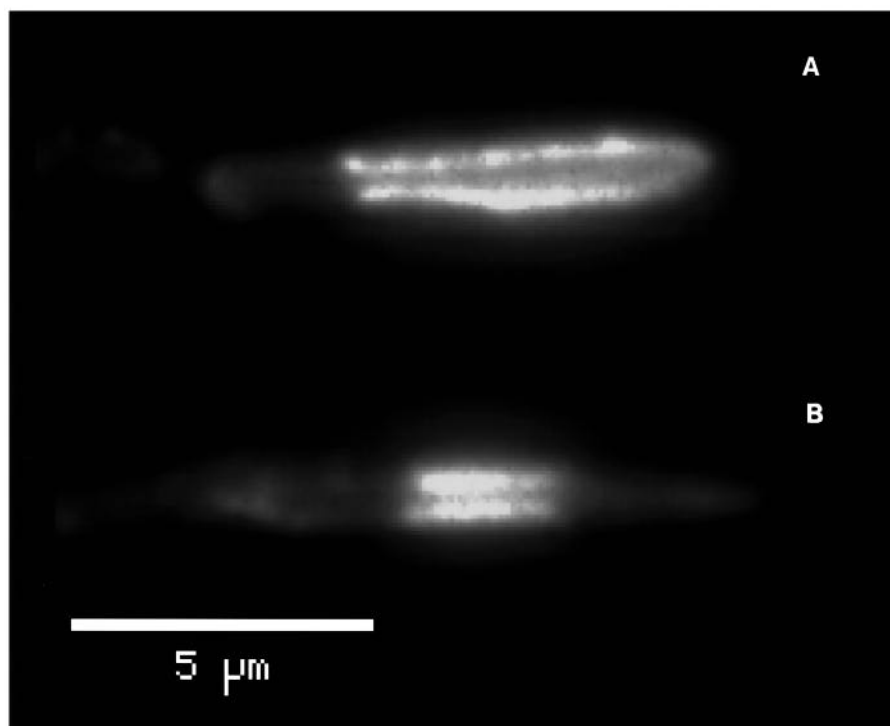
similar to that of capacitated cells shown in Fig. 2 (data not shown). In a control experiment, where intact sperm cells were bound to heparin-coated coverslips, SGalCer(C12-LRh) diffused over the acrosome region but did not enter or accumulate at the equatorial subdomain (data not shown).

### Enhanced incorporation of SGalCer(C12-LRh) causes blebbing of the plasma membrane

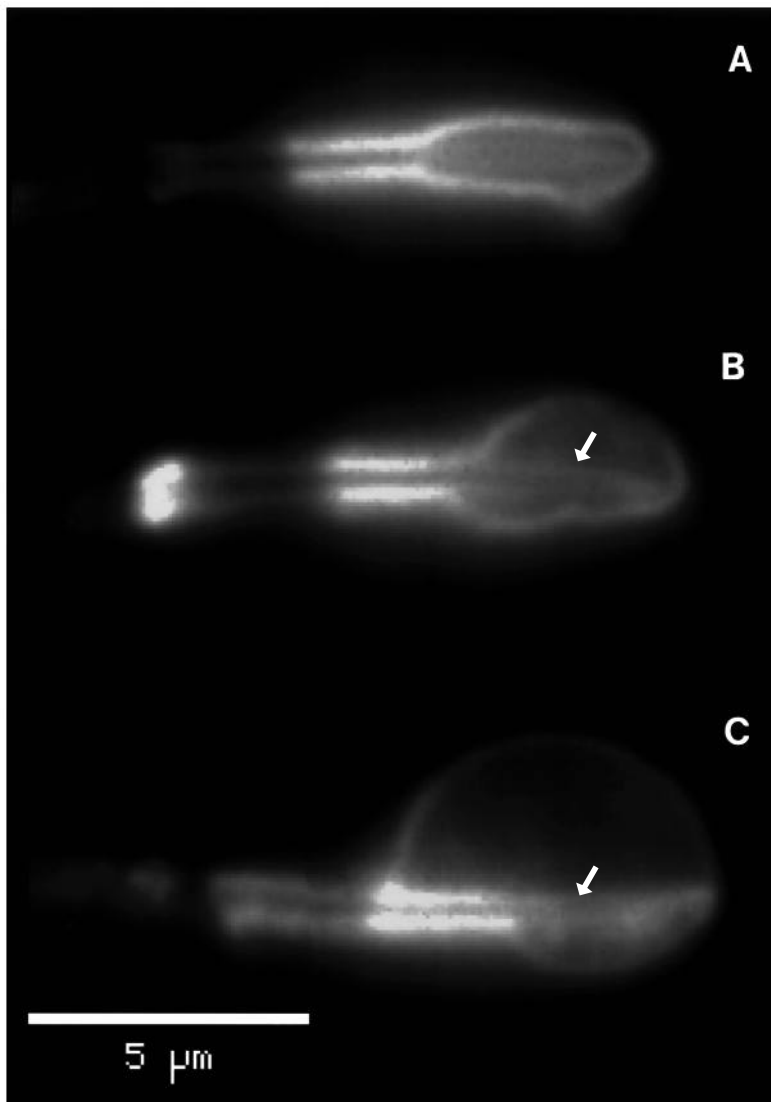
Sperm incubated with a 10-fold concentration of fluorescent glycolipids for 2 hours (instead of 10 minutes) showed enhanced incorporation of fluorescent glycolipids (up to 10% of the total lipids in the plasma membrane instead of 0.2% under normal conditions) accompanied by apical and pre-equatorial plasma membrane swelling (Fig. 4). Despite its blebbed appearance the plasma membranes remained continuous and intact, and the cells were able to exclude Hoechst 33258 stain, indicating that even such a massive incorporation of lipids did not disrupt the plasma membrane lipid bilayer.

### Glycolipid redistribution and the acrosome reaction

It is important to emphasize that the acrosome reaction of sperm cells induced by physiological levels of  $\text{Ca}^{2+}$  did not begin until the fluorescent glycolipids migrated to the equatorial subdomain (e.g. see Fig. 5). In contrast, addition of 1  $\mu\text{M}$   $\text{Ca}^{2+}$  ionophore A23187 induced the acrosome reaction without this migration (see Fig. 6A,B). The patchy pattern of fluorescence seen at the former acrosomal position during the ionophore-induced acrosome reaction indicates that the fluorescent lipids are trapped in mixed vesicles generated after multiple fusion of the plasma membrane with the underlying outer acrosomal membrane (Fig. 6B). In contrast, the mixed vesicles emerging from sperm cells initiating the acrosome reaction in the absence of ionophore were essentially non-fluorescent. Here the label appeared in the equatorial subdomain



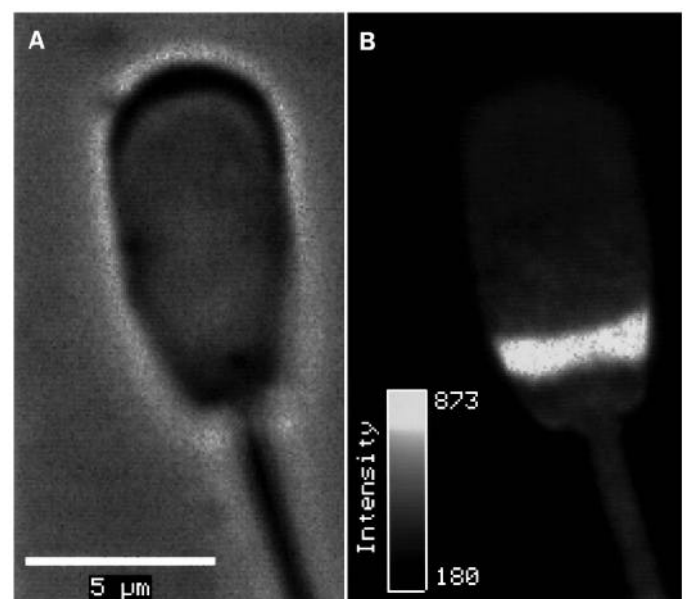
**Fig. 3.** Surface fluorescence of incorporated SGalCer(C12-LRh) in intact spermatozoa. Lateral distribution of SGalCer(C12-LRh) in sperm cells oriented with their edges parallel to the axis of the microscope. (A) Freshly ejaculated sperm cell; (B) a sperm cell after capacitation for 2 hours in the presence of 2 mM  $\text{CaCl}_2$ . The maximum/minimum fluorescence intensity ratios were approx. 50.

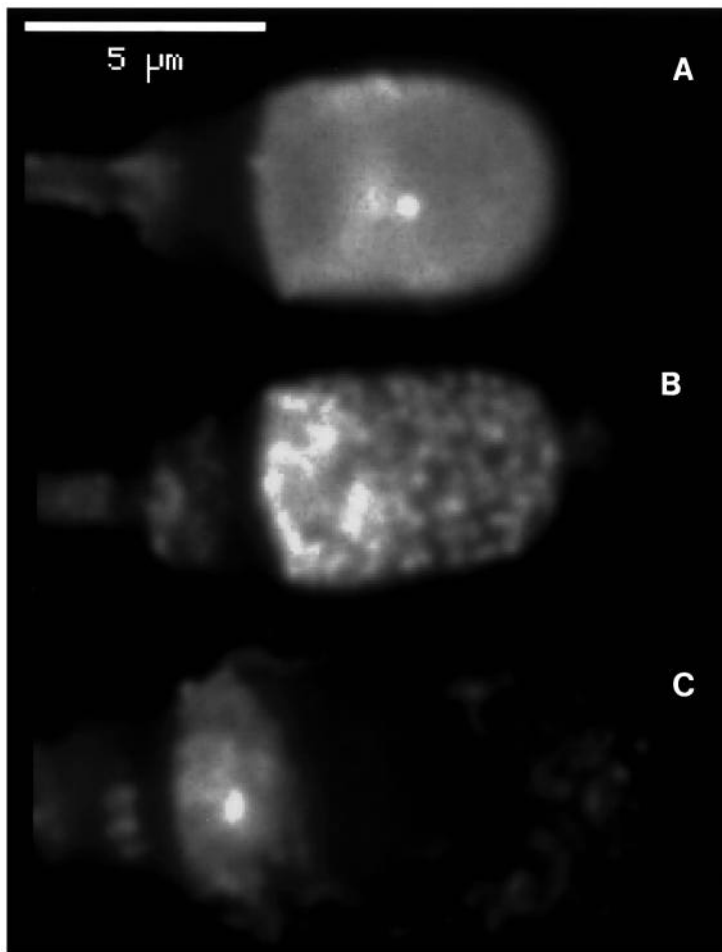


**Fig. 4.** Induction of sperm plasma membrane blebbing after long term incubations with high concentrations of SGalCer(C12-LRh)-containing vesicles. The flat faces of the sperm heads were oriented parallel to the optical axis of the microscope. (A-C) The progressive formation of a large plasma membrane bleb that loses contact with the underlying sperm structures. Sperm cells were incubated with the vesicles for (A) 30 minutes; (B) 1 hour; (C) 2 hours. The arrows indicate out of focus membrane fluorescence of the site of the plasma membrane that remained attached to intracellular structures at the edge of the sperm heads. The cells remained Hoechst 33258 negative.

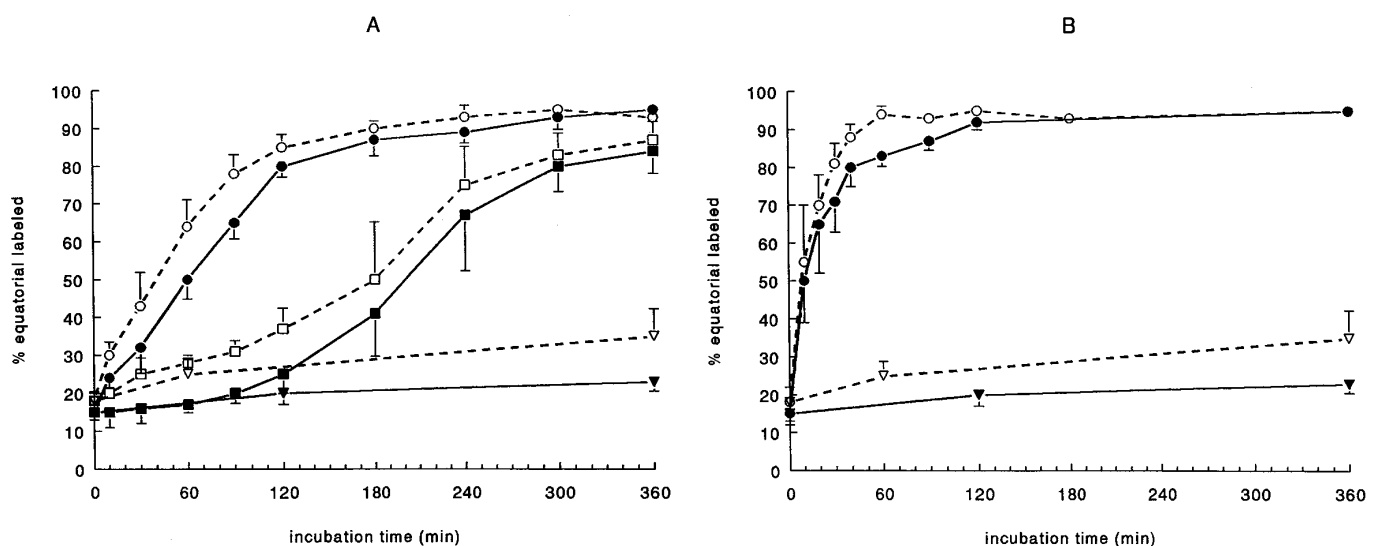
of the sperm head (Fig. 6C), indicating that SGalCer(C12-LRh) had migrated to this subdomain prior to the acrosome reaction. Similar results were obtained when sperm cells were capacitated with BSA or allowed to bind to zona-coated coverslips. The acrosome reaction, however, could not be triggered when sperm cells were bound to heparin-coated coverslips, probably because the lipid label failed to concentrate in the equatorial subdomain. It is of note that, upon labelling sperm cells with fluorescent glycolipids after capacitation *in vitro*, the acrosome reaction was triggered instantaneously (not shown). This probably reflects the destabilized state of the plasma membrane, which is a characteristic of capacitated sperm

**Fig. 5.** Complete migration of SGalCer(C12-LRh) to the equatorial subdomains on a sperm cell with an intact acrosome and plasma membrane. Phase-contrast micrograph (A) and fluorescence micrograph (B) of one boar sperm cell labelled with SGalCer(C12-LRh) after exposure to 2 mM  $\text{CaCl}_2$  for 3 hours. This sperm cell was labelled at the distal end of the equatorial subdomain (B) and its acrosome was still intact, since the normal apical ridge structure is clearly visible (A; Gadella et al., 1991).





**Fig. 6.** Acrosome reactions after the incorporation of SGalCer(C12-LRh) into the plasma membrane. The sperm heads were oriented with their flat face perpendicular to the optical axis. (A) A sperm cell with an intact acrosome in the presence of 2 mM  $\text{CaCl}_2$ . (B) The same cell undergoing an acrosome reaction after the addition of 1  $\mu\text{M}$   $\text{Ca}^{2+}$  ionophore A21387 (micrograph taken after an incubation of 15 minutes). (C) A sperm cell undergoing the acrosome reaction in the presence of 2 mM  $\text{CaCl}_2$  (4 hour incubation) without the addition of ionophore. The fluorescence intensity of the mixed vesicles in situ relative to that of the equatorial subdomain was 0.7 for (B) and 0.06 for (C). Bar, 5  $\mu\text{m}$ . Note: following the acrosome reaction (B,C) SGalCer(C12-LRh) may diffuse via the remaining equatorial outer acrosomal membrane into the inner acrosomal membrane. If this phenomenon is restricted to the equatorial subdomain it could enhance the fluorescence intensity of this area as analysed here by a maximum of 200%.



**Fig. 7.** Kinetics of the migration of the incorporated fluorescent glycolipids. The percentage of sperm cells with equatorial lipid labelling is expressed as a function of time. The migration of SGalCer(C12-LRh) and GalCer(C12-LRh) to the equatorial segment was monitored during different sperm capacitation experiments in vitro (A), and during sperm zona binding (B). In each sample  $3 \times 400$  sperm cells were counted. Sperm cells loaded with SGalCer(C12-LRh) are indicated by continuous lines with filled symbols ( $\nabla$ ,  $\blacksquare$ ,  $\bullet$ ), experiments with GalCer(C12-LRh) are indicated by broken lines and open symbols ( $\nabla$ ,  $\square$ ,  $\circ$ ). Mean values  $\pm$  s.d. of three independent experiments are expressed. (A) Washed sperm cells ( $\nabla$ ,  $\nabla$ ); sperm cells incubated with 4% (w/v) BSA ( $\blacksquare$ ,  $\square$ ); or with 2 mM  $\text{CaCl}_2$  ( $\bullet$ ,  $\circ$ ). (B) Washed sperm cells ( $\nabla$ ,  $\nabla$ ); sperm cells bound to zona coverslips ( $\bullet$ ,  $\circ$ ). Only cells with intact acrosomes and plasma membranes were analysed and only vital sperm cells were able to bind to the coated coverslips.



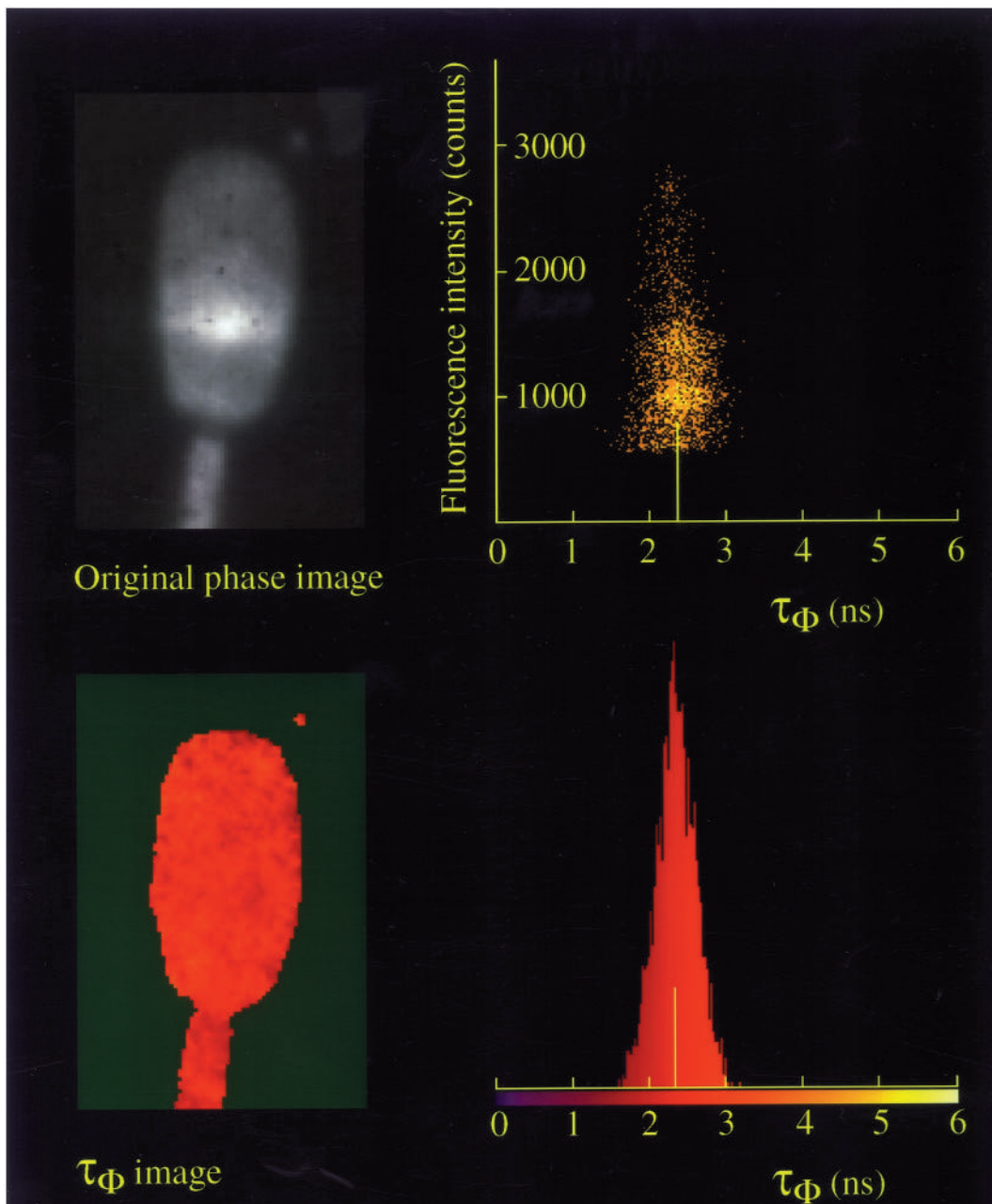
(Yanagimachi, 1994). After the acrosome reaction sperm cells were labelled mainly at the equatorial subdomain (see also Arts et al., 1993).

### Kinetics of glycolipid redistribution

The kinetics of SGalCer(C12-LRh) migration from the apical to the equatorial subdomain of the sperm head plasma membrane are shown in Fig. 7. After 2 hours of  $\text{Ca}^{2+}$ -induced capacitation approx. 85% of the sperm cells were labelled in the equatorial subdomain (Fig. 7A). Without the addition of  $\text{Ca}^{2+}$  the apical distribution of SGalCer(C12-LRh) did not change for at least 6 hours, showing that the observed migration of the fluorescent probe is dependent on extracellular  $\text{Ca}^{2+}$ . During BSA-induced capacitation a similar shift of SGalCer(C12-LRh) to an equatorial position was seen, albeit

at a slower rate (Fig. 7A). Similar experiments using GalCer(C12-LRh) showed comparable distribution patterns before and after capacitation but did not exactly match those of SGalCer(C12-LRh) (cf. Fig. 2), and the migration was at a slightly faster rate (Fig. 7A).

Sperm cells binding to coverslips coated with a zona protein matrix showed a much faster glycolipid redistribution (Fig. 7B). It is important to note that sperm cells not binding to the zona matrix (approx. 80%) were removed after 10 minutes. By this time 50% of the bound sperm cells were already labelled at the equatorial subdomain. This percentage increased rapidly, with between 80% and 90% showing redistribution within one hour (Fig. 7B). The time course of GalCer(C12-LRh) migration was again slightly faster than that of SGalCer(C12-LRh) (Fig. 7B).



**Fig. 8.** Analysis of the fluorescence lifetime of the incorporated SGalCer(C12-LRh) in the boar sperm head of an intact sperm cell. The upper left panel demonstrates the original digital fluorescence intensity distribution over the sperm head surface. The lower left panel depicts the pseudo-coloured in situ fluorescence lifetime distribution on the same sperm. The distribution of fluorescence intensity versus the distribution of fluorescence lifetime ( $\tau_\Phi$ ) of each analysed pixel is expressed in the upper right histogram. The temporal distribution of the fluorescence lifetime of the analysed pixels is given in the lower right panel.

### Redistribution of SGalCer(C12-LRh) does not occur on fixed spermatozoa

In control experiments SGalCer(C12-LRh)-labelled sperm cells were fixed in a cacodylate buffer containing 2% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde, and incubated with R, O4 or the Z-antibody (multivalent anti-sulphogalactolipid antibodies; B. M. Gadella et al., 1994). Neither the fixation nor the subsequent immuno-incubation procedures affected the lateral distribution of the fluorescent lipids. Lipid-labelled sperm samples that were fixed and incubated under *in vitro* capacitation conditions failed to show lateral redistributions of lipid fluorescence.

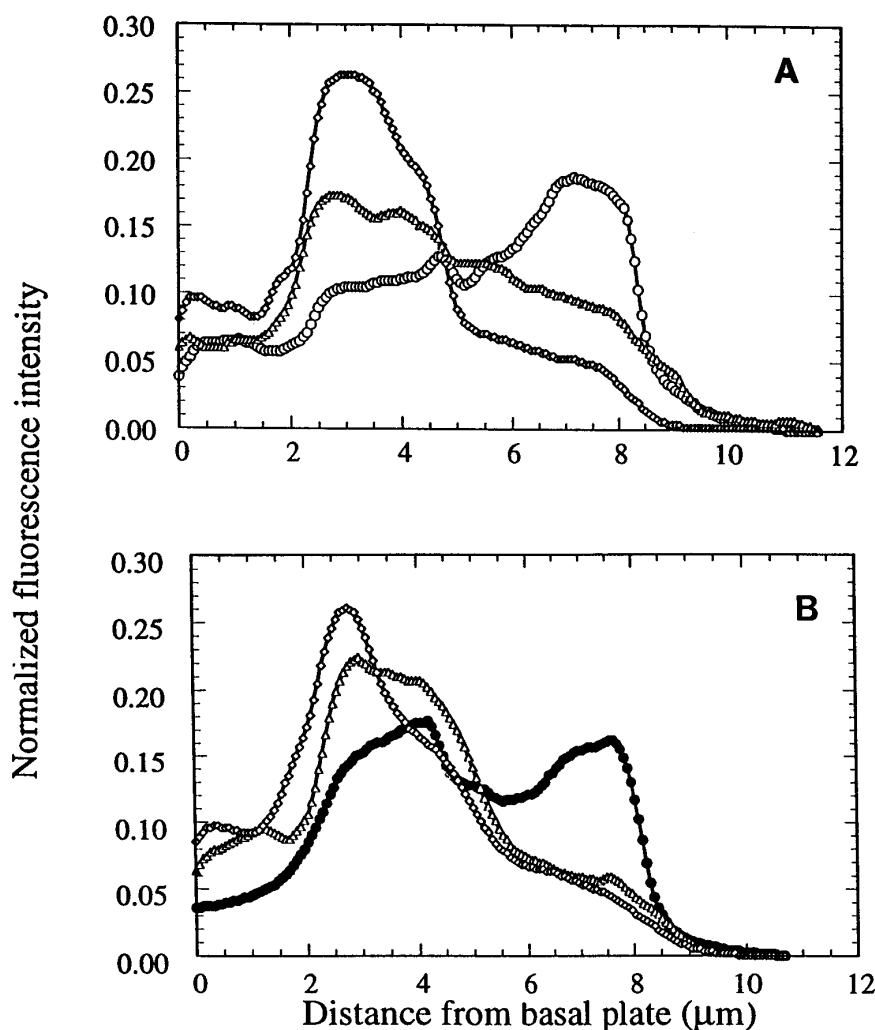
### Fluorescence lifetime imaging microscopy

The fluorescence lifetime of the incorporated probes in the different areas of the sperm plasma membrane was analysed with FLIM (T. W. J. Gadella et al., 1993, 1995; B. M. Gadella et al., 1994) and was found to be constant in all the subdomains of the sperm head, although the intensities of the fluorescence varied widely in the different regions. Thus the detected intensity differences were not due to subdomain-specific excited-state kinetics (i.e. quenching) of the fluorescent probe (lissamine rhodamine). A representative example of such an analysis is shown in Fig. 8 for SGalCer(C12-LRh); similar

patterns were observed for GalCer(C12-LRh). The fact that fluorescence lifetimes remained constant despite the five- to six-fold variation of fluorescence intensity provides strong evidence that the fluorescence intensity measured *in situ* correlates linearly with the concentration of incorporated fluorescent probe. A detailed analytical report of this novel microscopic technique is published elsewhere (T. W. J. Gadella et al., 1993, 1995).

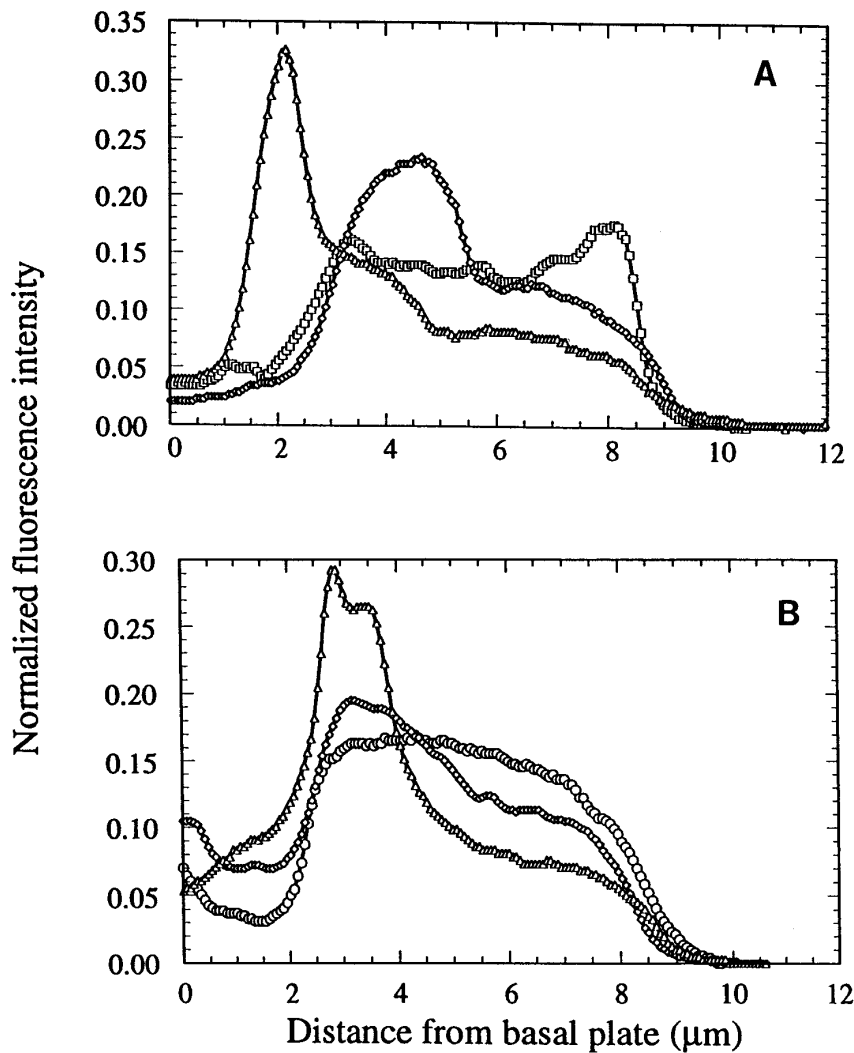
### Quantitative analysis of fluorescent tagged glycolipid distribution

To quantify the distribution of the probe, the digital images of fluorescence intensity over the sperm head were image processed (for procedure details see B. M. Gadella et al., 1994). The quantitative distribution of SGalCer(C12-LRh) and GalCer(C12-LRh) on freshly ejaculated sperm cells and the migration of these lipids to the equatorial subdomain of the plasma membrane are shown in Fig. 9A and B, respectively. Although the initial distribution of the two lipids was different, the migration of GalCer(C12-LRh) to the distal area of the equatorial segment of the sperm head plasma membrane was similar to that of SGalCer(C12-LRh). The redistribution of fluorescent glycolipids following sperm binding to zona matrix-coated coverslips is quantified in Fig. 10. After ten minutes



**Fig. 9.** Quantitative redistribution of incorporated fluorescent glycolipids over the sperm head plasma membrane during capacitation *in vitro*. The lateral distribution of the fluorescent tagged glycolipids after incorporation in the sperm head plasma membrane is expressed as a function of the distance from the basal plate. (A) Different SGalCer(C12-LRh) labelling patterns observed on a sperm cell immediately after ejaculation ( $\circ$ ), and on two sperm cells during capacitation *in vitro* with 2 mM  $\text{CaCl}_2$  (for 90 minutes  $\triangle$ , for 4 hours  $\diamond$ ). (B) Different GalCer(C12-LRh)-labelling patterns on a sperm cell immediately after ejaculation ( $\bullet$ ), and two sperm cells that are capacitating *in vitro* in 2 mM  $\text{CaCl}_2$  (for 90 minutes  $\triangle$ , for 4 hours  $\diamond$ ). The sperm cells analysed had intact acrosomes and plasma membranes.





**Fig. 10.** Quantitative redistribution of the plasma membrane-incorporated fluorescent glycolipids over the sperm head plasma membrane after zona matrix binding. The relative distribution of fluorescence is expressed as a function of the distance from the basal plate. (A) Redistribution of SGalCer(C12-LRh) after sperm zona binding ( $\square$ , 10 minutes;  $\diamond$ , 30 minutes;  $\triangle$ , 60 minutes). (B) Redistribution of GalCer(C12-LRh) after zona binding ( $\circ$ , 10 minutes;  $\diamond$ , 30 minutes;  $\triangle$ , 60 minutes). Only sperm cells with intact acrosomes and plasma membranes were analysed.

**Table 1.** Quantitative distribution of fluorescent glycolipids, SGalCer(C12-LRh) and GalCer(C12-LRh), over boar sperm heads after their incorporation into freshly ejaculated sperm cells and during various treatments

Probe	Treatment	Relative distribution of the probe over the sperm head (% of total fluorescence per head)				
		Apical	Pre-equat.	Equat.	Distal-equat	Post-Equat.
SGal(C12-LRh)	Control	44	26	17	9	5
	Ca <sup>2+</sup>	2	7	34	48	9
	BSA	10	13	45	25	17
	ZP	8	4	40	44	6
Gal(C12-LRh)	Control	35	20	32	8	5
	Ca <sup>2+</sup>	4	10	50	30	6
	BSA	8	10	43	32	17
	ZP	5	8	20	57	10

Control, freshly ejaculated sperm cells with an apical labelling pattern; 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. Mean values are indicated ( $n = 5$ ); s.e.m. < 10%; equat., equatorial.

SGalCer(C12-LRh) was distributed in the plasma membrane covering the acrosome, but within an hour the label was concentrated in the equatorial subdomain (Fig. 10A). The distribution pattern of incorporated GalCer(C12-LRh) under the same conditions is very similar but not identical (Fig. 10B). The

labelling patterns of representative sperm cells were analysed at the beginning and end of these experiments. From the generated line plots we calculated the relative amount of incorporated fluorescent label per subdomain of the sperm head (Table 1).

## DISCUSSION

This paper describes the lateral distribution of incorporated SGalCer(C12-LRh) in the plasma membrane of living spermatozoa and redistribution during *in vitro* capacitation and zona binding. The results compare favourably with immunolocalization of SGalAAG on fixed boar sperm cells (B. M. Gadella et al., 1994). In addition this paper provides evidence that the immunolocalization truly reflected lateral distribution of SGalAAG on sperm cells: (i) post-fixation or post-labelling with multivalent anti-sulphogalactolipid antibodies did not alter the lateral distribution of incorporated SGalCer(C12-LRh); (ii) incorporated lipids also failed to be redistributed when fixed sperm cells were subjected to conditions that induced capacitation and glycolipid redistribution in unfixed cells.

For a detailed discussion of the current models explaining the lateral polarity of the sperm plasma membrane we refer to a separate paper (B. M. Gadella et al., 1994; see also Holt, 1984; Peterson and Russell, 1985). The changing patterns of fluorescence seen in the present work indicate that the migration of glycolipids from the apical to the equatorial subdomain during *in vitro* capacitation and zona binding occurs prior to the acrosome reaction, since following the acrosome reaction, the mixed vesicles of outer acrosomal and plasma membranes contain only trace amounts of fluorescent glycolipids (see Fig. 6C). A similar rearrangement of transmembrane proteins in the boar sperm head plasma membrane during  $\text{Ca}^{2+}$ -induced capacitation *in vitro* was reported by Aguas and Pinto da Silva (1989). In addition, by using an anionic-lipid binding probe (polymyxin) Bearer and Friend (1982) showed that lipid domains are modified prior to the acrosome reaction in guinea-pig sperm. In retrospect, it is probable that the detection of this membrane-impermeable probe reflects the distribution of SGalAAG in the surface membrane, since this sulpholipid is the most prominent anionic lipid in the outer leaflet of mammalian sperm plasma membranes (Bearer and Friend, 1982; B. M. Gadella et al., 1993).

The observation that all sperm cells with an apical glycolipid distribution had intact acrosomes supports the hypothesis that SGalAAG prevents fusion of the plasma membrane with intracellular membranes (B. M. Gadella et al., 1991, 1994). This idea is supported by physicochemical data indicating that sulphogalactolipids stabilize the lamellar phase of lipid bilayers (Mannock et al., 1985; Wolf et al., 1986; Curatolo, 1987). In other words, the observed efflux of SGalAAG from the apical and pre-equatorial subdomains of the sperm plasma membrane may destabilize this membrane, thereby facilitating its subsequent fusions with the underlying outer acrosomal membrane. In this context it is interesting to note that incubation of spermatozoa with arylsulphatase A (a seminal plasma enzyme that desulphates SGalAAG on intact spermatozoa; B. M. Gadella et al., 1993) both causes a migration of glycolipids from apical to equatorial subdomains of uncapacitated sperm cells (B. M. Gadella et al., 1994) and triggers the degenerative acrosome reaction (Gadella et al., 1991). Our current knowledge suggests that it is the movement of the SGalAAG and GalAAG from the apical area of the sperm plasma membrane that is instrumental in initiating acrosome reactions, rather than the formation of hexagonal II phases induced by

the generated GalAAG as previously proposed (Gadella et al., 1991).

The demonstration of lateral movement and efflux of glycolipids from the apical to the equatorial segments of the plasma membrane implies that other membrane lipids travel in the opposite direction. One likely candidate for such a reversed flux is phosphatidylcholine. Since phosphatidylcholine contains polyunsaturated fatty acid chains (C22:6; Seliv-onchick et al., 1980; Nikolopoulou et al., 1986; Parks and Lynch, 1992), movement towards the apical region would increase the membrane fluidity in this region, thus facilitating acrosomal exocytosis. Previous work has demonstrated that the various subdomains of mouse and ram sperm plasma membranes have distinct lipid fluidity characteristics (Wolf and Voglmayer, 1984; Wolf et al., 1986, 1988, 1990). Preliminary experiments indicate that SGalAAG (one of the prominent lipids in the plasma membrane with a restricted topology in the outer leaflet; B. M. Gadella et al., 1993) can bind free extracellular  $\text{Ca}^{2+}$ , enhancing the membrane fluidity (Tupper et al., 1992, 1994). This finding might explain the observed  $\text{Ca}^{2+}$ -induced redistribution of SGalAAG. The movement of membrane proteins into the apical subdomain also appears to be important both in the acrosome reaction (e.g. the ESA152 antigen on ram spermatozoa; McKinnon et al., 1991) and for sperm binding to the zona (e.g. the 2B1 antigen on rat spermatozoa; Jones et al., 1990).

The induction of the acrosome reaction in the presence of both  $\text{Ca}^{2+}$  and the  $\text{Ca}^{2+}$  ionophore A23187 is not preceded by migration of incorporated fluorescent glycolipids (compare Figs 5 and 6) and hence differs significantly from the physiological sequence of events. This illustrates that the acrosome reaction is triggered by elevated intracellular  $\text{Ca}^{2+}$  levels induced by the ionophore (see also Roldan and Harrison, 1989; Roldan and Frago, 1993). In a physiological situation these elevated intracellular  $\text{Ca}^{2+}$  levels are triggered after binding of zona agonists to a specific sperm plasma membrane receptor (Wassarman, 1987, 1990; Roldan and Harrison, 1989; Saling, 1991; Ward et al., 1992; Roldan and Frago, 1993). This report provides evidence that the acrosome reaction (by a receptor activation) takes place only after the translocation of SGalAAG into the equatorial region. We therefore speculate that a simultaneous migration and/or aggregation of the receptors involved in  $\text{Ca}^{2+}$  entry or a change in their lipid environment (such as the efflux of apical glycolipids or the influx of other lipids) sensitizes the agonist-dependent pathway. The SGalAAG redistribution to the equatorial subdomain might also be functional in later events that lead to fertilization, since: (i) SGalAAG seems to be involved in zona binding via a SGalAAG binding protein (SLIP; Tanphaichitr et al., 1993); and (ii) this subdomain is specifically involved in fusion of the sperm with the oocyte plasma membrane (Yanagimachi, 1994).

In conclusion, we have demonstrated glycolipid polarity in the sperm plasma membrane following the incorporation of fluorescent-tagged glycolipids and show redistribution of these glycolipid probes during capacitation and zona binding of sperm cells. FLIM proved to be an essential tool for the analysis of the subdomain polarity of the incorporated fluorescent glycolipids, allowing us to quantitatively relate the intensity of fluorescence to the local concentration of the probe in the sperm head plasma membrane. The efflux of SGalAAG

from the apical subdomain of the sperm plasma membrane is a prerequisite for the physiological acrosome reaction and reflects an important capacitation event that may well be essential for mammalian fertilization. The data strongly support the concept that the subdomain organization of the sperm plasma membrane is dependent on the phase behaviour of the lipids within the membrane. We anticipate that the incorporation of fluorescent lipids into living cells becomes a powerful technique to detect membrane changes during sperm manipulation (e.g. during cryopreservation or in vitro fertilization) and will help to reveal the role of lipid polarity dynamics in sperm physiology and its function in fertilization.

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