Diffusion Barriers in Ram and Boar Sperm Plasma Membranes: Directionality of Lipid Diffusion Across the Posterior Ring

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ABSTRACT

The plasma membrane of mammalian spermatozoa, like that of other differentiated cells, is compartmentalized into discrete regions or domains that are biochemically and functionally distinct from one another. Physical structures within the membrane, such as the posterior ring at the juncture of the sperm head and tail, have long been thought to act as diffusion barriers to help segregate important molecules required for fertilization within specific domains and to regulate migration of molecules between domains. In this investigation, we used a quantitative photobleaching technique (video-FRAP) to assess the efficacy of the posterior ring as a barrier to exchange of lipids between the postacrosomal and midpiece plasma membranes. A lipid reporter probe (1,1'-diadecyl-3,3',3'-tetramethylindocarbocyanine; DiIC$_{12}$) was incorporated into the plasma membrane of live ram and boar spermatozoa, and the directionality of its diffusion across the posterior ring was measured by line-profile analysis. Results showed that DiIC$_{12}$ was able to traverse the posterior ring from the direction of the postacrosomal plasma membrane and to diffuse onto the midpiece plasma membrane. These results suggest that the posterior ring is not an immutable barrier to lipid exchange in mature spermatozoa and that there are other mechanisms for maintaining in-plane lipid asymmetry, such as differential phase behavior and interaction with the submembranous cytoskeleton.

fertilization, sperm, sperm capacitation

INTRODUCTION

A characteristic feature of differentiated cells is segregation of selected protein and lipid components of the plasma membrane into compartments or domains that are biochemically and functionally distinct from one another. Well-known examples are the apical and basolateral surfaces of epithelial cells [1], inner and outer segments of retinol rod cells [2], cell body versus axonal plasma membranes of neurones [3], and head and tail membranes of spermatozoa [4, 5]. Within these compartments, lipid “rafts” and protein “microdomains” are present that range in size from tens of molecules to several hundred nanometers in diameter and are highly dynamic structures responding to external and internal stimuli such as temperature, agonists, and cytoskeletal rearrangements [6, 7]. Elucidating the basic mechanisms that generate these lateral heterogeneities in cell membranes and maintain them over relatively large distances against the randomizing forces of diffusion is fundamental to understanding many aspects of cell differentiation and function.

Cells employ several complementary strategies to prevent uncontrolled mixing of membrane lipids and proteins, the most widespread being interaction with the cytoskeleton and extracellular matrix, spontaneous self-association, affinity for immobilized membrane constituents, selective internalization, and corolling between “fences” or diffusion barriers [8–10]. Diffusion barriers are of growing significance in current concepts of membrane organization in which proteins are viewed as having short-range diffusion within compartments and effect long-range diffusion by intercompartmental “jumps” (the membrane skeleton fence model [11, 12]). Lipids also show diffusion boundaries, possibly because of nonideal mixing depending on the temperature. This is readily demonstrable in vitro in model membrane systems by differential scanning calorimetry and fluorescence quenching techniques [13, 14], but in live cells it is usually inferred from fluorescence recovery after photobleaching (FRAP) measurements and fusion of monocomponent liposomes with specific regions of the plasma membrane. Thus, in Xenopus eggs the diffusion coefficient for the fluorescent lipid probe HEDAF in the animal pole was $1.5 \times 10^{-8}$ cm$^2$/sec, whereas in the vegetal pole it was $7.6 \times 10^{-8}$ cm$^2$/sec [15]. After fertilization, this discrepancy increased 100-fold. In cultured rat neurones, fluorescent NBD-DOPE liposomes fuse with the axonal plasma membrane but not with the cell bodies [16], and in acrosome-reacted human spermatozoa phosphatidylserine liposomes adhere specifically to the equatorial segment region [17].

Intramembranous diffusion barriers also have a structural basis, particularly at sites of intercellular contact. The well-known paradigm is the zona occludens, which prevents intermixing of proteins and lipids between the apical and lateral plasma membranes of epithelial cells, at least in the exoplasmic leaflet of the bilayer [18]. Similarly, confinement of sodium channels to the nodal region of axons has been attributed to the very close contact in the paranodal domain between loops of the myelin sheath and the axonal plasma membrane [19]. Within single cells, arrays of intramembranous particles frequently correlate with domain boundaries. In nonmating gametes of Chlamydomonas reinhardtii, a “necklace” of particles at the base of the flagellum is thought to prevent free diffusion of agglutinins from the cell body [20], and in the plasma membrane of the connecting cilium of retinal rod cells, the position of intramembranous particles correlates with separation of rhodopsin between the inner and outer segments [2]. Thus, both lipid and protein components of cell membranes are subject to diffusion boundary phenomena.
Mammalian spermatozoa possess a complex plasma membrane that is subdivided into five macrodomains (acrosome, equatorial segment, postacrosome, midpiece, and principal piece), each containing a unique complement of proteins and lipids [21, 22]. These macrodomains are established early on during differentiation in the testis coincident with the formation of electron-dense structures within in the plasma membrane at the junction of the head and tail (the posterior ring) and between the midpiece and principal piece (the annulus). It has been hypothesized that during spermatid elongation the posterior ring behaves as a molecular filter to prevent membrane components inserted onto the sperm head from spreading onto the tail, and vice versa [23]. In mature spermatozoa, the posterior ring and annulus have long been assumed to play an important role in maintaining segregation of surface membrane components and in regulating migration of specific antigens during epididymal maturation and capacitation [24–26].

In this investigation, we examined the functionality of the posterior ring as a barrier to lateral diffusion of lipids between the head and tail regions of mature ram and boar spermatozoa. Using 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiIC12) as a reporter probe, we devised a quantitative video-FRAP procedure that measures directionality of recovery following photobleaching. Contrary to established beliefs, our results suggest that lipids are able to freely exchange between the postacrosomal and midpiece plasma membranes and that the posterior ring is not an immutable barrier to diffusion.

**MATERIALS AND METHODS**

**Preparation and Labeling of Spermatozoa with DiIC12**

Ejaculated spermatozoa were collected from three boars (Large White) and three rams (Suffolk) maintained at the Babraham Institute. Boar semen (sperm-rich fraction) was filtered through four layers of surgical gauze before use. Sperm morphology and motility was checked after dilution into PBS by phase contrast microscopy. Only samples showing good forward motility (>60%) were used in experiments.

For labeling with DiIC12 (Molecular Probes, Eugene, OR), whole semen was diluted 1:10 (v/v) with a modified Krebs-Ringer phosphate glucose medium (KRPG [27]) and 500 μl was layered over 500 μl of 0.28 M sucrose/10 mM Heps, pH 7.2 (sucrose medium). After centrifugation at 250 × g for 10 min, supernatants (seminal plasma and KRPG) were removed and the sperm pellets were resuspended in 500 μl of KRPG. From this suspension, 20 μl of washed spermatozoa was mixed with 200 μl of sucrose medium containing 2.5% ethanol and 25 μg/ml DiIC12. The DiIC12 was stored as a stock solution of 5 mg/ml in 100% ethanol at −20°C and diluted 1:20 (v/v) into sucrose medium/5% ethanol immediately before use. Any precipitated dye was removed by centrifugation. Further dilutions were made into sucrose medium/2.5% ethanol. Preliminary experiments showed that DiIC12 remained largely soluble in sucrose/2.5% ethanol mixtures, whereas it precipitated strongly in salt-containing medium such as KRPG, even in the presence of 2.5% ethanol. Spermatozoa/DiIC12 suspensions were incubated at 34°C for 20 min, and unbound dye was removed by washing spermatozoa twice by centrifugation in 1 ml of sucrose medium. Spermatozoa were finally resuspended in 200 μl of sucrose medium containing 20% KRPG and 0.1% sodium azide in preparation for FRAP analysis. Uptake of DiIC12 was assessed by epifluorescence illumination on a Nikon LABOPHOT-2 photomicroscope using filter block G (emission, 590 nm). For FRAP analysis, spermatozoa were drawn by capillary action into microslides (50 μm path length, rectangular cross section; Camlab Ltd., Cambridge, UK), sealed with Cristoseal (Hawksley, Lancing, UK), and secured on a temperature-controlled microscope warm stage set at 20°C.

**Video-FRAP Analysis**

A modified Nikon OPTIPHOT-2 fluorescence photomicroscope equipped with two epi-illumination attachments was used throughout this investigation [28]. One attachment allowed spermatozoa to be viewed by full-field illumination from an ultraviolet light source, and the second attachment permitted point illumination by a laser beam of Gaussian cross-sectional intensity generated from a water-cooled 15-W argon ion laser (Spectra-Physics Ltd., Hemel Hempstead, UK). Images were collected via a cooled CCD video camera (SP-Eye; Photonic Sciences, Cambridge, UK) mounted on the vertical camera port. The signal from the camera was fed into a Matrox Metror frame grabber (Matrox, Dorval, PQ, Canada), and the images were analyzed using Image Pro (Media Cybernetics, MD). A sequence of 20 images was taken over a period of 6 sec using an on-chip integration time of 120 msec/frame. Measurements were conducted in the following way. Several frames were collected with the laser illumination set to the monitoring beam (prebleach levels). The laser power was then increased ~1000-fold for 50 msec (bleach level) and then returned to the monitoring level for collection of the remaining frames (postbleach recovery). Diffusion information was calculated from thick-line profiles (i.e., a line profile averaged over the width of the bleached spot) for each of the 20 frames. The line profile was usually aligned along the anterior-posterior axis of the sperm and was typically 150 pixels long out of an image size of 768 × 576 pixels, giving a bleach spot width of 40 pixels (2 μm). Data from three regions within the line profile were then used to determine diffusion coefficients for the “left” and “right” sides of the bleach position. The three regions were 20 pixels to the “left” of the center of the bleach, 20 pixels to the “right”, and 30 pixels well removed from the bleach region for use in normalizing the fluorescence intensity with time. This technique yields two data sets, each with 20 points (one per frame), that represent recovery of fluorescence from opposing directions along the line profiles. Measured intensity was corrected as follows:

\[
I_{\text{measure}} = 0.785 \cdot I_{\text{bleach}} + 0.215 \cdot I_{\text{norm}},
\]

where \(I_{\text{bleach}}\) is the intensity of the bleached region and \(I_{\text{norm}}\) is the normalizing intensity. Rearranging gives

\[
I_{\text{bleach}} = (I_{\text{measure}}/0.785) - 0.274 \cdot I_{\text{norm}}.
\]

These data are then normalized for full-field photobleaching by the following equation:

\[
I = I_{\text{bleach}}/(I_{\text{norm}} \cdot 0.785).
\]

These data sets were then analyzed using the standard FRAP equations [28] to give a recovery time (\(t_D\)), diffusion coefficient (D), and percentage recovery (%R).

**RESULTS**

**Structural Basis for the Posterior Ring in Spermatozoa**

The posterior ring (also known as the striated or nuclear ring) is defined as “a circumferential line of fusion of the
plasmalemma with the outer and inner membranes of the nuclear envelope” [29] (Fig. 1). It is present in all mammalian spermatozoa and appears during the elongation stage of round spermatids at the position where the manchette (a transient cylindrical structure composed of microtubules) encounters the plasma membrane. As the nucleus elongates and the cytoplasm is displaced distally, the posterior ring defines the limits of the postnuclear sheath internally and on the surface forms a clear boundary between the postacrosomal and midpiece domains [5, 30, 31]. It is regarded as proteinaceous based on its electron density in transmission electron micrographs.

Validation of Analytical Procedures for Assessing Directionality of DiIC12 Diffusion

For reasons described previously [28], only live spermatozoa showing uniform fluorescence over the head and tail plasma membranes were analyzed by video-FRAP. Before addition of sodium azide, these uniformly stained spermatozoa were vigorously motile, whereas spermatozoa that stained strongly on the acrosome and midpiece (the “dead-pattern” spermatozoa [28]) were immotile. To validate the procedures described in Materials and Methods for measuring quantitatively the recovery and directionality of DiIC12 diffusion, preliminary measurements were performed on the plasma membrane overlying the sperm head. In previous work using ODAF as a lipid reporter probe and a 2.1-μm-diameter laser beam, there were no significant differences in diffusion coefficients between different sites within the anterior acrosomal region; thus, this region is regarded as an essentially homogenous macrodomain [28]. Similar conclusions were reached from an analysis of multiple sites along the plasma membrane overlying the midpiece (unpublished results). Because diffusion within the plasma membrane overlying the acrosome is essentially two-dimensional, recovery will be multidirectional. When the focused laser beam was positioned over the center of the anterior acrosome, the bleached area showed ~98% recovery within a recovery period of 6 sec (Fig. 2, a–e). Measurements of the recovery of fluorescence along the thick-line profile x–y bisecting the bleached area at 16 different time points are plotted in Figure 3a. Taking the first recorded image following the initial Gaussian bleach wave as zero time, recovery was equal and multidirectional with D values for left- and right-line profiles measured as 4.84 × 10⁻⁹ cm²/sec and 3.33 × 10⁻⁹ cm²/sec, respectively. Summation of pixel points along opposing directions of diffusion x–z (left line) and y–z (right line, Fig. 3b) gave recovery curves that did not deviate significantly from one another, confirming the visual impression that recovery is symmetrical from all directions.

FIG. 2. Video frames of ram spermatozoa stained with DiIC12 and subjected to large-area photobleaching. a and f Spermatozoa before photobleaching, b–e The focused laser beam has been centered over the acrosome, followed by photobleaching. Note symmetrical recovery of DiIC12, g–j The focused laser beam has been positioned so that it straddles the postacrosome and the equatorial segment. The partial eclipse effect is caused by good recovery of DiIC12 within the equatorial segment but poor recovery within the postacrosome. Individual frames represent intervals of 450 msec. ×1600.

A contrasting situation is shown in Figure 2, f–j. This spermatozoon stained uniformly over the plasma membrane with DiIC12 and hence was classified as a live cell. However, the postacrosomal plasma membrane contained a larger immobile fraction relative to that on the acrosome, as shown clearly in Figure 2, f and g, in which the focused laser beam was positioned so that it overlapped both the equatorial segment region of the acrosome and the postacrosomal plasma membrane with its center at the boundary between them. Following photobleaching, recovery began immediately within the equatorial segment due to diffusion of DiIC12 from the direction of the anterior acrosome but stopped abruptly at the junction with the postacrosome. Recovery within the equatorial segment plasma membrane was calculated to be 75% in contrast to only 34% in the postacrosomal plasma membrane. The result was a partial eclipse effect (Fig. 2, h–j) that persisted for as long as 5 min of observation. A quantitative analysis of summed pixel points along the anterior-posterior axis revealed that left- and right-line profiles deviated substantially from each other (Fig. 3, c and d), reflecting differences in the sizes of immobile phases.

Spermatozoa are well known to be resistant to ethanol and to retain their motility in ethanol concentrations as high as 5% [27]. Chronic exposure to ethanol, however, has been reported to suppress capacitation, although the effects are reversible and are not mediated by any solvent action [32, 33]. To verify that short exposure to ethanol during the labeling protocol did not perturb the plasma membrane, ram spermatozoa were loaded with DiIC12 by back-exchanging it from BSA-DiIC12 in KRPG alone (without ethanol) for 30 min at 20°C. Subsequent FRAP analysis revealed that D values on the acrosome were not significantly different.
FIG. 3. Individual fluorescence recovery curves and line profile summation analysis of the spermatozoa shown in Figure 2. a and c) Fluorescence recovery across the photobleached areas in Figure 2, a–e, and Figure 2, f–j, respectively. Each line is separated by 450 msec. b and d) Respective line profile analyses of the fluorescence intensity along the bisecting lines x–y, with z as the midpoint for calculation of left (△–△) and right (■–■) profiles. Where lines are closely parallel or touch, recovery is considered to be symmetrical (as in b), but where lines diverge recovery is considered to be asymmetrical (as in d).

from those labeled in the presence of ethanol (unpublished results). This finding suggests that the functionality of the plasma membrane was not affected by acute exposure to ethanol under the conditions described.

Does the Posterior Ring Act as a Functional Barrier to Prevent Free Exchange of DiIC\textsubscript{12} Between the Midpiece and Postacrosomal Plasma Membranes?

Having validated a method for measuring directional diffusion of DiIC\textsubscript{12}, we next investigated whether or not the probe was freely exchangeable between the postacrosomal and midpiece plasma membranes and could cross the posterior ring. For this purpose, the focused laser beam was positioned over the anterior midpiece of live DiIC\textsubscript{12}-stained spermatozoa, with the edge of the beam touching the neck region. In theory, if DiIC\textsubscript{12} diffuses across the posterior ring from the direction of the postacrosomal region as well as from the posterior midpiece, recovery would be symmetrical and left and right line profiles would converge. If, however, the posterior ring behaves as a barrier to diffusion, then recovery would only be possible from the direction of the posterior midpiece and left and right line profiles would diverge. Between 40 and 50 ejaculated spermatozoa from ram and boar were analyzed in this way, and the experiments illustrated in Figures 4 and 5 are representative of the data as a whole.

The majority (~70%) of spermatozoa in both species produced symmetrical or nearly symmetrical recovery curves that, when analyzed by line profile summation, showed no significant differences in the directionality of recovery along the anterior-posterior axis of the bleached area. Curves either overlapped or intersected with each other (Figs. 4a and 5a). For the remaining spermatozoa, right and left recovery profiles did not always superimpose but ran closely parallel to one another (Fig. 4, b and c; Fig. 5, b and c). These anomalies may be caused by a variety of factors that are difficult to control such as 1) nonuniform incorporation of DiIC\textsubscript{12} into the plasma membrane (similar distortions also arose if very small particles of DiIC\textsubscript{12} attached randomly to the plasma membrane near the neck region, causing intense points of fluorescence), 2) inherent differences in D values between the plasma membrane overlying the postacrosome and the midpiece, and 3) heterogeneity among spermatozoa due to intrinsic factors such as aging. In both species, however, there were no significant differences between left and right recovery profiles with respect to %R and calculated D values (Table 1).

DISCUSSION

This work has shown that there is free diffusion of DiIC\textsubscript{12} from the postacrosomal plasma membrane to the midpiece plasma membrane of live ram and boar spermatozoa, suggesting that the posterior ring is not a barrier to exchange of lipid molecules. Other mechanisms must be present, therefore, to maintain the compartmentalization of membrane components between the head and tail macrodomains of mammalian spermatozoa.
**Diffusion Boundaries and the Problem of Antigen Migration in Sperm Plasma Membranes**

Unlike somatic cells, spermatozoa are transcriptionally inactive and cannot respond to agonists by synthesizing new proteins. At the same time, however, they must regulate their fertilizing capacity until they are within the vicinity of the egg and must avoid spurious interactions with other cell types. Two of the ways in which spermatozoa achieve these goals are 1) important molecules required for egg recognition and fusion are synthesized as precursors in the testis and processed later on in development at the appropriate place and time and 2) selected molecules are sequestered onto areas of the surface membrane where they are inactive, but in response to appropriate signals, they reposition to regions where they become active, either by interaction with novel lipids and proteins within a different membrane domain or because they reach a critical concentration necessary for functionality. These two pathways may run concurrently or sequentially and may be interdependent. Four sperm glycoproteins that show both of the above phenomena are fertilin and PH20 (guinea pig [34]), which redistribute from the acrosome to the postacrosome in the caput epididymidis, CE9 (rat), which spreads from the principal piece of the tail onto the midpiece during passage through the corpus epididymidis [35, 36], and 2B1 (rat), which migrates from the tail to the acrosomal domain concomitant with capacitation in the female tract [37]. All four of these glycoproteins undergo endoproteolytic cleavage as a prelude to their migration. Membrane glycolipids also undergo repositioning. A sulfogalactolipid on boar spermatozoa redistributes from the apical ridge of the acrosome to the equatorial segment during capacitation [38]. A common feature of all the above examples is that the protein or lipid moves laterally within the plane of the bilayer, usually across domain boundaries and frequently against large concentration gradients. Because many of these antigens have putative roles in egg recognition and membrane fusion (e.g., fertilin, PH20, 2B1), the mechanisms that regulate their compartmentalization and subsequent migration are clearly of major significance for the acquisition of fertilizing capacity by spermatozoa.

**TABLE 1.** Diffusion coefficients (D) and percentage recoveries (%R) of DiIC12 on the midpiece region of ram and boar spermatozoa subjected to video-FRAP analysis to determine directionality of recovery across the posterior ring.

<table>
<thead>
<tr>
<th>Spermatozoa</th>
<th>Profile</th>
<th>D (×10⁻⁹ cm²/sec)</th>
<th>%R ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boar (n = 9)</td>
<td>Left</td>
<td>1.8 ± 0.4</td>
<td>89.1 ± 13.1</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>2.1 ± 0.6</td>
<td>79.3 ± 8.4</td>
</tr>
<tr>
<td>Ram (n = 11)</td>
<td>Left</td>
<td>16.2 ± 5.1</td>
<td>79.2 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>15.3 ± 4.1</td>
<td>81.2 ± 5.6</td>
</tr>
</tbody>
</table>

* Values for D and %R represent mean ± SEM. Differences between left and right profiles are not significant (P > 0.05).
A long-established and attractive hypothesis, based on the paradigm of epithelial cell tight junctions, is that the posterior ring and annulus function as diffusion barriers to compartmentalize the surface membrane into head, midpiece, and principal piece. Both structures have a morphological basis [5], and their position in the plasma membrane of the mature spermatozoon coincides with distribution boundaries between many surface antigens and with the location of underlying organelles, e.g., mitochondria in the midpiece and the fibrous sheath in the principal piece. The results from classic FRAP analysis have supported this view. The D values for lipid reporter probes DiIC$_{14}$ and ODAF are 3- to 4-fold higher over the head plasma membrane than over the tail plasma membrane [22, 39]. Percentage recoveries are also significantly lower on the midpiece than on the postacrosome or principal piece (~55% vs. ~80%), suggesting that barriers separate or at least delineate in-plane lipid inhomogeneities. To date however, there has been no quantitative assessment of the diffusion of lipids and proteins across the posterior ring or annulus in spermatozoa in real time. To address this question, we adapted the technique of video-FRAP analysis for measuring the directionality of recovery of DiIC$_{12}$ following large-area photobleaching. Where no obvious boundary exists (such as within the acrosomal region), recovery is symmetrical from all directions, but where a boundary occurs (such as between the acrosome and postacrosome in the spermatozoon; Fig. 2, f–j), diffusion is asymmetric, as indicated by skewed recovery curves (Fig. 3). When this technique was applied to the posterior ring of live ram and boar spermatozoa, however, we were unable find evidence for a barrier to diffusion of DiIC$_{12}$ from the postacrosome to the midpiece. Recovery curves were consistently symmetrical, indicating bidirectional diffusion. It is not possible to say whether DiIC$_{12}$ can diffuse in the reverse direction or not (i.e., from midpiece to postacrosome); recovery analysis of the postacrosomal region is difficult because of the tapering shape of the sperm head in this region. Assessment of the barrier function of the annulus is also problematic because of the weak fluorescence signal from the membrane overlying the principal piece, which narrows rapidly in a posterior direction. However, Cardullo and Wolf [22] reported diffusion of DiIC$_{14}$ from the direction of both the postacrosome and the principal piece of ram spermatozoa following photobleaching of the whole midpiece region (10–12 µm long). Although qualitative, this observation implies that, like the posterior ring, the annulus is not a barrier to exchange of lipids between adjacent regions. Three provisos to this conclusion are 1) different classes of lipids may vary in their ability to diffuse across the posterior ring or annulus, 2) the barriers may function only during early stages of spermiogenesis in the testis when, in combination with vectorial transport and antigen retrieval mechanisms, they could behave as molecular sieves to facilitate formation of different surface compartments in elongating spermatids [23], and 3) the barriers may only ‘fence in’ large molecules such as proteins that interact more strongly with the cytoskeleton than do lipids.

**Compartmentalization of Lipids in Sperm Plasma Membranes**

If, as our data suggest, physical structures such as the posterior ring (and possibly the annulus) do not prevent exchange of lipids between different surface domains in the plasma membrane of mature spermatozoa, what other mechanisms could be responsible for maintaining in-plane lipid asymmetry? Specifically, how could the situation shown in Figure 2, f–j, arise?

Evidence for lateral heterogeneity of lipids in sperm plasma membranes is derived from several sources. First, not only are there differences in diffusion coefficients for Di and ODAF probes between membrane domains, but these domains also respond independently of each other to external stresses. Thus, ODAF diffusion on the acrosome of bull spermatozoa increases fivefold between 20°C and 37°C, whereas on the midpiece the increase is less than twofold [28]. Second, freeze-fracture work and quantitative fluorescence image analyses with filipin have demonstrated cholesterol-rich and cholesterol-poor areas on both the micrometer and nanometer scales [5, 40]. Of particular interest is an array of sterol particles immediately below the equatorial segment. Third, antibodies to glycolipids have shown that some are spatially restricted (e.g., sulphogalactolipid on the apical ridge of boar spermatozoa [38]). Fourth, monoclonal liposomes frequently fuse with specific membrane domains and, depending on the conditions, do not spread into other areas. Arts et al. [17] observed fusion of phosphatidylserine liposomes with the equatorial segment of acrosome-reacted, but not acrosome-intact, human spermatozoa. Fifth, differential scanning calorimetry of sperm membrane preparations has revealed that ~50% of lipids are immobile, suggesting the presence of gel phase domains [26]. Collectively, these observations indicate that there are significant compositional and/or organizational differences in lipids between the major surface macrodomains of spermatozoa.

The forces responsible for these lipid diffusion boundaries are currently unclear [28]. The most likely candidates are the submembranous cytoskeleton, thermodynamically driven phase separations caused by the high proportion of unsaturated plasmalogens, variations in cholesterol concentrations, and formation of nonlamellar hexagonal II phase structures. As least two of these forces may be responsible for the observed lipid diffusion boundary between the postacrosome and equatorial segment plasma membranes. In ejaculated ram spermatozoa, vimentin and F-actin are concentrated beneath the membrane in the equatorial segment [41, 42], and a cholesterol-rich layer is found in the postacrosome immediately below and adjacent to the equatorial segment [40, 43]. Freeze-fracture and surface replica studies have shown this cholesterol-rich layer to contain arrays of particles and nanometer size pits that delineate a sharp boundary with the equatorial segment [40] (cf. the particle arrays at the base of the flagellum of *Chlamydomonas reinhardtii* [20] and between inner and outer segments of retinol rod cells [2]). It is not known whether these structures represent lipid rafts, but whatever their composition they signify substantial differences in molecular organization between membrane domains where we have observed asymmetrical recovery following large-area FRAP. The appearance of a large immobile fraction (~66%) in the postacrosome of the spermatozoon in Figure 2, f–j, explains to a large extent the skewed recovery curves and apparent boundary effect, although the possibility cannot be excluded that there was some exchange of the mobile fraction between the two domains that was undetectable with current instrumentation. We do not know precisely what causes this situation, but one explanation is that the spermatozoa in question were in the process of losing their viability and that degenerative changes to the plasma membrane were spreading anteriorly from the direction of the tail. If so, it
emphasizes once again the segregated nature of the sperm plasma membrane.

We have shown, using a quantitative video-FRAP technique, that despite its location and structure the posterior ring is not an immutable barrier to lipid diffusion between the midpiece and postacrosomal domains of spermatozoa. It seems more likely that differential phase behavior, interaction with integral membrane proteins, and the influence of the cytoskeleton are the major factors in determining lateral heterogeneity of lipids in sperm plasma membranes.

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