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· *Review* ·

Supramolecular organization of the sperm plasma membrane during maturation and capacitation

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Abstract

Aim: In the present study, a variety of high resolution microscopy techniques were used to visualize the organization and motion of lipids and proteins in the sperm's plasma membrane. We have addressed questions such as the presence of diffusion barriers, confinement of molecules to specific surface domains, polarized diffusion and the role of cholesterol in regulating lipid rafts and signal transduction during capacitation. **Methods:** Atomic force microscopy identified a novel region (EqSS) within the equatorial segment of bovine, porcine and ovine spermatozoa that was enriched in constitutively phosphorylated proteins. The EqSS was assembled during epididymal maturation. Fluorescence imaging techniques were then used to follow molecular diffusion on the sperm head. **Results:** Single lipid molecules were freely exchangeable throughout the plasma membrane and showed no evidence for confinement within domains. Large lipid aggregates, however, did not cross over the boundary between the post-acrosome and equatorial segment suggesting the presence of a molecular filter between these two domains. **Conclusion:** A small reduction in membrane cholesterol enlarges or increases lipid rafts concomitant with phosphorylation of intracellular proteins. Excessive removal of cholesterol, however, disorganizes rafts with a cessation of phosphorylation. These techniques are forcing a revision of long-held views on how lipids and proteins in sperm membranes are assembled into larger complexes that mediate recognition and fusion with the egg. *(Asian J Androl 2007 July; 9: 438–444)*

Keywords: cholesterol; lipid rafts; single molecules; sperm membranes

1 Introduction

During their passage from the testis to the site of fertilization in the oviducts, mammalian spermatozoa encounter a wide range of fluids of very different origins and composition (e.g. compare testicular fluid with seminal plasma and these with oviduct secretions). These fluids have a major influence on post-testicular developmental processes, such as maturation and capacitation, and as a result spermatozoa are transformed from an immotile, infertile state to a vigorously active cell with the ability to bind specifically to, and ultimately fuse with, the egg. Despite much research effort over many years the processes involved remain problematic. As a working hypothesis we suggest that post-testicular development of spermatozoa should be viewed as a hierarchy of signaling processes, initiated by different agonists within the different fluid environments, which proceeds in a step-wise fashion until the spermatozoon reaches the site of fertilization in a fully competent state. This scenario ensures that not only do sperm complete all their developmental stages in the correct sequence but also that they do not respond to external signals prematurely.

Central to the above hypothesis is the organization of the sperm's plasma membrane. Like many terminally differentiated cells, the sperm's surface membrane is highly compartmentalized with the result that many lipids and proteins are spatially restricted [1]. It is now known from work on other cell types that agonist bind-

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ing and receptor activation frequently involves assembly of multi-molecular complexes, which transduce external signals across the plasma membrane into the cytoplasm where they are amplified to elicit a response (e.g. the immunological synapse) [2]. Because spermatozoa do not synthesize new membrane proteins, formation of signaling complexes would require spatially separated molecules to come together, possibly from different regions of the cell. These complexes might, in turn, show polarized migration from regions where they are inactive to areas where they become fully functional. This is the concept of "being in the appropriate place, at the appropriate time and in the correct form" [3]. Both the complexes and their individual components will be subject to the randomizing forces of diffusion yet in many instances they show polarized migration by mechanisms that are unknown but might involve the cytoskeleton [4].

A long-standing goal in our laboratory has been to elucidate the basic properties of sperm plasma membranes in relation to their composition, compartmentalization and developmental competence. For this purpose, we have applied a range of high resolution microscopy and biophysical techniques to visualize and measure lipid and protein diffusion in different membrane domains under varying experimental conditions. We have addressed problems such as the existence of intra-membranous barriers, diffusion of antigens against large concentration gradients, formation of multi-component complexes following agonist binding and the role of lipid rafts as signaling platforms.

2 Materials and methods

The materials used in the experiments described below, together with details of the methods used, are described in the following publications: atomic force microscopy (AFM [5]), fluorescence recovery after photobleaching (FRAP [6–8]), fluorescence loss in photobleaching (FLIP [9]), single particle fluorescence imaging (SPFI [9]), single molecule tracking (SMT [10]) and lipid raft isolation [11]. Except where stated otherwise, experiments have been carried out predominantly on spermatozoa from boars and rams.

3 Results

3.1 Atomic force microscopy (AFM)

AFM of ejaculated ovine, porcine and bovine spermatozoa revealed significant differences in surface topography of the plasma membrane between the anterior acrosome (Ac), equatorial segment (EqS) and post-acrosome (PAc) (Figure 1, [5]). In all three species the PAc plasma membrane has a rough uneven surface without any apparent regularity in the undulations. At the boundary with the EqS, however, an abrupt change takes place with a clearly defined line (in boar and bull) or necklace of rectangular depressions (in ram) between the two domains. Within the EqS, the plasma membrane becomes relatively smooth except for a semi-circular or crescent-shaped area designated the equatorial subsegment (EqSS), which has fine irregular corrugations that distinguish it from the surrounding smooth membrane. Anterior to the EqS there is again an abrupt change leading to the Ac plasma membrane, which has a rougher surface than that overlying the EqS. Therefore, there are significant and consistent differences in surface membrane topography between domains on the sperm head.

The EqSS is of particular interest as it has only been described recently [5]. It could not be reliably detected by AFM on ovine testicular spermatozoa, but was present

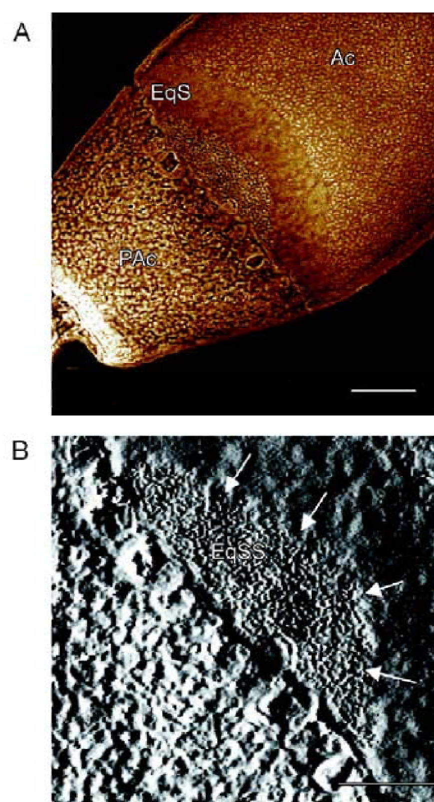

Figure 1. Atomic force microscopy (AFM) images of an ovine spermatozoon. (A): Low resolution picture to illustrate the difference in membrane topography between the postacrosome (PAc), equatorial segment (EqS) and acrosome (Ac). (B): Higher resolution of the equatorial subsegment (EqSS)–outlined by arrows. Bar = 1 µm. Adapted with permission from Ellis *et al.* [5], 2002. Copyright Elsevier.

on cauda epididymidal spermatozoa, suggesting that it is assembled during epididymal maturation. This result was confirmed by staining the EqSS with 4G10 monoclonal antibody (McAb), which is specific for phosphotyrosine residues. Intact live spermatozoa are not stained on the EqSS with 4G10 McAb; they have to be permeabilized by cold shock or fixation, suggesting that the structures causing the topographical features detected by AFM are intracellular. All permeabilized cauda spermatozoa were stained over the EqSS with 4G10 McAb. Less than 5% of testicular spermatozoa were positive. By contrast, in the boar approximately 80 % of testicular spermatozoa were stained with 4G10 McAb, but in this case the whole EqS was positive, whereas in mature cauda spermatozoa only the EqSS was stained with the antibody. In the boar, the EqSS was also reactive with Hsp70 antibody [12]. Therefore, during epididymal maturation phosphorylated proteins are either transported into the EqSS from elsewhere or else they are phosphorylated *in situ*. How they become restricted to the EqSS is a major puzzle, but the fact that they are so spatially discrete suggests specialized function.

To date, the major phosphorylated protein identified in the EqSS is sp38 (an inner acrosomal membrane protein [13]), with lesser amounts of phosphorylated F-actin capping protein and actin-associated proteins M1 and M2. These proteins are characteristically intracellular proteins. This is consistent with our earlier conclusions that the EqSS is created by a sub-plasma membrane structure. It is tempting to speculate that sp38 or the actin-associated proteins might have a role during sperm fusion with the oolema (e.g. in formation and relocation of fusion complexes).

3.2 Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching measures diffusion of tens of thousands of molecules in a membrane. The fluorescent probe may be a lipid or protein inserted exogenously into the bilayer to “report” on its properties or it may be attached to an endogenous component the behavior of which is assumed to be unaffected by the external molecule, frequently a protein such as an antibody or lectin. Using a variety of lipid reporters (ODAF, $DiIC_{12}$, NBD-PC, NBD-PE; molecular probes) and antibodies to surface antigens we have shown that:

1 Diffusion coefficients are 3–5 times faster on the head than on the tail in bovine, ovine, porcine, murine and human spermatozoa. The exception is the guinea pig in which diffusion coefficients are similar throughout the head and tail [6]. A noteworthy finding is that a significant (approximately 50 percent) immobile phase is present on the midpiece plasma membrane.

2 During epididymal maturation when the plasma membrane becomes more unsaturated owing to a decline in specific phospholipids, diffusion coefficients increase by 40% to 120% on the sperm head [8].

3 Removal of cholesterol by cyclodextrins to induce capacitation changes has relatively small, and mostly insignificant, effects on lipid diffusion in all regions [11]. Depletion of membrane cholesterol with very high levels (50–100 mmol/L) of cyclodextrins, however, eventually immobilizes lipid diffusion.

4 Diffusion coefficients for a glycosylphosphatidylinositol-anchored glycoprotein (2B1 or PH20 or SPAM1) on capacitated rat spermatozoa are not significantly different between the acrosome and midpiece arguing against mechanisms involving free diffusion and trapping for its polarized migration [4].

5 Peroxidation of endogenous unsaturated phospholipids has little or no effect on diffusion coefficients for ODAF but addition of hydroperoxides (e.g. cumene hydroperoxide) exogenously to a live sperm suspension causes immediate immobilization of all membrane components [8].

These data reinforce concepts of compartmentalization of the spermatozoon’s plasma membrane and although informative in a global sense, they provide few clues to the underlying mechanisms. The high immobile phase on the midpiece region is suggestive of an interaction with the cytoskeleton, but high resolution tracking techniques are required to tackle problems such as confined diffusion, directed flow and the presence of intramembranous barriers.

3.3 Are diffusion barriers present in sperm plasma membranes?

Physical structures within the sperm’s plasma membrane, known as the posterior ring and annulus, were described by electron microscopists over 30 years ago and traditionally have been thought to function as diffusion barriers to compartmentalize the membrane overlying the midpiece and principal piece. Additionally, the differences between the PAc, EqS and Ac as shown by AFM and FRAP hint that diffusion boundaries are present between domains on the sperm head. To test this hypothesis, we devised a video-FRAP system to measure the directionality of fluorescence recovery on the sperm tail following bleaching of a lipid reporter probe $DiIC_{12}$ (Figure 2 [9]). We have assumed that this probe behaves in the membrane as single molecules and that the tail is essentially a cylinder. Results showed that $DiIC_{12}$ diffused readily across both structures in an anterior to posterior direction (it was not determined if posterior to

anterior diffusion was similarly unimpeded but it is reasonable to presume that this would be the case). To investigate the presence of putative diffusion barriers on the sperm head, where diffusion would be 2-dimensional, an FLIP procedure was followed [9]. Using this technique, the same area is bleached repeatedly with several seconds recovery between bleaches. If a diffusion barrier is present, at say the EqS, and the bleach area is located on the anterior of the head, fluorescence in the PAc region will remain high, whereas that on the Ac will decline progressively. If a barrier is not present then fluorescence will bleach uniformly over the whole head. When a 2–3 micron diameter laser beam was focused on the anterior tip of the sperm head and bleached 6–7 times then $DiIC_{12}$ fluorescence decreased uniformly over the whole sperm head. The results were the same when the bleach spot was moved to the PAc. Therefore, there does not appear to be a barrier to diffusion of single lipid molecules on the sperm head.

3.4 Diffusion of lipid assembles in sperm plasma membranes

In keeping with other cells, it is likely that dynamic assemblies of molecules form in sperm membranes, either in response to external agonists or as a result of rearrangements of the cytoskeleton as part of more global signaling processes. In the course of our experiments with lipid reporters, it was found that the longer chain $DiIC_{16}$ did not incorporate uniformly into boar sperm plasma membranes but formed particles 0.3–1.0 µm in diameter. These particles probably represent tens to hundreds of $DiIC_{16}$ molecules and can be regarded as essentially homogeneous, although it is possible that endogenous lipids might be associated with them. Significantly, many of the particles exhibited rapid movement within the confines of the sperm head and remained fluorescent for 10–60 s depending on their size. AFM confirmed that the $DiIC_{16}$ particles were on the surface and not beneath the plasma membrane. The diffusion of the particles was followed by video tracking and analysis of their individual trajectories (Figure 3 [11]). We refer to this as single particle fluorescence imaging (SPFI). Only ejaculated or cauda epididymidal spermatozoa showed the phenomenon; $DiIC_{16}$ particles did not form in testicular spermatozoa. It was found that: (i) diffusing particles on the anterior acrosome moved freely into and out of the EqS but rarely (approximately 10% of the time) crossed the boundary onto the PAc; (ii) particles diffusing on the PAc were very slow and never entered the EqS; (iii) mean square displacement analysis revealed that particle diffusion was essentially random although within a domain diffusion coefficients fluctuated approximately fivefold.

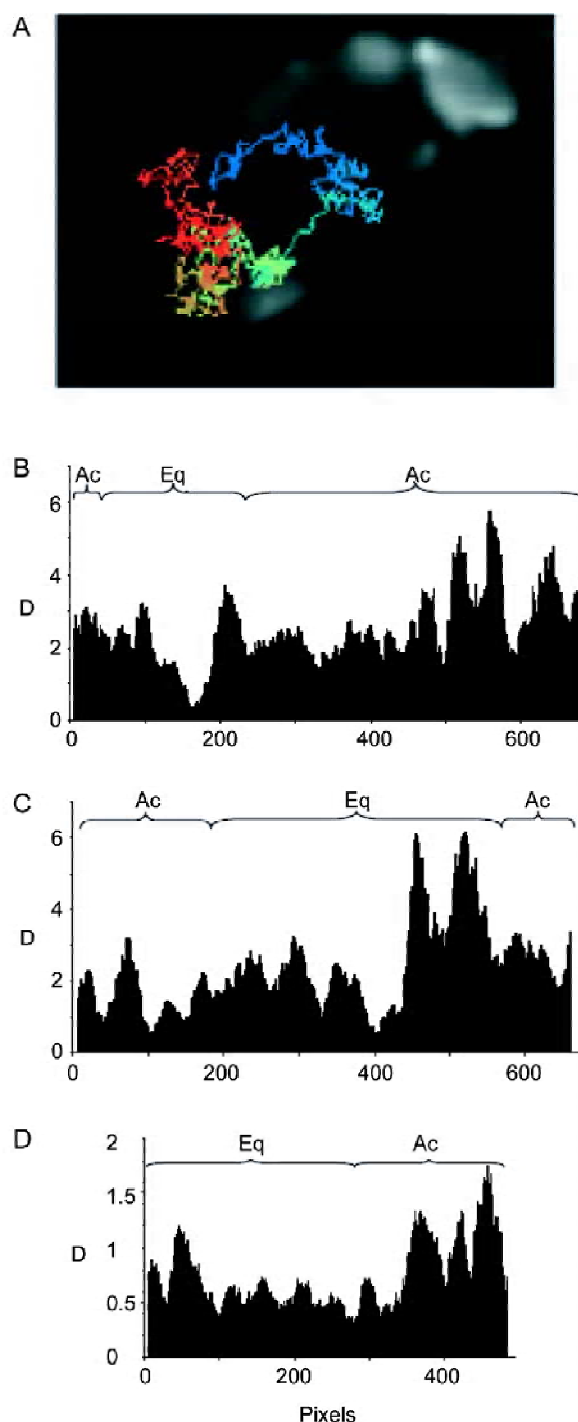

Figure 2. Trajectory of a lipid particle ($DiIC_{16}$) diffusing over the surface of a live porcine spermatozoon. (A): Colour changes are time-based beginning with blue and ending with brown. Particles of $DiIC_{16}$ exchange freely between the Ac and EqS but do not cross over onto the PAc. (B–D): Diffusion coefficients of the $DiIC_{16}$ particles fluctuate in a random fashion irrespective of their position in domains. Ac, anterior acrosome; Eq, EqS; D, diffusion. Adapted with permission from James *et al*., 2004 [9].

There was no obvious pattern to these fluctuations.

On the basis of these results we have proposed that a molecular filter is present in the plasma membrane between the PAc and the EqS domains [9]. Single molecules are free to exchange across this filter but larger complexes, such as represented by the $DiIC_{16}$ particles, are unable to do so. Only by disassembling on one side of the filter followed by individual components diffusing across it and reassembling on the other side would molecular complexes relocate from one domain to another. Various scenarios on this theme can be envisaged (e.g. after a lipid or protein molecule diffuses across the EqS it might re-associate into a different complex with new functional capabilities). It is not possible to say if the filter at the EqS is unidirectional or bidirectional as we have not observed sufficient numbers of moving particles in the PAc. The physical basis for such a filter is not known, but by extending the concept of the "fence and picket" model, as outlined by Kusumi *et al.* [14], it is possible that the EqS-PAc junction is created by a line or necklace of transmembrane proteins stabilized by the cytoskeleton. Because the state of the cytoskeleton (filamentous versus monomeric) is sensitive to intracellular pH, changes to proton pump activity in more distant areas of the cell membrane could influence the filter and, hence, polarized migration of protein complexes through it.

It is worth noting that diffusion coefficients for single lipid molecules (ODAF, $DiIC_{12}$ and NBD-PC) on the acrosome of boar spermatozoa were approximately 30 times faster than the $DiIC_{16}$ lipid particles (compare values of 3.0–3.5 $\mu m^2/s$ for ODAF to 0.1–0.2 $\mu m^2/s$ for $DiIC_{16}$ particles (units given as $\mu m^2/s$ are equivalent to 10^{-8} cm^2/s used in early publications).

3.5 Diffusion of single molecules in sperm plasma membranes

FRAP, FLIP and SPFI measure diffusion of tens of thousands, or at best hundreds, of molecules in a membrane. They cannot, therefore, provide the resolution necessary to investigate the dynamics of complex assembly following agonist binding or the formation/dissolution of lipid rafts. Given the presence of lipid rafts in sperm membranes (see below) and the importance of cholesterol efflux in activating protein kinases and intracellular phosphorylation cascades during capacitation, it

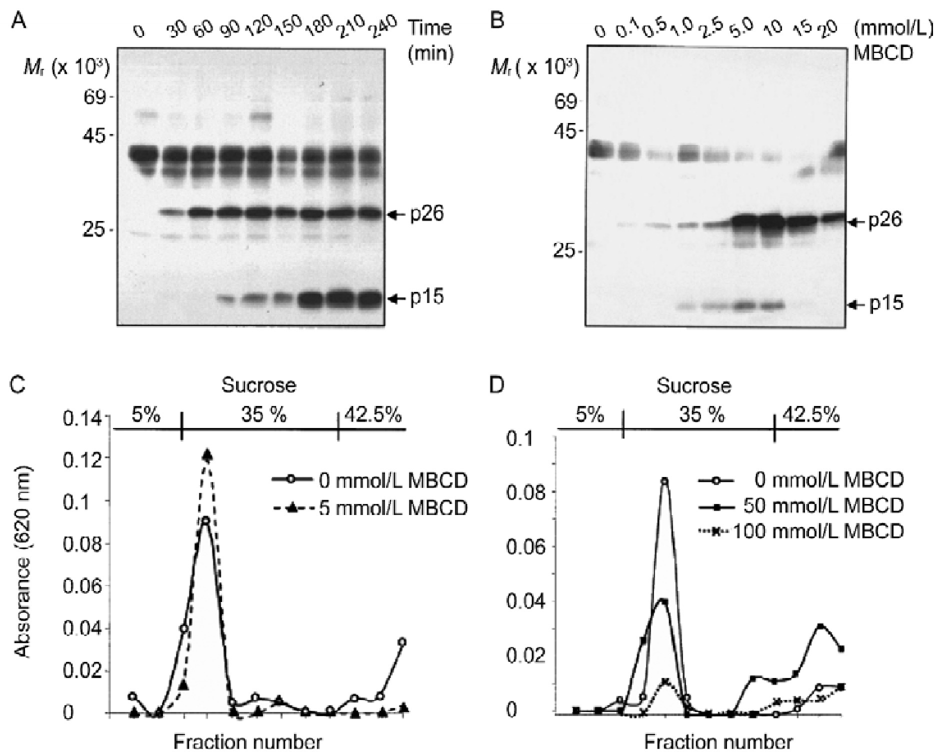

Figure 3. A reduction in plasma membrane cholesterol with 5 mmol/L cyclodextrin (MBCD) initiates phosphorylation of intracellular proteins in porcine spermatozoa with a concomitant increase in size/number of lipid rafts (A, C). Excessive removal of cholesterol with high levels of MBCD, however, disrupts rafts and leads to a decrease in phosphorylation (B, D). Modified with permission from Shadan *et al.* 2004 [11].

is necessary to understand diffusion at the level of single molecules. This level of resolution is now achievable with total internal reflection fluorescence microscopes, sensitive charge-coupled device cameras, stable fluorophores and sophisticated tracking software. When combined with a nanopipette delivery system, which enables labeled probes to be applied to precise areas of the membrane under interrogation, single molecule tracking (SMT) becomes a powerful tool for understanding, for example, lipid rafts, diffusion barriers, transient confinement zones and anomalous diffusion.

Using Atto^{647}-WGA lectin as a general probe for membrane glycoproteins we have applied SMT to different surface domains of boar spermatozoa. Experiments in progress indicate that diffusion coefficients on the anterior Ac are several-fold faster than on the PAc and that trajectories are essentially random. One reason for the inherently lower diffusion rates in the PAc is that WGA lectin might bind to a different class of glycoproteins that are not present on the Ac. This possibility cannot be excluded entirely but seems unlikely as both FRAP and SPFI also recorded lower diffusion coefficients for lipid molecules and lipid particles in the PAc. The fact that DiIC16, a saturated probe that partitions preferentially into liquid ordered phases, stains the PAc in a uniformly dense manner suggests fundamental differences in membrane organization that may involve proteins of the membrane skeleton, actin, destrin and thymosin-β10, which, depending on the species, frequently localize to the PAc and EqS [15].

3.6 Evidence for multimolecular assemblies (e.g. lipid rafts) in sperm plasma membranes

A cold Triton-X100 insoluble, low density fraction was first demonstrated in sea urchin spermatozoa by Ohta *et al.* [16] and interpreted as containing lipid rafts. Since then, lipid rafts have been reported in murine, guinea pig, human and porcine spermatozoa [11, 17, 18]. Rafts are enriched in cholesterol, sphingomyelin, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins, and in the inner leaflet protein kinases (e.g. Src kinases). Cholesterol is also found in non-raft regions and has a major influence on the miscibility of different phospholipids, contributing to the separation of liquid ordered (l_o) and liquid disordered (l_d) phases in artificial bilayers and biological membranes. A reduction in membrane cholesterol is known to be one of the key first steps in initiating signaling cascades during sperm capacitation and accounts for the requirement for macromolecules like bovine serum albumin (BSA) in capacitation media. The discovery by Visconti *et al.* [19] that treatment of sperm with cyclodextrins (which selectively extract cholesterol from membranes) initiates tyrosine phosphorylation of intracellular (usually flagellar) proteins concomitant with capacitation revolutionized studies on this phenomenon as it provided an objective and reproducible assay. Since then, specific tyrosine phosphorylated proteins have been described in murine, bovine, porcine, human and ovine spermatozoa following capacitation. Removal of cholesterol from immature testicular spermatozoa, however, does not induce tyrosine phosphorylation [11], a finding consistent with the hypothesis that these cells have not yet assembled all the downstream signaling pathways necessary to respond to activation of receptors in the plasma membrane. In porcine spermatozoa incubated with methyl-β-cyclodextrin it was found that removal of low amounts of cholesterol actually increased the proportion of lipids rafts in the plasma membrane and it was only when spermatozoa were treated with very high concentrations (50–100 mmol/L) of cyclodextrin to remove > 70% of the cholesterol that rafts dispersed and tyrosine phosphorylation was inhibited. Significantly, sphingomyelinase treatment of whole porcine spermatozoa induced tyrosine phosphorylation without overall loss of cholesterol. Sphingomyelinase converts sphingomyelin to sphingosine-1-phosphate and ceramide, both of which are known signaling molecules [20]. However, a secondary effect of ceramide is to displace cholesterol from rafts into non-raft regions with parallel recruitment of protein kinases into the raft. This is highly suggestive of dynamic assembly of signaling platforms in membranes in response to external agonists. In support of this conclusion we have observed a change in the distribution of GM1 gangliosides in the plasma membrane of murine and porcine spermatozoa following cyclodextrin treatment. Initially, FITC-cholera toxin β-subunit bound to the sperm tail but after capacitation it appeared over the acrosomal region. Other workers have described the appearance of a sulfogalactolipid, known as SLIP1, and zona binding proteins on the anterior or rostral ridge of the acrosome following capacitation with BSA [21, 22]. One explanation for this phenomenon is that it reflects anterior migration of lipid rafts.

4 Discussion and conclusion

Collectively, the high resolution microscopy techniques described above have extended our knowledge of the supramolecular organization of the sperm's plasma membrane well beyond that provided by the traditional procedures of freeze-fracture, scanning and transmission electron microscopy. Using AFM we have discovered a new morphological region, the EqSS, in Artiodactylia spermatozoa, which is assembled during epididymal maturation and has an unusual concentration of Hsp70 and

phosphorylated proteins, the latter of unknown function. FRAP, SPFI, and SMT are providing insights into the dynamics of lipid and protein diffusion in the plasma membrane with important implications for the organization of putative diffusion barriers, assembly of multi-molecular complexes following agonist binding and behavior of signaling centers, such as lipid rafts. It is anticipated that future developments in SMT techniques will enable several molecules in the membrane to be labeled and followed simultaneously, providing quantitative information on, for example, their diffusion rates and association times with other molecules.

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