Capacitation dependent changes in the sperm plasma membrane influence porcine gamete interaction

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Capacitatie-afhankelijke veranderingen in de spermaplasmamembraan beïnvloeden de gameetinteractie bij het varken

(met een samenvatting in het Nederlands)

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Contents

Preface

More than 300 years ago, Antoni van Leeuwenhoek [1] discovered the sperm cell and postulated that these 'small animals' bear everything to create a new individual, while the oocyte was just a sort of cultivation medium. Nowadays we know that the sperm cell and the oocyte both bear half of the information to form a newborn. The finding of Antoni van Leeuwenhoek initiated fertilization research and this thesis elaborates on this process.

Mature sperm cells carry all elements to fertilize the oocyte. However, freshly ejaculated sperm cells are not able to fertilize the oocyte. In the early 50's, Chang [2] and Austin [3] reported independently at approximately the same time that sperm cells needed to be activated to become fully fertile. This process, called capacitation, normally occurs in the female genital tract but could also be achieved in vitro. Adequate temperature and media containing bicarbonate and other components, were essential to fully capacitate sperm cells outside the female genital tract (i.e. under in vitro conditions). These capacitation media made it possible to fertilize mammalian oocytes in the laboratory, better known as in vitro fertilization (IVF). IVF enabled many infertile couples to overcome their fertility problems and become parents. Another fertilization technique, artificial insemination (AI) is now frequently used in the breeding industry where it more or less has replaced natural mating in some domestic species (e.g. porcine, bovine). Nevertheless, fertility problems are not completely overcome by AI or conventional IVF. New techniques, like intra cytoplasmic sperm injection (ICSI), that cross most natural borders have recently been developed. One single sperm cell is drawn into a pipette and subsequently injected into the cytoplasm of the oocyte. Offspring from ICSI pregnancies do look normal, although long term effects have not been studied yet. Passing most barriers that sperm cells face during natural mating may be dangerous, since natural selection of the male gamete does not occur. The selection during ICSI is done under the microscope based only on sperm morphology and motility, but we do not know what these parameters tell us about the quality of the selected cell.

As mentioned above, capacitation is the activation of sperm cells in the female genital tract which can also be achieved in vitro. Capacitation media for several species have been designed by trial and error. Although the principle of capacitation has been detected long ago, information about what happens at the molecular level has only become available very recently. A number of transitions in the sperm cell and at the level of its plasma membrane have been elucidated, however, the picture is far from complete. This thesis describes capacitation induced changes of proteins and lipids in the plasma membrane of the sperm cell. These capacitation events are most likely involved in the primary sperm-oocyte interaction or events following this interaction.

Although many researchers have contributed to discover molecules and mechanisms involved in sperm-oocyte interaction, the exact process is unclear. A few proteins have been postulated to play a role in sperm-oocyte interaction, however, the role of these proteins is still uncertain. As an example, knock-out mice that lack expression of some of these proteins were still fully fertile. This thesis focuses on an animal model to study sperm-oocyte interaction, namely the porcine. The starting material of an interaction assay were the two parts that are involved in the primary sperm-oocyte interaction: the sperm plasma membrane and the extracellular matrix of the oocyte. The porcine system is used as a model, since large amounts of

both sperm cells and oocytes can relatively easily be obtained, which is necessary to set up such interaction assays. By using isolated sperm plasma membranes instead of whole sperm homogenates and zona pellucida (i.e. egg extracellular matrix) fragments instead of solubilized zona pellucida proteins, an interaction system has been created to resemble physiological sperm-oocyte interaction as closely as possible. The possibility that the data presented in this thesis may contribute to the identification of the primary zona pellucida receptors and allow researchers to investigate the downstream events that occur after sperm zona-binding will be discussed.

Chapter 1

Dynamics of the mammalian sperm plasma membrane in the process of fertilization

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submitted for publication

Abstract

Sexual reproduction requires the fusion of sperm cell and oocyte during fertilization to produce the diploid zygote. In mammals complex changes in the plasma membrane of the sperm cell are involved in this process. Sperm cells have unusual membranes compared to those of somatic cells. After leaving the testes, sperm cells cease plasma membrane lipid and protein synthesis, and vesicle mediated transport. Biophysical studies reveal that lipids and proteins are organized into lateral regions of the sperm head surface. A delicate reorientation and modification of plasma membrane molecules take place in the female tract when sperm cells are activated by so-called capacitation factors. These surface changes enable the sperm cell to bind to the extracellular matrix of the egg (zona pellucida). The zona pellucida primes the sperm cell to initiate the acrosome reaction, which is an exocytotic process that makes available the enzymatic machinery required for sperm penetration through the zona pellucida. After complete penetration the sperm cell meets the plasma membrane of the egg cell (oolemma). A specific set of molecules is involved in a disintegrin-integrin type of anchoring of the two gametes which is completed by fusion of the two gametes plasma membranes. The fertilized egg is activated and zygote formation is preluding the development of a new living organism. In this review we focus on the involvement of processes that occur at the sperm plasma membrane in the sequence of events that lead to successful fertilization. For this purpose, dynamics in adhesive and fusion properties, molecular composition, and architecture of the sperm plasma membrane, as well as membrane derived signalling are reviewed.

1. Introduction

The earliest event in life is the meeting of the sperm cell with the egg. Enormous numbers of sperm cells are deposited in the female genital tract, but only one sperm cell will successfully fertilize the egg. The fusion of sperm and egg leads to the recombination of fathers and mothers genetical information resulting in a new individual. Sperm-egg interaction and the subsequent fertilization are highly regulated processes. Ongoing research shows the important role of the sperm plasma membrane in mammalian fertilization. The plasma membrane is not only the border of the sperm cell, but it appears to be a very dynamic structure. Here, we review the sperm-egg interaction mainly focussed on the sperm cell and particularly its plasma membrane.

Already during the formation of sperm cells in the testis (spermatogenesis, for review see [4,5]), the plasma membrane and other specific structures are prepared to react adequately to the female genital tract and the oocyte. Spermatogenesis in mammals starts at puberty by forming sperm cells in the testis from stem cells (type A spermatogonia) arranged at the basal lamina in seminiferous tubuli. Type A spermatogonia divide several times to form spermatocytes and subsequent spermatids after meiosis. During the subsequent divisions the cells migrate towards the lumen. Round spermatids are transformed into highly differentiated and polarized cells (Fig. 1A). The flagellum is the first structure to be developed during spermatogenesis which coincides with the recruitment of mitochondria from the cytoplasm to form a helical pattern around the midpiece of the flagellum (Fig. 1B). More or less simultaneously the acrosome (a large secretory vesicle in the sperm head) is formed: the perinuclear Golgi apparatus produces small

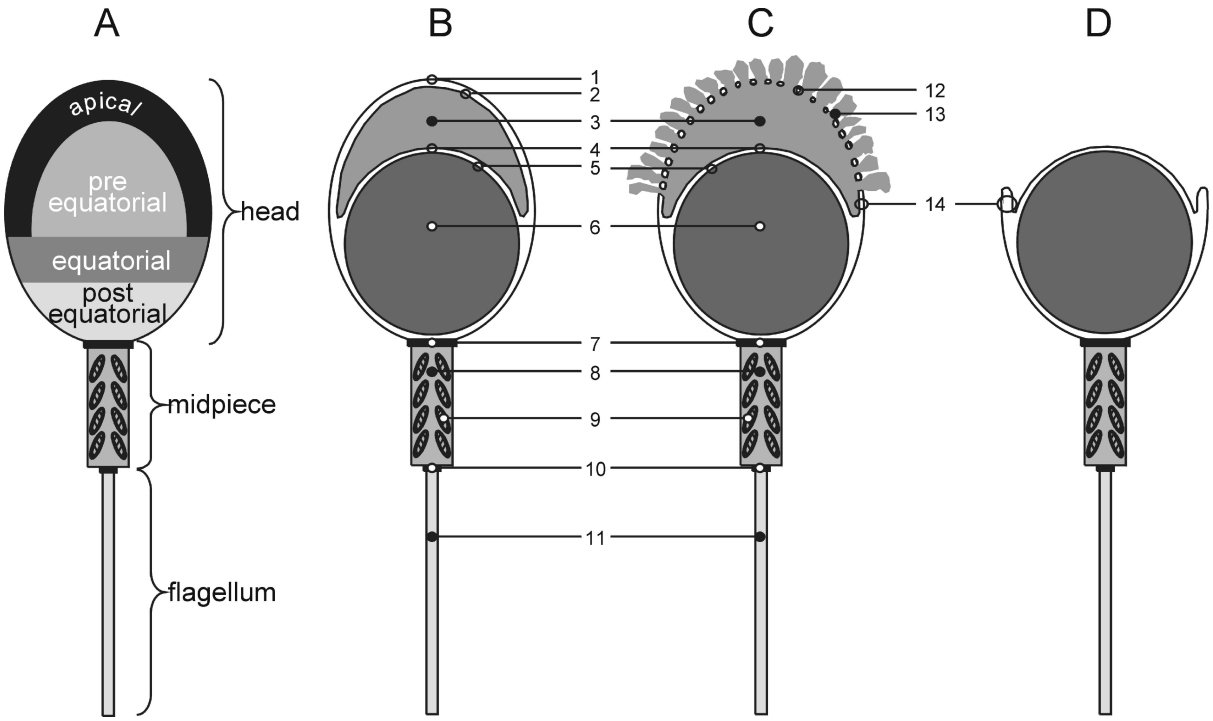

Figure 1. Sperm cells are polarized cells with a head, flagellum and midpiece (A, schematic surface drawing). The sperm head can be subdivided in four regions: apical, pre-equatorial, equatorial and post-equatorial regions. The acrosome (large secretory vesicle, 3) is situated apical to the nucleus (B). After binding of the sperm cell to the oocyte with its apical plasma membrane, the plasma membrane fuses with the underlying outer acrosomal membrane at multiple sites (C). The acrosomal content (hydrolytic enzymes) will be secreted, which enables the sperm cell to digest the egg extracellular matrix (zona pellucida). After the acrosome reaction has been completed, the inner acrosomal membrane forms a continuum with the remaining plasma membrane (D). This hairpin structure is involved in the primary binding of the sperm cell to the oolemma. Note that the representations B, C and D are cross sections through a flattened cell. 1: plasma membrane; 2: outer acrosomal membrane; 3: acrosomal content; 4: inner acrosomal membrane; 5: nuclear envelope; 6: nucleus containing highly condensed DNA; 7: posterior ring; 8: midpiece; 9: mitochondrion; 10: annular ring; 11: flagellum; 12: mixed vesicle (i.e. plasma membrane fused with outer acrosomal membrane); 13: acrosomal secretion; 14: hairpin structure.

condensing vacuoles that contain dense material (proacrosomal granules), which will form the acrosomal vesicle. The acrosomal vesicle spreads out over the nucleus, while the Golgi apparatus contributes more and more material to the developing acrosome. The plasma membrane approaches the nucleus due to cytoplasm redistribution, with the acrosome in between both structures, inducing polarity in the developing spermatid. The DNA of the nucleus starts to condense by changes in histones and other specific basic proteins that associate with the DNA. Concomitantly, the cell elongates as the cytoplasm is stretched out along the flagellum. The acrosome stops growing and the Golgi complex migrates to the caudal site of the spermatid. The cell volume of spermatids is reduced to approximately 25% of original volume, due to water loss, cytoplasm loss just before sperm release and the separation of a cytoplasmic package (residual body). Residual bodies are formed at the time sperm releases from the epithelium. These residual bodies contain packed RNA and organelles such as the Golgi apparatus, endoplasmic reticulum (ER), lysozomes and peroxysomes.

Due to loss of most cell organelles and DNA transcription, spermatozoa lack protein expression and vesicular transport. This implies that the plasma membrane is a stable and metabolically inert structure, since protein, phospholipid, cholesterol, and other components of the plasma membrane cannot be newly synthesized. However, the plasma membrane of released sperm cells is not yet fully matured (for review see [4]). During the transit of the sperm cell through the epididymis, the plasma membrane changes for example by the release, modification and adsorption of proteins and lipids. The role of these surface alterations is not fully understood, although some adsorbed proteins are involved in sperm-oocyte binding. In most mammalian species, sperm cells are fully matured when they reach the end of the cauda of the epididymis [4].

The mature sperm cell has three highly specialized regions (Fig.1A): (i) the sperm head, involved in sperm-oocyte interaction; (ii) the midpiece with mitochondria, involved in energy production; (iii) the flagellum, involved in motility. The sperm head plasma membrane is separated from the midpiece plasma membrane by the posterior ring and this latter domain is separated from the flagellum plasma membrane by the annular ring (Fig. 1B: structural elements 7 and 10, respectively). The sperm head contains, besides a very low amount of cytosol, the nucleus and the acrosome (Fig. 1B). The acrosome is a large vesicle containing hydrolytic enzymes, necessary for the penetration of the oocyte extracellular matrix (zona pellucida, ZP) [6]. Furthermore, the acrosome-overlying plasma membrane is separated from the post-acrosomal membrane by the equatorial segment. These borders can be observed by electron microscopy [7]. Freeze fracture studies of the mammalian sperm plasma membrane indicated that the different domains all contain different concentrations and distributions of intramembranous particles, which represent transmembrane proteins (for review see [4]). This lateral polarized distribution could also be observed using specific lectins and antibodies [8].

Sperm-oocyte interaction can be subdivided in a sequence of events (Fig. 2) (for review see [6]). Ejaculated sperm cells need to be activated in the female genital tract in a process called capacitation (Fig. 2A). Sperm cells become hypermotile during capacitation and only capacitated sperm cells bind to the ZP in a species specific manner (Fig. 2B). Binding of the sperm cell to the oocyte subsequently induces various signals in the sperm cell. The concerted signals trigger a Ca^{2+} influx and the plasma membrane fuses at multiple sites with the outer acrosomal membrane (acrosome reaction, Fig. 1C and 2C) [9]. The acrosomal content, mainly hydrolytic enzymes, starts to disperse and digest the ZP (Fig. 2D). During the acrosome reaction the apical plasma membrane and the outer acrosomal membrane form ‘mixed’ vesicles that disperse (Fig. 1C). Throughout this process the inner acrosomal membrane becomes a part of the outer barrier of the cell and will form a continuous membrane structure with the plasma membrane which looks like a hairpin structure (Fig. 1D) [10]. Consequently, the inner acrosomal membrane is exposed to and binds to the ZP (secondary ZP binding). Hypermotile sperm cells that have reacted properly upon binding to the ZP penetrate the ZP and enter the space between the ZP and the egg plasma membrane (perivitelline space) (Fig. 2D). Here the sperm cell binds to the egg plasma membrane (oolemma) with its tip. After binding of the sperm cell to the oolemma with its tip, the sperm head binds laterally with its equatorial region in which the hairpin membrane structure is involved (Fig. 2E). After lateral binding, the sperm plasma membrane and the oolemma fuse and, subsequently, the sperm cell is incorporated in the oocyte (Fig. 2F).

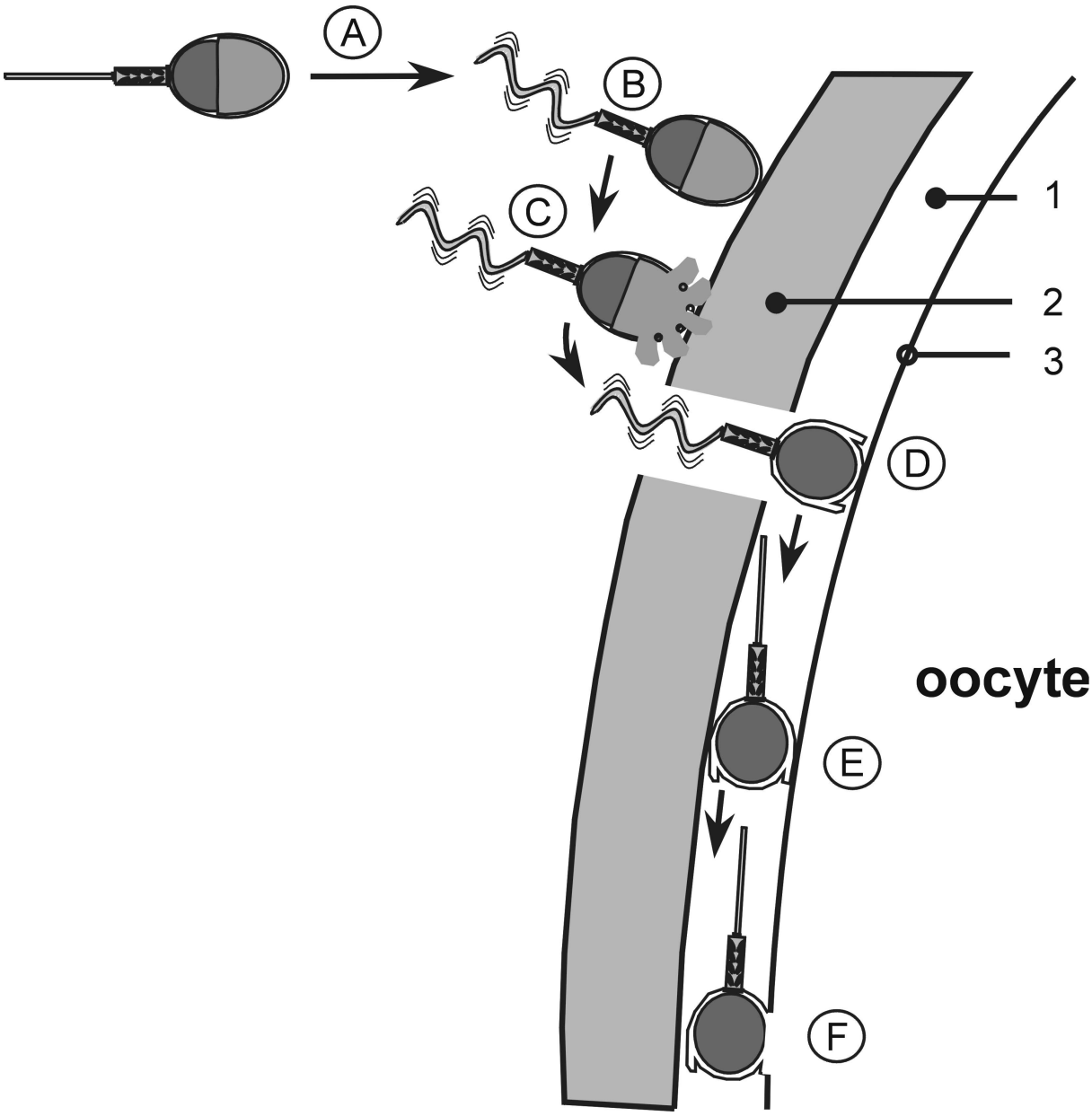

Figure 2. Sequence of mammalian fertilization. A: freshly ejaculated sperm cells are activated in the female genital tract during a process called capacitation. B: capacitated sperm cells are hypermotile and are able to bind yo the egg extracellular matrix (zona pellucida, ZP). C: binding of sperm cells to the ZP triggers the acrosome reaction and acrosomal enzymes are secreted. D: hydrolytic enzymes secreted from the acrosome degrade the ZP and subsequent sperm cells penetrate the ZP, enter the perivitelline space and bind to the oolemma with the apical tip. E: Subsequent to apical tip binding, oolemma binding changes to the hairpin structure of the acrosome reacted sperm cell. F: after hair pin structure binding to the oolemma, the sperm cell fuses with the oocyte and the sperm cell is subsequently incorporated in the oocyte. 1: perivitelline space; 2: ZP; 3: oolemma (egg plasma membrane).

Only very recently we have gained insight into the heterogeneity of the plasma membrane. The diversification of the cell surface probably relates to physiological specializations of the plasma membrane. This has been demonstrated for many epithelial cells that have an apical plasma membrane which is in contact with a lumen and a basolateral plasma membrane in contact

with supporting cells and the blood circulation [11]. These two plasma membrane compartments have different physiological functions and also differ in molecular composition. Mixing of membrane components between the two plasma membrane compartments is prevented by tight junctions [12]. It also has become clear that the composition and lateral organization of the plasma membrane regulate the affinity for adhesion factors, the permeability for hydrophilic solutes, cell signalling and cell fusion events.

This review will place another cell type -namely the mammalian sperm cell- into the spotlight of membrane researchers. This single cell has a variety of plasma membrane specializations, and each of these has a unique role in the cascade of events that will lead to the ultimate goal of the sperm cell: the fusion with the plasma membrane of the oocyte. During the activation of the sperm cell (i.e. capacitation) in the female genital tract, dramatic reorganizations take place in the sperm plasma membrane in order to achieve the ability to fertilize the oocyte. The current knowledge on the dynamics and functions of the sperm plasma membrane organization in relation to the physiology of fertilization will be reviewed. Besides the physiological importance of this topic special attention will be paid to the potential of the sperm cell and its plasma membrane as a biological model system for future studies on the dynamical aspects in the regulation of membrane heterogeneity and its consequences for cell physiology.

2. The lipids of the sperm cell and their role in sperm-oocyte interaction

As mentioned in the introduction the mature sperm cell lacks a set of organelles important for the synthesis of lipids (e.g. the ER and the Golgi complex) and the breakdown of lipids (e.g. lysosomes and peroxisomes). In the mature sperm cell its surface membrane is not in contact with intracellular membranes because vesicle mediated membrane transport is blocked. The only exception to this is when the apical plasma membrane fuses with the underlying outer acrosomal membrane, which only takes place during the highly regulated acrosome reaction (Fig. 1). The unusual composition and organization of lipids in the sperm plasma membrane are probably reflections of these specific sperm properties.

2.1. Lipid composition

The lipid composition of the sperm plasma membrane of several mammalian species has been elucidated. Although there is considerable variation between different mammalian species, in general the plasma membrane contains approximately 70% phospholipids, 25% neutral lipids and 5% glycolipids (on molar base) [13].

2.1.1. Phospholipids

Phospholipids can be divided into phosphoglycerolipids and sphingomyelin. The phosphoglycerolipids vary in molecular structure because of different polar head groups at the sn-3 position of the glycerol backbone (phospholipid classes). Each phospholipid class comprises a number of molecular species due to different aliphatic acyl-, alkyl or alk-/-enyl chains attached to either the sn-1 or sn-2 positions of the glycerol backbone (phospholipid species). Apart from some variations among different mammalian species [14], the phospholipid class composition of sperm cells is generally comparable with that of somatic cell types. For example, human sperm

cells contain 50% phosphocholineglycerides (PC), 30% phosphoethanolamineglycerides (PE), 12.5% sphingomyelin (SM), 3% phosphatidylserine (PS), 2.5% cardiolipin (CL) and about 2% of phosphatidylinositol (PI) [13]. Contrarily, the molecular species composition of PC and PE, and perhaps also of the other phospholipid classes, is quite unique for sperm cells. The sn-2 position of the glycerol backbone of these phospholipids is predominantly esterified with long chain polyunsaturated fatty acids (almost exclusively docosahexanoic acid, 22:6 and docosapentanoic acid, 22:5). Furthermore, at the sn-1 position they contain predominantly saturated aliphatic chains with a carbon chain length of 16 atoms, of which approximately 55% is attached as a vinylether (plasmenylcholine or plasmenylethanolamine) and 25% as a saturated alkyl-group (phosphatidalcholine or phosphatidalethanolamine, respectively) [15,16]. Only 20% of the PC and PE contain the normal sn-1 ester linkage of a fatty acid. During capacitation the PC levels may increase due to methylation of PE [17].

2.1.2. Neutral lipids

Major variations in neutral lipid composition of sperm membranes can be found between different species, individual males but also among different ejaculates. The major variable factor is the amount of cholesterol in the sperm plasma membrane. Human sperm cells for instance contain rather high amounts of cholesterol (25 mol % of total lipids) whereas boar sperm cells contain much less cholesterol (14 mol %). The cellular sterol content seems to be related with the length of capacitation [6]. In fact, it has been demonstrated that cholesterol is depleted from the plasma membrane of sperm cells upon capacitation (see section 2.3.1). Besides cholesterol low amounts of desmosterol, cholesterol sulfate, and cholesterol esters can be found [13].

2.1.3. Glycolipids

Glycolipids are normally formed by addition of a glycosidic head group to ceramide. The only exception in vertebrates is the glycolipid seminolipid which is found only in mammals, in sperm cells and Schwann cells (for review see [5]). About 98% of the seminolipid has the structure 1-O-hexadecyl, 2-O-hexadecanoyl, 3-3' sulfogalactosyl glycerol. Besides seminolipid only trace amounts of other glycolipids can be found in mammalian sperm cells [18]. After ejaculation seminolipid can be desulfated by secreted arylsulfatases that originate from the accessory sex glands [19]. Both seminolipid and its desulfated counterpart are believed to participate in certain fertilization processes (see sections 2.2.1, 2.3.3, 2.5 and 4.2).

2.2. Lipid organization

2.2.1. Lateral membrane topology

The structure of the sperm cell and the functional division of its surface into lateral domains and subdomains are summarized in Fig. 1. In the 70's first clues were found for a lateral heterogeneity of the topology of surface molecules in the plasma membrane of the sperm head. Lectins bound heterogeneously to the surface of the sperm head (see section 3.1.3). Furthermore, freeze fracture replicas revealed that integral membrane proteins were polarly distributed among different regions of the plasma membrane of the sperm head as well as other parts of the sperm cell (for review see [4]). The delicate surface organization of the sperm cell alters upon capacitation. This is partly

due to decoating and the removal of glycocalyx components [20] and adsorption of new components from the female genital fluids and partly a result of enzymatic modifications of glycocalyx components [21,22]. In addition, a lateral reorganization of transmembrane proteins takes place within the sperm head plasma membrane [23].

All these changes have been observed in the sperm head plasma membrane, which makes the interpretation of the lateral heterogeneity difficult: (i) The head plasma membrane is separated from the midpiece and tail plasma membrane by linear arrangements of trans membrane proteins forming the so-called posterior ring [4]. However, such structural barriers are not present in the sperm head plasma membrane and therefore lateral separation of sperm head plasma membrane components must be accomplished by other means [23-25]. (ii) One other possible explanation for the polar distribution of the integral membrane proteins could be that they are attached to the polarized sperm cytoskeleton (Fig. 3). However, the lateral organization of membrane proteins alters dramatically upon capacitation whereas changes in the cytoskeleton have not been observed

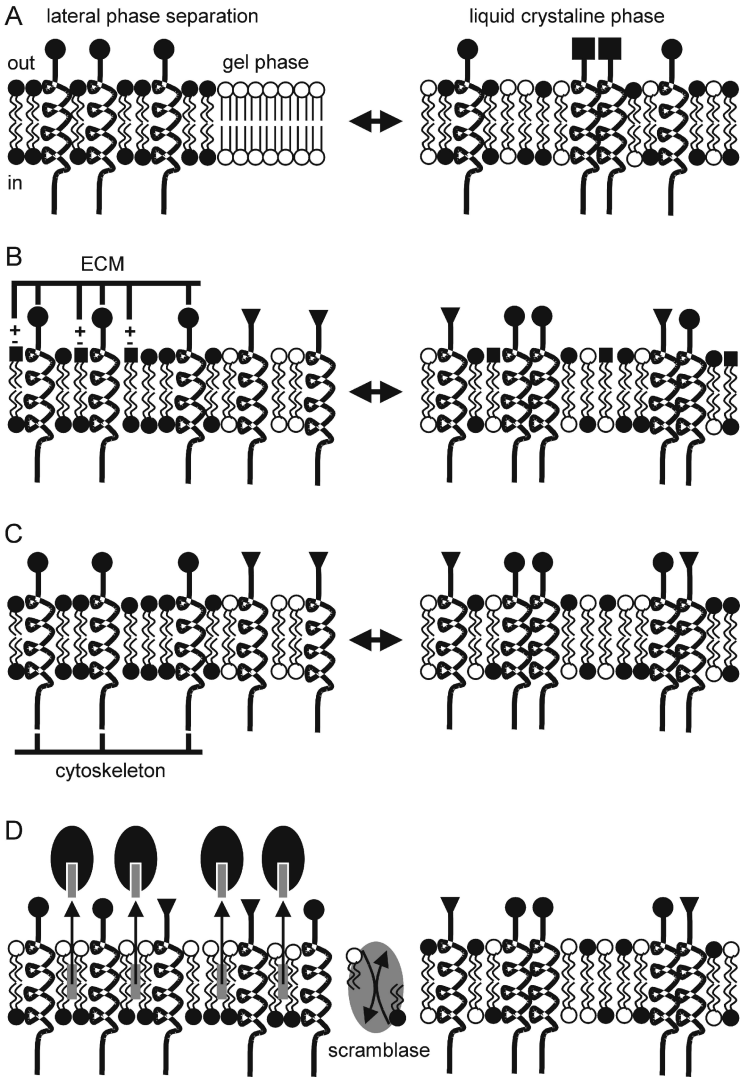

Figure 3. Current models for lateral membrane polarity dynamics upon sperm capacitation.
A: Lateral phase separation of gel phase (excluding freely diffusable membrane proteins) and liquid crystalline phase (aggregations of transmembrane proteins). Melting of gel phase preferring lipids results in lateral mixing of membrane lipids and free membrane proteins.
B: The extracellular matrix (ECM) of the sperm cell is very heterogeneous and is organized into lateral domains. Therefore, the ECM may create membrane domains via electrostatic interactions with glycolipids or with membrane proteins. Disruption of the ECM will allow mixing of lipids and proteins between the two lateral domains.
C: A similar scenario as described for B may be valid for the polarly organized cytoskeleton of the sperm cell.
D: Processes A-C may induce dimerization of transmembrane proteins (or dissociation of transmembrane protein aggregates). Dimerization is probably further facilitated by increased membrane fluidity due to the bicarbonate induced efflux of membrane cholesterol (gray rectangle) and the scrambling of bilayer phospholipid asymmetry.

[4,26]. A more likely explanation for the lateral heterogeneity in this membrane is provided by the differential electrostatic interactions of the membrane components with the lateral polarized glycocalyx (Fig. 3). A strong indication for the validity of this model is that capacitation induces remodelling of the glycocalyx which explains the dynamic alterations in lateral topology of the trans membrane proteins. Moreover, the glycocalyx may induce lateral polarity changes of lipids in the sperm plasma membrane which in turn may induce topological rearrangements of freely diffusable transmembrane proteins. Evidence has been provided that the lipids in the sperm head plasma membrane are indeed also organized into lateral membrane domains. Lateral polarity of lipids in the sperm plasma membrane was first detected using probes that complex unesterified sterols (filipin [27]) or anionic phospholipids (polymyxin [24]). The lipid-probe particles were visualized on freeze-fracture replicas and, although the underlying phenomenon is still not understood, the particles were polarly organized in the sperm head plasma membrane. Filipin complexes were distributed over the entire head plasma membrane surface, although a lower density of particles was detected in the post-equatorial subdomain in fresh sperm cells. After capacitation the post-equatorial region was devoid of filipin complexes and a slight increase in complexed filipin was found in the apical head plasma membrane [24]. Weak polymyxin B labelling was found on freshly ejaculated sperm cells whereas, after capacitation, a strong increase of labelling was detectable in the apical ridge region of the sperm head surface [28]. Most likely polymyxin B labels PS, an anionic phospholipid that is localized in the inner lipid leaflet of the sperm cell but that becomes partly exposed at the plasma membrane exterior during capacitation [29]. This phenomenon appears to be restricted to the apical area of the sperm head (see section 2.2.2 and [30]).

In our lab we have investigated the topology of seminolipid in the plasma membrane of fixed and living sperm cells using indirect immunofluorescent labelling techniques and vesicle mediated loading of a fluorescent acylated seminolipid analog, respectively [31,32]. Both techniques revealed that seminolipid is highly concentrated in the apical ridge region of the sperm head plasma membrane in control cells, whereas, in capacitated cells almost the entire seminolipid fraction migrates towards the equatorial region of the sperm head plasma membrane (Fig. 4). As is valid for most other glycolipids this lipid is confined to the outer lipid leaflet of the sperm plasma membrane [19] and is most likely in contact with the glycocalyx components (e.g. by linkage to its binding protein SLIP, see section 2.3.4). Reorganizations in the lateral polarity of lipids in the sperm head may therefore very well originate from altered seminolipid glycocalyx interactions [31].

It has been suggested that the lipid segregation and reorientation phenomena in the sperm plasma membrane could also be explained by lipid phase transitions (freezing of lipids from the fluid liquid crystalline phase to the frozen gel phase, see Fig. 3) [33]. However, these lipid phase transitions were only observed after cooling and they emerged within the subdomains [24,34]. The small circular areas in which the lipids were in the gel phase had a surface of maximally only 0.04 μm^2, which is very small in comparison with the average size of the subdomains (12 μm^2). The absence of the small gel phase structures in the head plasma membrane of capacitating sperm cells indicate that lipid phase separations do not occur under these conditions and that another phenomenon must drive the (re-)organizations of lateral lipid subdomains in the sperm plasma

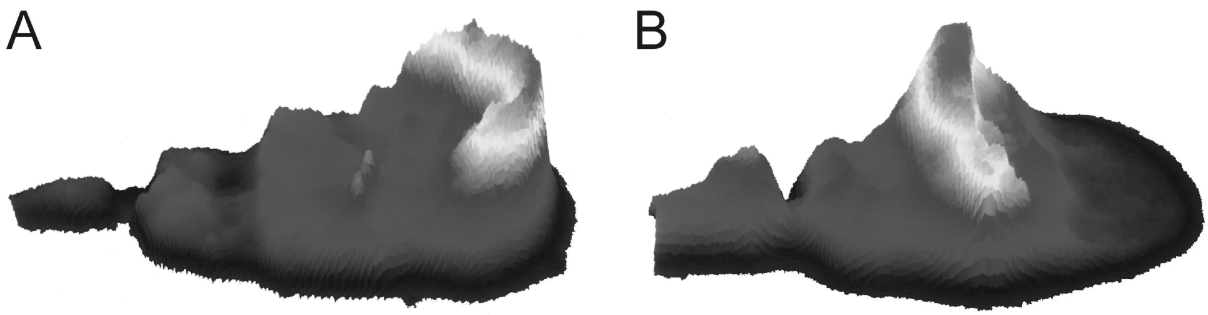

Figure 4. Lateral distribution of fluorescent glycolipids after their incorporation into the plasma membrane on boar sperm cells with intact acrosomes and plasma membranes. A: The distribution of SGalCer(C12-LRh) (a fluorescent analog of seminolipid) in the plasma membrane of a freshly ejaculated sperm cell. B: Idem, but after capacitation in vitro in a Tyrode's based medium containing 2 mM $CaCl_2$ for a period of 2 h. The distribution of the C12-LRh lipid label intensity was measured in situ on the sperm surface with an epifluorescence microscope (Rhodamine filter setting) connected to a CCD camera and an image analyser [32]. The in situ biophysical behaviour of the fluorescence probe was analysed by fluorescence lifetime imaging microscopy [31]. This enabled quantification in situ of the fluorescent probe at the surface of the sperm cell as indicated here in peak height and in warm colour table. Cells were stained with Hoechst 33258 and a peanut agglutinin-FITC conjugate in order to assess the intactness of the plasma membrane and the acrosome, respectively. The length of the sperm head is approximately 8 μm.

membrane. Although lateral diffusion properties of lipids and free membrane proteins vary considerably between the different subdomains of the sperm head plasma membrane [35], this is not due to lipid phase separation phenomena, but probably a reflection of the different subdomain lipid compositions due to regionalisation of the glycocalyx (see section 2.2.3).

Conclusively, we believe that the polarized organization of the glycocalyx is important for the lateral lipid heterogeneity in the sperm head plasma membrane. However, the capacitation dependent reorganization in lipid polarity most likely reflects changes in the glycocalyx as well as a collapse of the phospholipid asymmetry (see section 2.2.2.). Recently it has been demonstrated that lateral membrane specializations called membrane rafts can function as a focal point for cellular signalling [36]. It is well possible that the dynamic lateral organization of the sperm plasma membrane enables the cell to respond appropriately to the variable stimuli it will encounter throughout its voyage to the oocyte.

2.2.2. Membrane bilayer topology

Like plasma membranes of somatic cells the lipids in the sperm plasma membrane are asymmetrically distributed over the lipid bilayer. Reliable methods have revealed that the choline phospholipids SM and, to a lesser extent, PC are mainly found in the outer lipid leaflet [30,37]. The aminophospholipids PE and especially PS are located in the inner lipid leaflet probably due to active inward transport by an aminophospholipid translocase. Some older studies report equal distributions of PE and PS in the sperm plasma membrane bilayer [38,39]. In those studies protocols were used that had been well established to measure lipid asymmetry in erythrocytes, such as phospholipases or TNBS-labelling at low temperature [40]. These methods are, however, very detrimental for the highly sensitive sperm plasma membrane organization [30]. Even in

freshly ejaculated sperm samples, a proportion of 7-15% of the cells have a deteriorated plasma membrane and this proportion will only increase during sperm activation (see section 3.1). Therefore, it has now become standard to detect lipid asymmetry for sperm cells in a flow cytometer for living cells only [30,41].

We recently performed a set of tests to see whether lipid asymmetry was affected by in vitro capacitation [29]. In order to compare the lipid asymmetry in control cells with capacitating sperm cells it is important to work at physiological temperature (38.5˚C, pig body temperature) and to block endogenous phospholipases (see section 2.4 and [30]). Scrambling of lipid asymmetry was observed: after 2 h a marked increase of PC and SM translocation into the inner leaflet was observed from 14 to 30% of total PC and 2 to 23% of total SM, for control and capacitated cells respectively. Furthermore the inward movement of PE and PS were considerably slowed down in capacitated cells (up to 10 times). The activation of a scramblase would explain the increased inward translocation of PC and SM, but also the decrease in the net PS and PE translocation rates. Recently we detected that PS and PE are only exposed at the apical head plasma membrane using annexin V-FITC [42] and Ro1100-FITC [43] as probes for PS and PE exposure respectively [29,30,44]. Probably a similar observation has been done almost 20 years ago by Bearer and Friend who studied the anionic phospholipid distributions with polymyxin B in control (only faintly labelled) and capacitating sperm cells (where intense labelling took place at the apical plasma membrane) [28].

The scrambling of phospholipids is under control of a bicarbonate mediated signalling pathway. Bicarbonate directly activates a sperm specific adenylate cyclase and thereby switches on protein kinase A (PKA). Most likely PKA on its turn directly or indirectly activates the proposed scramblase resulting in the altered phospholipid asymmetry of the capacitating life sperm cells (see section 3.1.1 and Fig. 5). At any rate the scrambling is dose dependent on bicarbonate levels (half maximal response at 7 mM) and can be mimicked in absence of bicarbonate with phosphodiesterase inhibitors (inhibition of cAMP breakdown, Fig. 5), PKA activators, protein phosphatase 1 and 2a inhibitors and by addition of cAMP analogs.

Merocyanine is believed to monitor disordered lipid acyl-chain packing of membranes [45]. The probe shows only faint fluorescence in non-capacitated sperm cells where the lipid asymmetry is maximal, whereas, after scrambling it becomes more intercalated into the membrane which results in higher fluorescence. The use of the probe was first described for other cell types but confirmed for sperm cells by Harrison et al. [46]. These studies clearly showed that only a discrete subpopulation of living sperm cells actually picked up high merocyanine fluorescence. The number of positively responding living cells corresponded very well with the proportion of sperm cells that showed aminophospholipid exposure at the apical sperm head region [29,44]. In fact, the merocyanine response mirrored the scrambling responses, because it was dependent on the same bicarbonate induced signalling cascade [47]. Moreover, the merocyanine response turned out to be independent of activation of tyrosine kinases and protein kinase C (PKC), indicating that a signalling cross talk with tyrosine kinase (normally occurring during sperm capacitation) has no influence on the lipid asymmetry regulation.

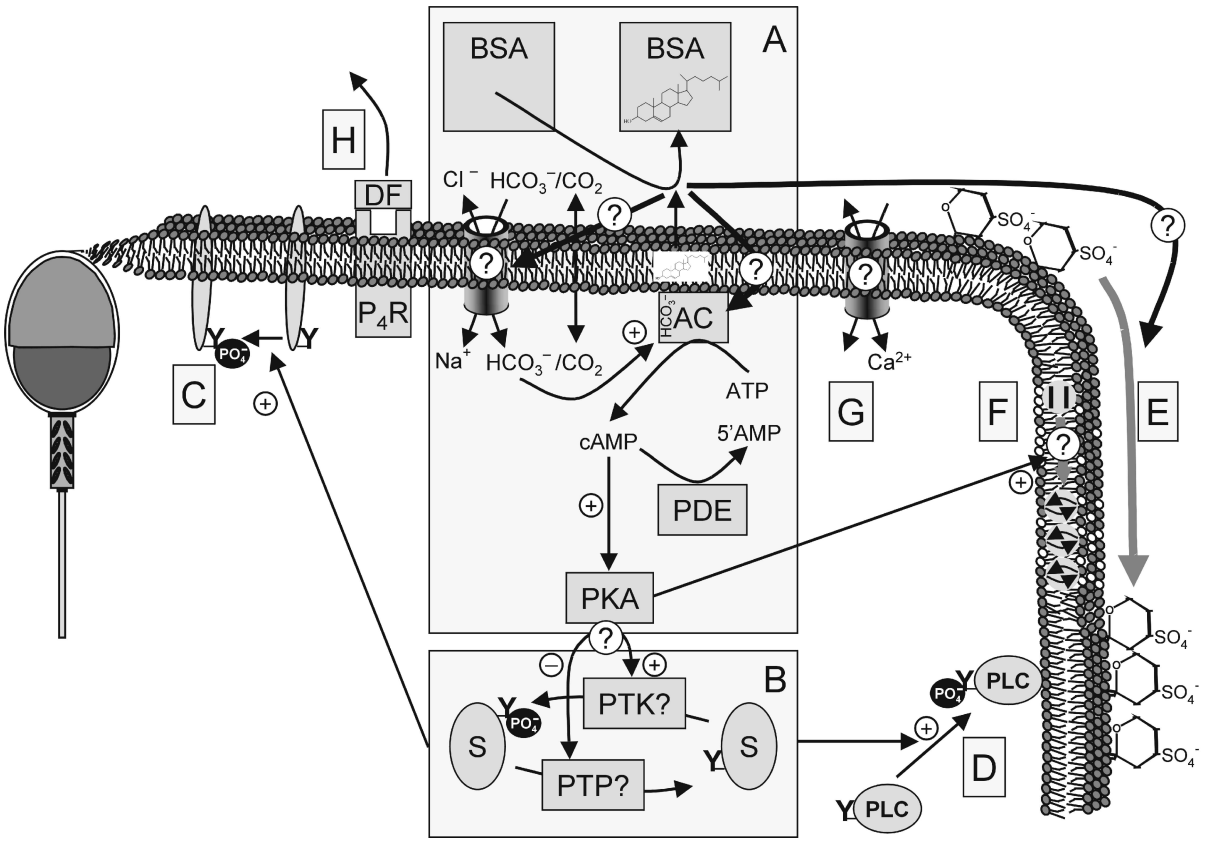

Figure 5. Proposed sequences of mammalian sperm capacitation. A: Bicarbonate may enter sperm cells via ion channels or via diffusion as carbon dioxide. Intracellular bicarbonate switches on adenylate cyclase (AC) and concomitant production of cAMP activates PKA. The role of cholesterol efflux in the activation of PKA is unclear. Cholesterol efflux may induce increased bicarbonate entry or may affect AC. B: PKA induces tyrosine (Y) phosphorylation of several substrates (S) most likely via activation of protein tyrosine kinases (PTK) or inhibition of protein tyrosine phosphatases (PTP). C: Sperm-ZP binding proteins and other plasma membrane proteins become tyrosine phosphorylated via the bicarbonate induced activation of PKA. D: Cytosolic PLC is tyrosine phosphorylated via the bicarbonate-PKA pathway. Tyrosine phosphorylated PLC is subsequently translocated to the plasma membrane. E: PKA activation induces plasma membrane changes like lateral redistribution of seminolipid and translocation of aminophospholipids. F: Aminophospholipids are translocated by the PKA-dependent activation of a postulated scramblase. Most likely the efflux of cholesterol is involved in these plasma membrane transitions. G: The entry of small amounts of calcium into sperm cells plays possibly an important role in capacitation. H: Decapacitation factors (DF) are removed from the sperm cell surface, uncovering receptors like the postulated progesterone receptor (P_4R).

2.2.3. Membrane fluidity

As mentioned above the plasma membrane of mammalian sperm cells has a pronounced domain organization (see section 2.2.1 and Fig. 1A). It is not clear whether or not this situation also applies for the lateral fluidity of lipids in the plasma membrane. Theoretically, the sperm plasma membrane should be very fluid due to the unusually high proportion of long chain polyunsaturated fatty acids of the main phospholipids. This prediction is supported by Laurdan fluorescence spectroscopy revealing a single liquid crystalline lipid phase and a lack of thermotropic phase transitions in the plasma membrane of living human sperm cells [48]. Other

biophysical techniques however, such as differential scanning calorimetry, electron spin resonance, and Fourier transform infrared spectroscopy, have detected thermotropic phase transition temperatures in membrane vesicles and lipid extracts from various mammalian sperm cells [14,33,49]. The multiple transition temperatures detected are consistent with the sperm plasma membrane's specializations into lateral subdomains. In fact, it has been suggested that the coexisting gel and fluid phases of lipids are a major driving force in the organization of the lateral membrane heterogeneity (Fig. 3A) [33]. However, it should be kept in mind that these studies have been performed on isolated membranes and vesicle membranes reconstituted from lipid extracts, which is not necessarily indicative for the intact plasma membrane in the living sperm cell [50].

One technique which can be employed to detect the lateral fluidity organization in the plasma membrane of the living sperm cell is fluorescence recovery after photobleaching (FRAP), in which a fluorescent reporter probe is inserted into the bilayer. The probe diffuses over the membrane surface but a well-defined spot on the surface is illuminated with a high-powered laser beam. Probe molecules that are in the laser spot are bleached (photo destructed) by the high energy of the laser excitation light pulse. The still intact probes from the unbleached environment of the bleached spot can now diffuse into the bleached spot. The rate of fluorescence recovery is proportional to the lateral fluidity of the membrane, whereas, the proportion of mobile lipids in the bleached spot can be calculated by the proportion of total fluorescence recovery. The technique is highly dependent on the probe that is used. For instance, Wolf and colleagues used 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanin (DiI16) stains to monitor lateral diffusion properties of ram and rodent sperm cells [51,52]. With this probe marked differences were found in lateral diffusion rates within the different regions of the sperm plasma membrane. Moreover rather low recovery percentages of 40-50%, indicative for the existence of gel phase domains, were found. Both fluorescence recovery rates as well as recovery percentages within the sperm membrane specializations changed upon sperm maturation as well as sperm capacitation. Interestingly, Jones and colleagues demonstrated using sperm of bull and other species that DiI16 probes only stained deteriorated cells, whereas, the probe did not stain living cells [50]. In those studies a more successful reporter probe 5-(*N*-octadecanoyl) aminofluorescein (ODAF) for FRAP studies was used. ODAF stains living cells as well as deteriorated cells [50]. It is unlikely that ODAF stained intracellular membranes of living sperm cells because the entire cell became homogeneously labelled (in case of intracellular labelling more pronounced fluorescence would be expected in the acrosomal region as well as the midpiece). However, in deteriorated cells such labelling patterns were found which was also the case for the DiI16 probe [50,53]. With ODAF the recovery percentages were approximately 90% in the head plasma membrane of living sperm cells [50,53]. The ODAF results are in agreement with the data obtained with Laurdan fluorescence spectroscopy on living sperm cells and on the lack of gel phase patches detectable in EM specimens of capacitating sperm cells (see 2.2.1). With this respect it is interesting for andrologists that cooling of sperm specimen for cryopreservation may lead to phase transitions as well as protein clustering [54]. This is believed to be one of the major causes of cryo-damage in sperm cells besides crystallization of water [55].

In our view the group of Jones has solved the contradiction that existed in literature about the presence and absence of a gel phase in sperm cells. In living sperm cells the gel phase does not exist, whereas, deterioration, cooling or fixation leads to rigidification of the membrane and low percentages of photobleaching recovery will be detected [50]. Similar artifacts probably occurred in the biophysical studies on extraction and reconstitution of sperm membranes explaining the detection of lipid transition temperatures [14,56]. It should be noted that the ODAF studies clearly demonstrated major differences in lateral diffusion rates within regions of the sperm head plasma membrane as well as in the tail and midpiece. This observation is in line with the lateral polarity detected for anionic phospholipids, cholesterol and glycolipids in sperm cells. The lateral diffusion rates of lipids in the different subdomains changes upon sperm maturation [57,58] as well as capacitation [35,59], although the latter has to be established with FRAP studies using the ODAF probe. Probably the alterations in lateral diffusion rates are due to the migration of certain lipids from one subdomain to another (Fig. 3 and 4) as well as the capacitation dependent scrambling of phospholipids in the apical plasma membrane of the sperm cell (Fig. 3 and 5).

2.3. Lipid protein interactions

Lipid molecules can be extracted or exchanged from the sperm plasma membrane by specific lipoproteins and lipid transfer proteins (carrier facilitated lipid transport). This process seems to play an important role in the modification of the lipid composition at the extracellular site of the sperm plasma membrane during capacitation [60,61]. Another type of protein lipid interaction, relevant for the fertilizing capacity of sperm cells, is the protein lipid interaction that results in linkage of the protein to a membrane. This can be in the form of covalent protein linkages to fatty acid chains (e.g. myristoylated proteins), GPI anchors or via electrostatic protein interactions with lipid head groups (via the phosphocholine head group of PC or the glycosidic moiety of glycolipids).

2.3.1. Lipoprotein mediated cholesterol transport

The observation that albumin should be included in in vitro fertilization (IVF) media in order to get efficient embryo production led Langlais and Roberts to propose a capacitation model leading to the acrosome reaction based on the possibility that lipoproteins extract sterols from the sperm plasma membrane [62]. More recently it has been established that cholesterol is indeed an important molecule in the sperm plasma membrane. The cholesterol content of sperm cells capacitated in vitro in a medium containing albumin decreased markedly (up to 40%) in various mammalian species [63] (Flesch et al., unpublished results). We recently discovered that this albumin-mediated decrease in cholesterol content only occurred when sperm cells were treated in a bicarbonate enriched capacitation medium, but not in absence of bicarbonate under otherwise similar conditions. This decrease in cholesterol content has also implications for the cholesterol topology in the sperm plasma membrane [34], which could indicate that albumin-mediated cholesterol extraction only occurs in restricted surface areas of the sperm cell. The extraction of cholesterol by albumin was rather specific because the phospholipid content remained identical (Flesch et al., unpublished results). Only sperm cells with a low concentration of cholesterol gave

a positive merocyanine response and a partial scrambling of phospholipid asymmetry indicating changes in the plasma membrane architecture (Flesch et al., unpublished results), whereas immature or cholesterol-rich sperm cells present in the same suspension did not show these events. This may explain some differences between studies on cholesterol in boar and human sperm [64,65] and sperm of mice or other rodents [66,67]. Sperm of rodents cannot be collected in ejaculated form because it is secreted together with spermicidal coagulation plug components [68]. Therefore, rodent sperm specimens have to be collected by aspirating the epididymis, and a relative large fraction of sperm cells from this site is still immature (i.e. with relatively high cholesterol levels). It has been shown that albumin has an additive effect on the bicarbonate mediated changes in membrane fluidity and the induction of a collapse in phospholipid asymmetry (see section 2.2.2). Therefore, we now believe that a subpopulation of well-matured sperm cells in ejaculated sperm samples already contains low enough cholesterol for bicarbonate-induced phospholipid scrambling. On the other hand albumin-mediated cholesterol extraction is required for the remaining non-responding sperm subpopulation to become responsive for bicarbonate-mediated changes in the plasma membrane architecture and signalling.

In this respect an interesting new approach of extracting cholesterol has recently been tested by Cross et al. [69] and by Visconti et al. [66]. They have used cyclodextrins to remove a large proportion of cholesterol from the sperm surface. Cyclodextrin has a very high affinity for cholesterol and, in contrast to albumin, it can extract cholesterol in absence of bicarbonate (i.e. in non-capacitating sperm cells). When cyclodextrin-treated sperm cells are incubated in a capacitation environment, a very rapid and pronounced signalling activation of PKA and protein tyrosine kinase (PTK) can be observed [66]. Taken together this demonstrates that only at relatively low cholesterol concentrations in sperm cell membranes activation of certain signalling pathways involved in fertilization processes can proceed. Scientific support for this can be found in a report where cholesterol has been described as modulator of receptor function in another cell type [70].

It is very well possible that the time required for optimal sperm capacitation relates to the slow efflux of cholesterol from the non-responding subpopulation of sperm cells. Further support for that idea is the fact that species with high cholesterol content (human and bull) require rather extended periods for optimal capacitation (resp. 8 and 6 h) whereas boar and ram sperm with lower cholesterol contents only require 1 or 2 h of capacitation [6]. The seminal plasma of human, stallion and perhaps other mammals contain prostasomes which are cholesterol rich vesicles secreted by the prostate [71,72]. These prostasomes block cholesterol efflux from sperm cells and probably serve to delay capacitation [71].

Although albumin may be a more physiological component than cyclodextrin to study cholesterol efflux from capacitating sperm cells, only a few groups have tried to determine cholesterol extracting lipoproteins from the female genital tract. With this respect Ravnik et al. [61] have reported a stimulating effect of human sperm capacitation by lipid transfer protein I, a key component of high density lipoproteins (HDL). The protein stimulates the acrosome reaction, is present in the female genital tract and originates from follicular fluid [73]. Therefore, the concentration of cholesterol extractor is optimal in the environment of the oocyte just after its ovulation. This scenario was predicted some time ago by Go and Wolf [74,75], who besides

cholesterol extraction also claimed a function in free fatty acid extraction for albumin. However, the importance of fatty acid extraction in sperm capacitation has never been investigated in further detail. Remarkably, also no attention has been paid in this respect to intracellular lipid transport proteins, despite the fact that the plasma membrane is in very close contact with intracellular membranes. In sperm cells, protein facilitated intracellular transport would be the way for transporting lipids and proteins from one membrane to another since vesicle mediated membrane transport is lacking in these cells.

2.3.2. Phosphatidylcholine binding proteins and capacitation

Bovine seminal plasma contains a series of proteins collectively called bovine seminal plasma proteins (BSP, A1/A2, A3, 30 Kda), which are secreted by the seminal vesicles [76]. These BSPs appear to have affinity for the sperm plasma membrane [77,78] and more specifically bind to phosphocholine moieties and PC liposomes [79,80]. In line with this, BSPs have been shown to induce PC efflux from epididymal sperm cells [81]. Furthermore, BSP complexes have been shown to support in vitro HDL-mediated cholesterol efflux from the sperm cell surface and capacitation of bull sperm [82]. The exact working mechanism of BSPs in this process is not clear, although it has been shown that BSP can bind to Apo A1 [83]. It appears that BSP binding to sperm cells is lost upon their interaction with epithelial cells of the oviduct [84]. Released sperm cells from this site are considered to be fully capacitated, competent to bind to the ZP and hypermotile [85-87].

2.3.3. GPI-anchored membrane proteins and zona binding

It is well known that membrane proteins can be covalently linked to membrane lipids. The most prominent way seems to be via the glycosylphosphatidylinositol anchor (GPI). For instance the protein PH20 later identified as a membrane bound hyaluronidase plays a role in the binding to and digestion of ZP structures [88]. Although 3 soluble isoforms of this protein have been localized in the acrosome, at least one 68 kDa isoform is GPI-linked to the plasma membrane of mouse sperm [89]. All forms may be important for successful penetration of the extracellular vestments that surround the egg prior to fertilization [88]. With respect to GPI-anchored proteins, Rooney et al. made interesting observations in seminal plasma of different mammalian species [90]. They observed that seminal plasma contained a stable population membrane free proteins that can be GPI-linked to the sperm cell surface (i.e. CD59, CD55 and Cdw 52). This type of sperm surface modification probably serves to make sperm cells resistant to complement attack in the female genital tract [90]. GPI protein modifications at the sperm surface may well be associated to polarity dynamics of the sperm plasma membrane.

2.3.4. Glycolipid binding proteins and zona binding

Male germ cells of mammals contain a sperm specific glycolipid called seminolipid (for review see [5]). In mature germ cells and sperm cells this glycolipid is exclusively present in the sperm plasma membrane [91] and only at the outer lipid leaflet of this membrane [19]. From the moment that seminolipid is surface oriented a seminolipid immobilizing protein (SLIP-1) is also found on male germ cells (Fig. 3B) [92,93]. The protein is 68 kDa large and therefore cannot

immobilize the entire seminolipid fraction (about 20% of the molar lipid fraction in the outer lipid leaflet of the sperm plasma membrane). Recently Tanphaichitr et al. have demonstrated that SLIP has a role in binding to the ZP [94]. Free seminolipid (i.e. the proportion of seminolipid that is not immobilized by SLIP) may also bind to the ZP by electrostatic interactions [95], which may serve to further stabilize the SLIP zona binding.

2.4. Lipid metabolism and signalling

Several reports have been published on the role of lipases in sperm capacitation and subsequently induced acrosome reaction (for review see [96]). The phospholipases cleave intact phospholipids into ceramide (sphingomyelinase), diacylglycerol (DAG) and alkylacylglycerol (phospholipase C), phosphatidic acid (phospholipase D) and in lysophospholipids and free fatty acids (phospholipase A2). These phospholipid catabolites serve as intracellular signalling molecules and activate protein phosphorylation cascades that are triggered in sperm cells during acrosomal exocytosis. Special attention has been paid to the activation of phospholipases A2, C and D in sperm cells whereas far less is known about sphingomyelinase although its activity increases upon capacitation [29]. Each of the following subsections will deal with the regulation of the activity of one of the sperm phospholipases and the subsequently induced lipid signalling. In these subsections it will also be discussed which protein phosphorylation cascades are activated by lipid signalling and whether they are involved in signalling cross-talks.

2.4.1. Phospholipase A2

Phospholipase A2 (PLA_2) is the enzyme which is responsible for cleaving intact phosphoglycerolipids (e.g. PC) into free fatty acids (liberated from the sn-2 position of the glycerol backbone) and lysophospholipids (e.g. lysophosphatidylcholine, LPC). Lysophospholipids and fatty acids seem to serve as co-activators of PKC, but their major role appear to relate to perturbation of the membrane structure (see section 2.5 and Fig. 6). Lysophospholipids may also modulate channel function by altering the mechanical properties of lipid bilayers [97,98], which might be required for influx of extracellular Ca^{2+}. In addition to this, it should be noted that Flaherty and Swann reported that proteases are not involved in the LPC mediated acrosome reaction in guinea pig sperm cells [99]. Fatty acids like arachidonic acid and docosahexanoic acid can be converted into leukotrienes, prostaglandins (by cyclooxygenase or lipoxygenase [100]), whereas lysophosphatidalcholine can be acetylated into platelet activating factor (PAF: 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phoshocholine) which are bioactive molecules [101]. The generation of secondary bioactive fatty acid metabolites (and the presence of responsible enzymes) in capacitating sperm cells has so far received little attention. Schaefer et al. reported that the acrosome reaction can be induced by prostaglandin E [102]. The formation and capability of PAF to induce the acrosome reaction during sperm capacitation have been reported [103,104].

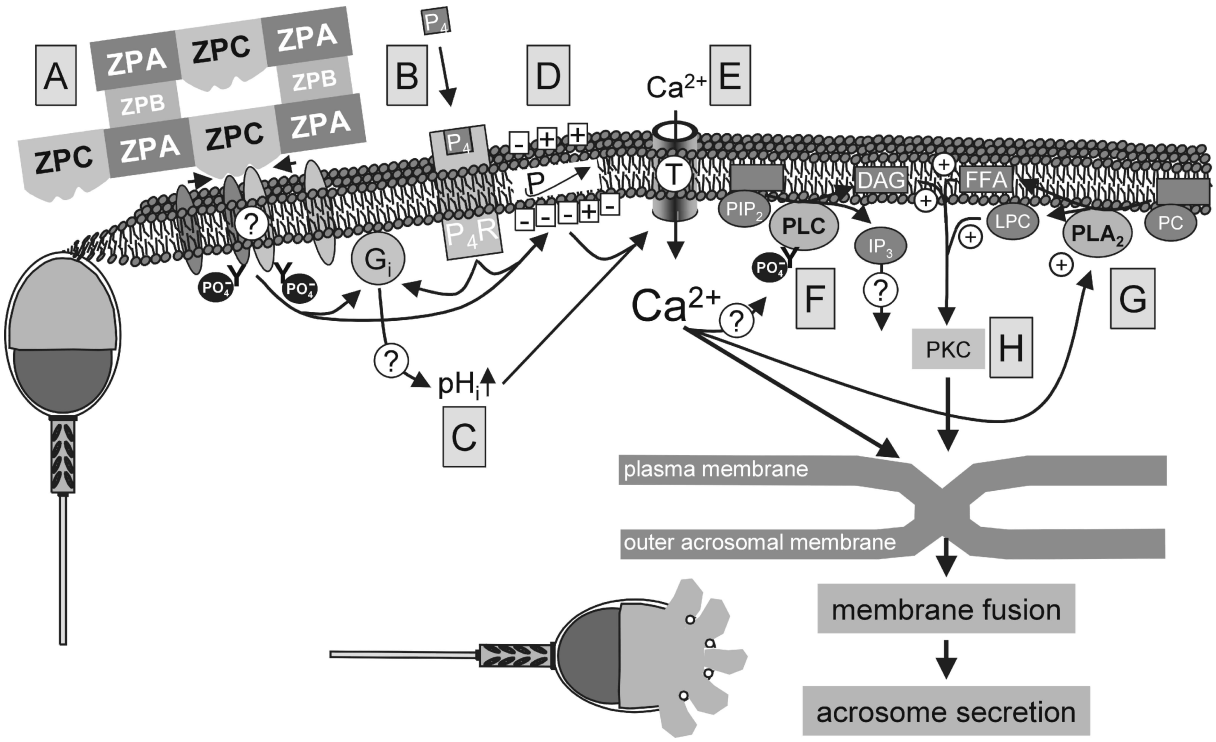

Figure 6. Proposed sequence of the zona pellucida and progesterone induced acrosome reaction. A: Zona pellucida (ZP) proteins (most likely ZPC) bind to sperm-ZP receptors, leading to aggregation and tyrosine (Y) phosphorylation. B: The direct environment of the ZP contains high levels of progesterone that can bind to its non-genomic receptor (P_4R) on the sperm surface. Both ZP and progesterone have a dual effect on sperm cells. C: The intracellular pH (pH_i) is increased via G-proteins (G_i) and D: the plasma membrane potential depolarizes. E: Both the increased pH_i and depolarization induce the entry of calcium via a T-type voltage dependent Ca^{2+} channel. F: The higher intracellular Ca^{2+} levels activate phospholipase C (PLC) that has been translocated to the plasma membrane during capacitation. PLC converts PIP_2 to diacylglycerol (DAG) and inositol- 1,4,5-trisphosphate (IP_3). G: Increased Ca^{2+} levels activate phospholipase A_2 (PLA_2), which degrades PC to LPC and free fatty acids (FFA). H: The role of IP_3 is unclear, but DAG, FFA and LPC activate protein kinase C (PKC). Both the increased intracellular Ca^{2+} levels and PKC activation are necessary for the fusion of the plasma membrane with the underlying acrosomal membrane, which leads to the subsequent secretion of acrosomal enzymes.

PLA_2 is activated in human [105] and boar spermatozoa [106] in response to progesterone. Enhanced activities were also induced by 15 mM bicarbonate [29]. Interestingly, reagents known to activate G-proteins (a transduction event involved in ZP-triggered acrosome reaction, Fig. 6) also induce PLA_2 [107]. PLA_2 is a large superfamily of enzymes (for review see [108]) divided into groups I to IX on bases of sequence information. In general the phospholipases A2 can be divided into secretory low molecular mass enzymes of 13-18 kDa, and cytosolic high molecular mass enzymes of 85 kDa forms and 29 kDa forms). All secretory enzymes require millimolar levels of Ca^{2+} for activation, whereas, some cytosolic forms do not require any Ca^{2+} and some require micromolar levels of this divalent cation. Information on which isoenzymes are present in sperm cells is far from complete. A 14-16 kDa sperm PLA_2 has been partially purified and characterized, and its N-terminal region revealed some similarities with members of the secretory group I and II PLA_2 (from snake venom and porcine/human pancreas), although the entire protein appears to represent a novel sequence [109]. Antibodies raised against cobra (Naja naja) venom

recognize a 16 kDa protein in SDS extracts from bull sperm cells [110], whereas, antibodies raised against porcine pancreas PLA_2 recognize a protein in hamster and human sperm cells [111]. Immunostainings with these antibodies revealed that PLA_2 was present in the acrosomal region as well as in other sperm compartments. No data is available on the ultrastructural localization of PLA_2 using immunoelectronmicroscopy on ultrathin sections. However, the observation that incubation of sperm cells with Fab fragments from the above mentioned antibodies blocked acrosomal exocytosis at least indicates that the PLA_2 is localized at the sperm surface and that its activity is involved in the acrosome reaction (Fig. 6) [112]. The larger cytosolic types of phospholipase have not yet been discovered in sperm cells. Homozygous male mice with a gene coding for mutated (non-active) 85 kDa type of PLA_2 showed normal fertility [113,114], which suggests that this cytosolic PLA_2 is not essential in sperm function including exocytosis. On the other hand bovine seminal plasma contains a 100 kDa isotype of secretory PLA_2 [115], although its relevance for sperm physiology has not been reported yet.

2.4.2. Phospholipase D

Phospholipase D (PLD) cleaves the polar head group from phospholipids leaving phosphatidic acid as lipid catabolite in the membrane. The formation of phosphatidic acid is only a minor pathway upon stimulation of sperm cells [116]. In fact the low amounts of phosphatidic acid were not a result of the induced PLD activity but rather a result of DAG kinase, an enzyme that phosphorylated DAG that was formed under capacitating conditions from phosphatidylinositol 4,5-bisphosphate (PIP_2) by the activation of phosphoinositidase C (PIC) [117,118]. The phosphorylation of DAG may be relevant for down regulating the PKC activation although phosphatidic acid has no effect on the time-course of exocytosis [119]. In conclusion, it is not likely that PLD is involved in lipid signalling in sperm cells.

2.4.3. Phospholipase C

Phospholipase C (PLC) cleaves DAG from the phosphorylated head group of phosphoglycerolipids. Both the role of formed DAG and phosphorylated head groups have been described extensively in the literature to serve as second lipid messengers in cell signalling. In contrast to PLA_2 the PLC isoforms are specific for the head groups of the phospholipids. Hydrolysis of PIP_2 and phosphatidylinositol 4-phosphate (PIP) is mediated by a specific PIC, whereas PC breakdown is mediated by a PC specific isoform of PLC (PC-PLC). Both PC-PLC and PIC activities have been identified in membrane preparations from bull sperm [120], but, although these enzymes seem to be localized in different membrane subfractions, no attempts have been made to localize these enzymes ultrastructurally. PC-PLC is activated in capacitating sperm cells [121] and is probably responsible for the bulk formation of DAG. PC-PLC appears to have no effect on PE and PI [122]. Another metabolic route to produce DAG is the degradation of phospholipids by concerted action of phospholipase D and phosphatidic acid phosphohydrolase, which was assumed to be active in sperm cells [123,124]. However, recent data has shown that this alternative route is quite unlikely in capacitating sperm cells (see section 2.4.2) [96]. On the other hand, sperm cells of a variety of mammalian species showed increased PIC activities upon incubations that induce the acrosome reaction, such as calcium ionophore

(A23187), progesterone or ZP proteins [116,125,126]. It is not well known which types of PIC are involved in this process (for review see [127]). There is some evidence that PIC-γ activation is modulated by tyrosine phosphorylation [128,129]. On the other hand there is no clear evidence for the involvement of a PIC-β that is activated by the pertussis toxin-insensitive G-protein [127]. The presence of other types of PICs has been suggested: sperm cells may have a PIC activated by a pertussis toxin-sensitive G-protein (G_o- or G_i-type), or a Ca^{2+}-dependent isoform, PIC-δ [127].

The DAG generated by either PC-PLC or PIC probably has a central role in downstream lipid signalling of the acrosome reaction [127]. The PC-PLC is like PIC stimulated by calcium ionophore (A23187), progesterone or ZP [121,126,130]. DAG is now known to activate sperm PKC [118] and PLA_2 [131] and also to have a positive effect on PC-specific PLC [121]. PKC is a serine/threonine kinase with various isoforms that have been classified into three groups: (i) the conventional type (cPKC: α, βI, βII and γ-isotypes), which are activated by Ca^{2+}, DAG, PS, free fatty acids and LPC [96], (ii) the second group of novel PKCs (nPKC: δ, ϵ, η, μ and θ isotypes), which are activated by DAG and PS but not by PLA_2 products, and are Ca^{2+}-independent, and (iii) the group of atypical PKC (aPKC: ζ, λ and I-isotypes), which are DAG and Ca^{2+}-independent but require PS and other activators such as free fatty acids [123]. Therefore, it should be noted that the scrambling of phospholipids (e.g. reorganization of PS [29]) also may affect the activation of PKC. Originally, no PKC activity was identified on ram sperm stimulated with calcium ionophore A23187 and phorbol esters (a classical way to induce PKC activity) [132]. However, phorbol esters have now been established to induce PKC activity in sperm cells of different mammalian species [133]. In fact, phorbol esters induced the acrosome reaction and PKC inhibitors were potent inhibitors of the ZP-induced acrosome reaction [134]. Recent work has shown that progesterone induces the activation of PKC and phosphorylation of various substrates, while both effects could be blocked by PKC inhibitors [118]. Several immunocytochemical studies have localized PKC in the sperm head and tail. Bull sperm have $PKC_{\beta I}$ throughout the acrosomal region [135], whereas human sperm cells have $PKC_{\beta II}$ in the equatorial region of the sperm head [136]. With the use of an antibody recognizing the PKC α,β,γ-isotypes, PKC was localized in equatorial region of the human sperm head and in the post-equatorial region of bull sperm [136,137]. In mice an unidentified PKC has been localized in the acrosomal region of the sperm head [129]. Treatment of bovine sperm with phorbol esters resulted in the Ca^{2+}-dependent translocation of PKC_{α} and $PKC_{\beta I}$ [135]. It has been suggested that PKC activation by phorbol esters and the stimulation of the acrosome reaction are Ca^{2+}-independent [136]. However, up till now it has been widely accepted that increased intracellular free Ca^{2+} levels are crucial for the acrosome reaction. Furthermore, the observations of Rottem et al. [136] do not correspond with the results of O'Toole et al. [118,138] who demonstrated that PKC mediated phosphorylation of protein substrates is inhibited by blocking Ca^{2+} entry with verapamil. Another observation that PKC activation controls Ca^{2+} fluxes over the plasma membrane [133] has been disputed by the findings of Bonaccorsi et al. [139] who demonstrated that the progesterone and the ZP-induced Ca^{2+} influx in the sperm cells are not affected by PKC inhibitors or phorbol esters. Roldan and Fraser [140] have demonstrated conclusively that PKC activation is down stream of Ca^{2+} influx.

None of the substrates of PKC have been identified so far although one putative substrate for PKC could be a 40 kDa mitogen-activated protein kinase (MAP-kinase [141]).

Besides its reputation as a signalling lipid, DAG may also perturb the plasma membrane structure and may facilitate membrane fusion directly (see section 2.5). The PIC mediated breakdown of PIP_2 not only results in the bioactive component DAG, but also in inositol 1,4,5-triphosphate (IP_3, Fig. 6). In somatic cells this phosphorylated head group can bind to specific IP_3 receptors on the ER and thereby mobilizes the intracellular Ca^{2+} pool (transport of Ca^{2+} ions from the ER lumen towards the cytosol). However, sperm cells do not contain ER and mobilizable intracellular Ca^{2+} pools have not yet been demonstrated. In addition, IP_3 has no effect on sperm activation or the acrosome reaction [96]. However, the generated IP_3 may have a function in intracellular Ca^{2+} elevation in oocytes [142]. In this context it is interesting to mention that sperm cells contain a protein called oscillin [143], which seems to bind to IP_3 receptors of the oocyte. By this coupling a very specific oscillation of Ca^{2+} transport and backward movements from the ER to the cytosol could be involved in oocyte activation [144]. Watson and colleagues have localized Ca^{2+} at the outer acrosomal region by electron microscopical techniques. It should be mentioned, however, that this Ca^{2+} is probably immobilized to proteins or crystalized as salt due to the acidic pH of this organelle [145].

2.4.4. Sphingomyelinase

Sphingomyelinase is involved in the generation of ceramide and phosphocholine from sphingomyelin. Its activity in sperm cells is only poorly characterized. Nevertheless, it was recently demonstrated that sphingomyelinase activities were up to 6 times enhanced by bicarbonate, whereas, PLA_2 and PLC were only moderate activated [29]. It is of interest to note that ceramide formation has been implied in the induction of apoptosis in several types of somatic cells [146]. In sperm cells classic apoptosis is impossible because gene expression and nuclear fragmentation are not possible on the completely compacted DNA in the sperm head. However, it is still possible that an apoptosis-like signalling cascade is involved in the induction of the acrosome reaction. In line with this is the discovery that the lipid asymmetry in the apical sperm head plasma membrane scrambles upon in vitro capacitation (see section 2.2.2). This process is induced by bicarbonate dependent activation of PKA [29].

2.4.5. PI3-kinases

A recent report suggested the presence of phosphoinositide 3 kinase (PI3-kinase) in sperm cells which converts PIP_2 into PIP_3. In analogy with somatic cell types [147,148] it was found that wortmannin (a PI3-kinase inhibitor) blocked exocytosis in sperm cells [129]. Although it is attractive to speculate that PI3-kinase plays an important role in the regulation of the acrosome reaction, information on the mechanism involved is still lacking.

2.5. Lipid metabolism and fusion

Besides their potential as second messenger molecules in cell signalling processes, phospholipid breakdown products are requisites for the promotion of non bilayer lipid membrane structures that occur during membrane fusions [149,150]. In line with this concept, ceramide, DAG and/or

phosphatidic acid formation in the inner leaflet of the sperm plasma membrane may be required for the acrosome reaction, because these phospholipid catabolites have only a small hydrophilic group combined with a wider hydrophobic group. As a consequence they may promote non-bilayer membrane structures surrounding and including the hemifusion micelle (an inverted micelle structure) at the spots where the fusions between the plasma membrane and the outer acrosomal membrane occur. On the other hand the formation of lysophospholipids and fatty acids promote the inward bending curvature of the outer leaflet of the sperm plasma membrane. In line with this is the observation made by Roldan and colleagues that LPC and LPI (outer leaflet lipids [30]) induce the acrosome reaction, whereas LPS (formed in the inner lipid leaflet of the sperm plasma membrane) prevents the acrosome reaction [103,151]. Similarly some fatty acids can enhance exocytosis, whereas others cannot [103]. The non-bilayer promoting catabolites are required to stabilize the hemifusion structure and to complete the fusions between the plasma membrane and the outer acrosomal membrane (for review see [152]). Veterinarian and human fertility clinics presently use an LPC test to observe the inducability of the acrosome reaction [153]. A similar scenario as described above for the acrosome reaction could be valid for the fusion event between the plasma membrane of the sperm head's equatorial region and the oolemma. However, the production of phospholipids catabolites should then be in the opposite lipid monolayer of the sperm plasma membrane for this intercellular fusion event. Now the outer leaflet of the plasma membrane from the adhered gamete will form hemifusion structures, whereas the inner leaflet will stabilize this structure and is involved in completion of this fusion. The sperm-specific glycolipid seminolipid localized exclusively in the outer leaflet of the sperm head plasma membrane [19] is also believed to play a role in the acrosome reaction and the fertilization fusion. In freshly ejaculated sperm cells the glycolipid is concentrated in sulfated form in the apical region probably preventing the acrosome reaction by stabilizing lamellar lipid bilayer of the plasma membrane [5] and by preventing Ca^{2+} influx over the plasma membrane (both requisites for the acrosome reaction [6,96,154]). However, after capacitation seminolipid migrates into the equatorial region of the plasma membrane of the sperm head (Fig. 4 and 5) and becomes partly desulfated by arylsulfatases [19,155]. The desulfoseminolipid has a shape of an inverted cone and, therefore, probably facilitates the formation of a hemifusion structure at the outer leaflet of the equatorial plasma membrane of the sperm head. It is well known that the fertilization fusion with the oolemma only occurs at this specific area of the site sperm surface. The cleavage of the sulfate moiety from seminolipid may also interfere with Ca^{2+} fluxes over the sperm plasma membrane [5]. Nevertheless, despite of all these considerations direct evidence for the involvement of lipid catabolites as non-bilayer lipids in the process of membrane fusion events remain to be presented.

2.6. Lipid peroxidation

Sperm cells are in contact with radicals on their journey to meet the oocyte. Mammalian sperm cells are sensitive for oxidative stress because of their high polyunsaturated fatty acid content (see section 2.2.3) and relative poor antioxidant defence [156,157]. In addition, these cells are constantly subjected to oxidative attack from the exoplasm where leukocytes secrete reactive oxygen species (ROS, [158]) but may also be capable to produce intracellular ROS [157,159].

The mechanism by which sperm cells would produce ROS is still unknown although two ROS generating systems have been proposed, sperm diaphorase [160] and NADPH oxidase [161]. These latter activities could well be attributed to contaminating neutrophils in semen [162]. Peroxidation is believed to regulate sperm function in two ways (i) mild peroxidation might benefit capacitation in vitro by redox dependent activating sperm specific PKA [163,164] and thereby also switching on tyrosine kinases [165] while superoxide anion induces sperm hypermotility and results in affinity for the ZP. (ii) Excessive peroxidation will result in sperm deterioration [166]. Although the detrimental effects of lipid peroxidation on sperm cells have been extensively described and reviewed [167], the beneficial effects of peroxidation of sperm capacitation are still poorly characterized and under present debate [167-170]. Peroxidative attack of tyrosine residues in proteins (formation of 3-nitrotyrosine, tyrosyl radicals and o,o'-dityrosine [171,172]) could have an effect on sperm tyrosine kinase induced signalling. However, the importance of peroxidation for effective fertilization remains to be investigated.

3. Proteins involved in sperm-oocyte interaction

Sperm cells are extremely specialized cells that are not able to synthesize proteins after spermatogenesis (lack of ribosomes and ER). Nevertheless, major post-translational modifications and relocations of sperm plasma membrane proteins (including the exchange of proteins from genital fluids and the sperm surface) take place prior to and during fertilization [6]. In this chapter the involvement of sperm plasma membrane proteins in mammalian fertilization will be reviewed.

3.1. Capacitation

Freshly ejaculated spermatozoa bind to the ZP only after activation in the female genital tract [6,173]. The activation of sperm cells (capacitation) is a widely investigated but very complicated subject. Obviously capacitation induces changes in the sperm plasma membrane resulting in its increased affinity for the ZP [6,174]. One of the complications in capacitation research is the difficulty to discriminate between induced changes at the plasma membrane and those in the remaining part of the sperm cell [175]. Furthermore, most research has been done on whole sperm cell populations although it has now been established that specific subpopulations of sperm cells react or do not react on the capacitation stimuli [46,175,176] or do deteriorate under these conditions [177,178]. Flow cytometry has proven to be a valuable tool to discriminate between different responses of sperm subpopulations to capacitation conditions [176]. Flow cytometric studies revealed that capacitated sperm cells should be kept at 38˚C during analysis, since cooling to only 30˚C already induces membrane deterioration in primed cells [29]. In this respect published data on the induction of the acrosome reaction under capacitating conditions should be interpreted with caution: they might be a result of post-capacitation temperature artifacts (e.g. in a flow cytometer which was not pre-equilibrated to physiological temperature in order to study sperm capacitation at 38.5˚C [29]).

3.1.1. Regulation of tyrosine phosphorylation

It has been shown for a number of mammalian species including man that a considerable number of proteins become tyrosine phosphorylated during in vitro capacitation [154]. It is not known how tyrosine phosphorylation is induced during in vivo capacitation (i.e. in the oviduct) because it is extremely difficult to evaluate oviductal influences on sperm cells in situ and at the proper period in the reproductive cycle [179]. However, in vitro capacitation protocols allow careful examination of the potential of various components in the capacitation media to induce protein tyrosine phosphorylation of sperm proteins. The main component is bicarbonate, and its omission not only inhibits tyrosine phosphorylation [180,181], but also diminishes the ability of sperm cells to bind to solubilized ZP proteins [174], and inhibits hypermotility induction of sperm cells [182]. A second component is albumin, and its role on cholesterol extraction is detailed in section 2.3.1. However, the role of albumin in bicarbonate induced tyrosine phosphorylation is debated [175,180,183,184]. A third capacitation component is the ion Ca^{2+}, although its direct role on protein tyrosine phosphorylation is difficult to examine (various processes need Ca^{2+}) and differs between various mammalian species [183,185]. In conclusion, bicarbonate seems to be the key player in triggering tyrosine phosphorylation of proteins in capacitating mammalian sperm.

The bicarbonate concentration is very low (<1 mM) at the site of sperm storage in the cauda epididymidis and sperm cells are confronted with much higher bicarbonate level (>15 mM) in the female genital tract indicating a possible in vivo role for bicarbonate [186]. Intracellular bicarbonate levels can be raised by bicarbonate entering the sperm cells via ion channels in the plasma membrane (Cl^-/HCO_3^- exchanger [26,187], Na^+/HCO_3^- exchanger [188]). An alternative explanation for the entrance of bicarbonate into the sperm cell is that CO_2/HCO_3^- levels are in equilibrium in the intracellular and extracellular compartments by gas diffusion through the sperm plasma membrane [186]. Carbonic anhydrase, which is present in the sperm head, could be involved in maintenance of high intracellular bicarbonate concentration by conversion of diffused CO_2 [189]. So how can the increased level of intracellular bicarbonate induce protein tyrosine phosphorylation? Most likely, bicarbonate is able to bind directly to a sperm specific adenylate cyclase and thereby activates the enzyme to produce increased levels of cAMP [190-192]. Increased cAMP levels activate cAMP dependent protein kinases (PKA), and the activated PKA induces protein tyrosine phosphorylation via a yet to be discovered sperm specific cross-talk signalling pathway: (i) mouse sperm capacitated with PKA specific inhibitors also reduced tyrosine phosphorylation whereas (ii) stimulation of PKA by active cAMP analogues [193] or by phosphodiesterase inhibitors induced tyrosine phosphorylation [194]. This may explain the supportive effect in the induction of sperm capacitation by including caffeine, a routine component in IVF media [6]. In vitro studies have shown signalling cross-talk between PKA and tyrosine phosphatases in somatic cells [195,196]. Inhibition of protein phosphatases that are specific for phosphoserine and phosphothreonine residues (PP1 and PP2A), resulted in increased numbers of capacitated sperm cells [197]. However, inhibitors for phosphotyrosine specific protein phosphatases have not been tested. Vanadate, known to inhibit tyrosine phosphatases as well as some other ATP dependent enzymes, has been shown to significantly inhibit the plateau phase of progesterone induced Ca^{2+} entry [198] (see also section 3.1.2), which may indicate that tyrosine phosphatases are involved in capacitation.

Tyrosine phosphorylation of sperm proteins is linked with increased ZP affinity [199], acrosome reaction [200] and hypermotility [201]. Only a few studies have been performed to investigate the tyrosine phosphorylation status of sperm plasma membrane proteins under capacitation conditions. Capacitation of boar sperm cells induced, in a bicarbonate dependent fashion, tyrosine phosphorylation of a plasma membrane specific set of proteins [175]. Interestingly our results indicate that two specific proteins from the apical sperm plasma membrane that became tyrosine phosphorylated upon capacitation in vitro showed high affinity for native zona pellucida material [202].

3.1.2. Ion channels

The initiation of the acrosome reaction is, like other membrane fusion events, dependent on a massive increase in intracellular Ca^{2+} levels of sperm cells [203,204]. Extracellular Ca^{2+} is required for sperm capacitation and the ability to undergo induced acrosome reaction [180,205]. Low levels of extracellular Ca^{2+} (90 μM) in combination with calcium ionophore induce capacitation but not the acrosome reaction in mouse sperm, whereas, higher levels of extracellular Ca^{2+} (1.8 mM) are appropriate to induce the acrosome reaction [206]. Addition of a calcium chelating agent (required to study the Ca^{2+}-independent sperm capacitation) in combination with Ca^{2+} imaging techniques [176,207,208], radioactive Ca^{2+} [209], and ion-selective microelectrodes [210] have shown the increase in intracellular Ca^{2+} concentration in sperm cells during in vitro capacitation.

It is not clear how extracellular Ca^{2+} passes the plasma membrane during sperm capacitation. The sperm plasma membrane contains a variety of Ca^{2+} channels: voltage dependent Ca^{2+} channel [211] (see section 3.3.4), Ca^{2+}-ATPase [212], Na^{+}/Ca^{2+} exchanger [213] and probably others. Besides these Ca^{2+} channels, the presence of IP_3-gated Ca^{2+} channels on acrosomes have been reported [214] as well as the presence of a Ca^{2+}-ATPase in the acrosome [215], suggesting that the acrosome could also serve as an intracellular sink for Ca^{2+}. However, acrosomal Ca^{2+} most likely is immobilized to proteins or as salt crystals due to the acidic pH of the acrosome [216]. It should be noted that sperm cells lack the ER as a reservoir of immobilizable Ca^{2+}, which is supported by the observation that IP_3 did not affect cytosolic Ca^{2+} levels in sperm cells (see section 2.4.3). Furthermore, mitochondria are not localized in the sperm head and most likely do not influence the Ca^{2+} levels at the acrosomal region of the sperm head. It has been postulated that free Ca^{2+} levels in the cytoplasm are kept low in freshly ejaculated sperm cells by calmodulin-sensitive Ca^{2+}-ATPase that inhibits capacitation. Inhibition of calmodulin or calmodulin-sensitive Ca^{2+}-ATPase leads to increased intracellular Ca^{2+} levels, which was reflected in a higher proportion of capacitated cells [209,217,218]. These findings suggest that the continuous removal of cytoplasmic Ca^{2+} by Ca^{2+}-ATPase is involved in the prevention of premature capacitation of sperm cells. Furthermore, inhibition of a Na^{+}/Ca^{2+} exchanger by a 10 kDa seminal plasma peptide (caltrin) prevents Ca^{2+} uptake by freshly ejaculated bovine sperm cells [213,219]. Probably bicarbonate is involved in elevation of the intracellular Ca^{2+} levels since anion channel blocker SITS reduced Ca^{2+} uptake [210]. PKC and voltage dependent Ca^{2+} channels also seem to be involved in increased Ca^{2+} uptake [208]. In this respect, depolarization of the plasma membrane potential indeed is prerequisite for the ZP-

induced acrosome reaction [220,221]. Na^+/K^+-ATPase could play a role in capacitation induced depolarization, since incubation of sperm cells in capacitation medium with low levels of sodium (less than 25 mM) inhibits capacitation [222]. This phenomenon could be explained by a requirement of intracellular sodium to activate Na^+/Ca^{2+} exchangers and thus to increase intracellular Ca^{2+} concentrations.

3.1.3. Glycocalyx

The glycocalyx could play a major role in intercellular gamete communication because it forms the extracellular coating of the sperm's surface. Besides this, the lateral polarized nature of the glycocalyx could be relevant for the lateral organization of plasma membrane molecules since the carbohydrate network is in direct contact with the sperm plasma membrane via integral membrane proteins and glycolipids (Fig. 3B).

The glycocalyx alters during capacitation as could be shown by lectin binding studies (for review see [6]). It is not known whether the changes in lectin binding reflect chemical modification of carbohydrate structures, (un-)covering of carbohydrate structures, or repositioning of glycoproteins or glycolipids. The repositioning of the glycolipids during capacitation has been shown (see section 2.2.1 and Fig. 3B) as well as the release of 'decapacitation factors' during capacitation [223,224] and other factors bound to the sperm plasma membrane [225]. It has been postulated that removal of decapacitation factors induces tyrosine kinase activity in transmembrane proteins (Fig. 7) [6]. Interaction of these transmembrane proteins with the ZP leads to aggregation of the transmembrane proteins and subsequently further increases the

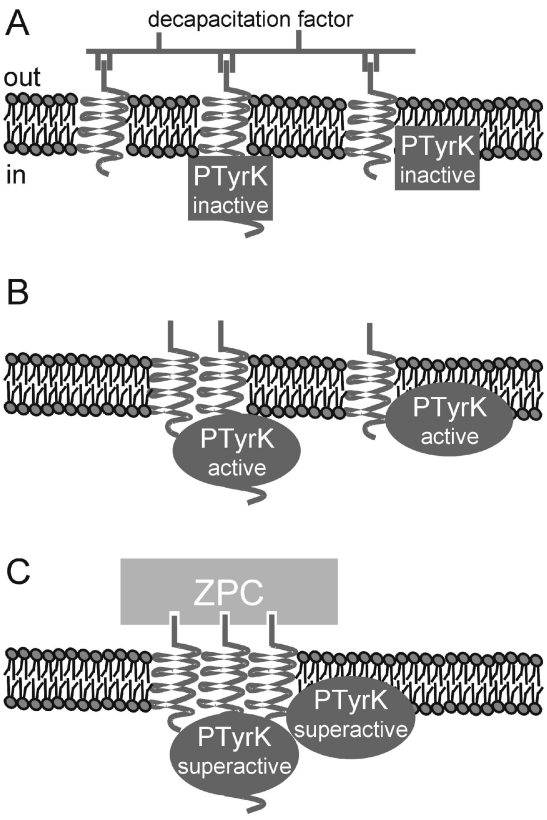

Figure 7. Model for regulation of tyrosine kinase activity by capacitation and zona binding.
A: Decapacitation factors are components of the extracellular matrix and interact with transmembrane proteins. The decapacitation-transmembrane protein interactions prevent tyrosine kinase activity. Tyrosine kinase domains may be in and/or coupled to the transmembrane proteins.
B: Upon capacitation, the decapacitation factor is removed which activates tyrosine kinases and may enable the sperm cell to bind to the ZP.
C: ZP binding to sperm ZP receptors (transmembrane tyrosine kinases) induces aggregation and further increases tyrosine kinase activity. (Adapted from [6]).

tyrosine activities. Related to the removal of surface coating components is the proposed uncovering of progesterone receptors on the sperm plasma membrane (see also section 3.3.5). For instance in dogs it has been demonstrated that >90% of the cauda epididymal sperm cells have affinity for progesterone, whereas, freshly ejaculated sperm do not have such affinity due to a coating factor that is secreted from the prostate [226]. However, subpopulations of sperm cells regained the affinity for progesterone when this factor is released during in vitro capacitation or by density gradient washing. The sperm cells that expose a functional progesterone receptor are the specific cells that initiate the acrosome reaction after a progesterone challenge [226-228] (see also section 3.3.5).

3.2. Zona pellucida binding

Sperm binding to the ZP is important in fertilization since it is considered to be a species specific recognition of the two gametes (in contrast to sperm interactions with the oolemma see section 4.1 and 4.2). Sperm binding to the ZP also evokes the acrosome reaction, which is required for successful penetration of the zona pellucida (and thus for fertilization). Despite the importance of sperm binding to the ZP, the proteins from the sperm plasma membrane involved in ZP recognition and adhesion have not been completely elucidated yet.

3.2.1. Zona pellucida

Nomenclature for zona proteins is inconsistent between the different mammalian species. Therefore, we have followed the protein nomenclature as proposed by Harris [229], who divided the proteins of the zona pellucida of a number of mammals into ZPA, ZPB and ZPC based on sequence variations and homologies.

Besides the ZP amino acid sequences of several species, also information about the ZP carbohydrate structure has been revealed [230,231]. The importance of N-linked and/or O-linked glycosidic residues on the ZP proteins in the primary sperm binding is still a subject of debate. Enzymatic removal or partly digestion of both O- and N-linked carbohydrates have been reported to abolish sperm-ZP binding [232-234]. ZPC seems to be the most important candidate to function as primary sperm receptor but also as inducer of the acrosome reaction [231,235]. However, indirect zona affinity experiments indicate that anti-ZPA could inhibit sperm-ZP binding, whereas, anti-ZPC did not inhibit the sperm-ZP binding in human sperm [236]. In porcine ZPB and ZPC seems to be involved in sperm binding to the zona pellucida [237-239]. Finally, ZPB has been shown to be the primary sperm receptor in the rabbit system [240,241]. Most likely, the complete native texture of the zona pellucida is required for optimal sperm binding [202].

3.2.2. Primary sperm-ZP binding

Recognition of the zona pellucida by the sperm cell can be subdivided in two phases: (i) the primary binding in which acrosome intact sperm cells bind with proteins that are situated only at the apical plasma membrane of the sperm head [6] (intra-acrosomal proteins are not exposed in acrosome intact cells, and proteins localized at other sites of the sperm surface will block zona penetration); (ii) the secondary binding in which the acrosome reacted sperm cell exposes a set

of intra-acrosomal proteins with high affinity for the zona pellucida (see section 3.2.3). Most likely, the putative primary ZP receptors are modified upon sperm capacitation since binding affinity of the sperm cell for the ZP increases dramatically upon capacitation [6,174]. The increased affinity of the sperm cell for the oocyte could also be a result of capacitation induced conformational changes of ZP receptors (including dimerization of receptors) or by decoating the ZP receptors. Sperm-ZP binding results almost instantaneously in the acrosome reaction [242]. The cellular signalling cascades involved in this process are not completely resolved. It seems plausible that an integral plasma membrane protein is involved with at its extracellular domain a ZP binding site and at its cytosolic domain a functional site for activation of intracellular signal transduction [243]. More than one entity may be involved in primary sperm-ZP binding as is based on results from knock-out mice lacking β1,4-galactosyltransferase [244] (see below).

The research on primary sperm-ZP binding has followed several strategies. One of them is coupling of solubilized sperm proteins or ZP proteins to a solid phase. The solid phase can be column material like sepharose, polystyrene, Western blot paper or in the case of ZP, intact ZP [245]. Proteins with affinity for the solid phase proteins can easily be separated from proteins with no affinity. A drawback from this method is that proteins have to be solubilized, which affects the quaternary structure of the zona proteins and may even lead to protein denaturation. Both effects can reduce the bioactivity (sperm binding) dramatically. Therefore, the method of choice would be to investigate zona binding with native zona material [245]. In extension primary zona binding should be investigated with isolated apical plasma membrane material as has been done with control and capacitation induced boar sperm material [202].

A second strategy is the inhibition of sperm-ZP binding by either solubilized sperm proteins or antibodies directed against sperm proteins [246-248]. A clear drawback of this method is that it does not provide direct evidence for the involvement of the protein under study in zona binding. Alternative phenomena such as sterical hindrance of the antibody or artifactual immuno-aggregation (antibody capping) may explain the decreased affinity for the ZP. The inhibition of sperm-ZP binding by solubilized sperm proteins is an elegant method to show the affinity of the sperm protein for the ZP [249]. Solubilized sperm proteins are sometimes used to show the inhibitory effect on IVF. In such studies it is important to remind that inhibitory effects may be at a different level of fertilization than primary zona binding (the tested protein could play a role in secondary binding [250], acrosome reaction, oolemma binding or fusion). These arguments are also valid for in vivo immunization experiments [246], since antigens can interfere at different stages of fertilization.

Although several proteins have been postulated as sperm ZP receptors (for review see [251]), only two important primary ZP receptor candidates are discussed here. Both proteins bind to ZP and are localized at the apical plasma membrane and were first described in mice. (i) A 95 kDa protein reported in several mammalian species [252-258]. The protein is phosphorylated on a tyrosine residue [180,255] and the degree of tyrosine phosphorylation probably increases during in vitro capacitation [253,258,259], although some contradictory findings have been published [180,193]. Binding of ZPC to sperm cells induces (additional) tyrosine phosphorylation [252,255,260] and the p95 protein is also assumed to be involved in acrosome reaction [261]. Sperm specific hexokinase is a tyrosine phosphorylated integral plasma membrane protein that

migrates on reducing gels as a 116 kDa protein and on non-reducing gels as a 95 kDa protein [262]. The possibility that p95 is the sperm specific hexokinase has been postulated [263], although this possibility has been debated [264]. (ii) The enzyme β1,4-galactosyltransferase has been proposed as (a part of) a ZP receptor in the sperm plasma membrane of a variety of mammalian species [244]. At least in mouse sperm cells β1,4-galactosyltransferase binds to ZPC [265] and subsequently the acrosome reaction is evoked by this binding [266]. Sperm cells from β1,4-galactosyltransferase deficient mice are still able to fertilize oocytes [267], although these sperm cells are less able to bind to ZPC and only insufficiently undergo the ZPC-primed acrosome reaction (see 3.3) compared to wild type sperm cells [267]. The general concept that sperm β1,4-galactosyltransferase binds to ZPC which provokes the acrosome reaction and is subsequently followed by sperm zona penetration is probably oversimplified. Most likely other molecules (sperm as well as ZP proteins) are also involved in the primary sperm-ZP interaction [243].

Small sperm-associated proteins like pig spermadhesins probably contribute to a firmer binding of sperm cells to the ZP [225], although the direct involvement in ZP adhesion of these proteins to the sperm cell at the site of fertilization has not yet been confirmed. In fact it has been shown that these tightly bound components are released from the sperm surface during sperm interactions with oviductal epithelia [268]. A more likely scenario is that spermadhesins are involved in suppressing antigenic activity against sperm cells [269] or are just required for interacting with the epithelial cells of the oviduct [270]. From studies in oviductal and primary cell cultures it appeared that sperm cells bind to the oviduct for a period of time. The released cells were capacitated and had high affinity for the ZP [85-87].

3.2.3. Secondary sperm-ZP binding

Intra-acrosomal proteins become exposed after the acrosome reaction and are involved in a more firm attachment of the sperm cell to the zona pellucida (secondary ZP binding) [271]. ZPA seems to be the secondary binding partner in the zona pellucida [272]. Several proteins have been identified as secondary ZP binding proteins: PH-20 [273,274], sp38 [275-277], acrosin [278], P-selectin [279,280]. Acrosin and PH-20 have both besides mentioned ZP affinity also proteinase [281] and hyaluronidase activities [89] respectively. Binding of these secondary ZP binding proteins may prevent the release of hyperactivated sperm cells from the ZP. Meanwhile their enzymatic activities are used to digest the ZP matrix followed by binding to a new substrate, ultimately enabling the sperm cell to pass the ZP [282].

3.3. Acrosome reaction

The acrosome reaction is initiated immediately after primary binding of a sperm cell to the zona pellucida of the oocyte [298]. The apical plasma membrane of the sperm head starts to fuse with the underlying outer acrosomal membrane at multiple sites resulting in the dispersal of the acrosomal content (Fig. 1 and 2) [299]. During the acrosome reaction hydrolytic and proteolytic enzymes are secreted in order to release, hydrolyse, and dissolve the ZP matrix locally in the immediate direction of the penetrating sperm cell which ultimately ensures the entrance of that sperm cell into the perivitelline space [300]. The acrosome reaction should not proceed prior to

ZP binding because the part of the enzymatic machinery of this organelle required for successful zona penetration will be lost. Preliminary acrosome reacted sperm cells are considered to be incompetent to fertilize the oocyte. Sperm cells that reach the perivitelline space are always acrosome reacted and able to fuse with the oocyte [301,302]. Importantly, the equatorial plasma membrane area of the sperm head has not participated in the fusions with the acrosomal outer membrane and seems to be the specific site involved in oolemma interaction (see chapter 4).

The acrosome reaction has been widely researched and a list of inducers is presented in Table 1. In some reports the acrosome reaction is considered as an indicator of sperm capacitation. Indeed capacitated sperm cells have a destabilised plasma membrane (see section 2.2) and therefore are sensitive to even small environmental stress when compared to non-

Table 1. The acrosome reaction can be induced in vitro by several agents. The mechanism of action of these agents is very divers, ranging from the physiological relevant activation of sperm ZP receptors by solubilized ZP proteins, to perturbation of membranes by ethanol.

inducer	**possible mechanism**	**reference**
protein		
solubilized ZP	receptor activation (physiological)	[283]
progesterone	receptor activation? (physiological?)	[284]
glycoconjugate	mimic ZP activation?	[285]
glycosamineglycan-sulfate	mimic ZP activation?	[286]
angiotensin II	receptor mediated?	[287]
atrial natriuretic peptide	receptor mediated?	[288]
trypsin inhibitor	?	[289]
sialic acid binding protein	?	[290]
lipid		
arachidonic acid	membrane perturbation? receptor mediated?	[291]
platelet activating factor	membrane perturbation? receptor mediated?	[292]
lysophospholipids	membrane perturbation? receptor mediated?	[153]
fluid		
follicular fluid	multiple? activators including progesterone	[293]
chemical agents		
calcium ionophore	introduction of Ca^{2+} into the cell	[294]
cAMP analogue	capacitation effect?	[295]
cGMP analogue	capacitation effect?	[296]
other		
ethanol	membrane perturbation	[294]
low temperature	‘cold shock’ membrane perturbation	[295]
electropermeabilization	introduction of Ca^{2+} into the cell	[297]

capacitated sperm plasma membranes [29]. Therefore, addition of Ca^{2+} in combination with calcium ionophore effectively induces fusion of the plasma membrane with the underlying acrosome membrane, whereas, non-capacitated sperm cells with much more rigid plasma membranes do not induce these membrane fusions under the same conditions [303]. The destabilized plasma membranes of capacitated sperm cells also make them more vulnerable to even slight temperature changes (slow cooling from 38.5˚C to 30˚C induces spontaneous acrosome reactions in capacitated but not in control sperm cells) [29]. However, when capacitated sperm cells are examined at physiological conditions they fail to induce the acrosome reaction [29]. It has been shown before that dramatic differences exist between spontaneous acrosome reaction (detrimental), calcium ionophore induced (by-passing physiological induced Ca^{2+} influx required for membrane fusion) and the physiologically induced acrosome reaction (by the zona pellucida) [199,304,305]. Nevertheless, useful clinical assays to predict the fertilizing potential of semen have been developed in which the calcium ionophore challenge is used to detect the relative amount of induced acrosome reaction indirectly indicating to the relative amount of capacitated sperm cells [306].

3.3.1. Receptor aggregation

Soluble ZPC induces the acrosome reaction in sperm cells of several mammalian species [283]. The acrosome reaction is probably induced by ZP receptor aggregation which is mediated by primary ZP binding (Fig. 6 and 7). In vitro experiments revealed that p95 and β1,4-galactosyltransferase may aggregate after ZP binding: ZP peptides bind to p95 and subsequently addition of anti-ZP antibodies results in aggregation [307]. Anti-β1,4-galactosyltransferase Fab fragments and subsequently addition of antibodies against these Fab fragments resulted in aggregation of β1,4-galactosyltransferase [308]. Although these experiments were performed in vitro in both cases the ZP binding event did not induce the acrosome reaction, whereas, the antibody induced aggregation coincided with the induction of the acrosome reaction. Aggregation of proteinase inhibitor bound to the mouse sperm surface, in the absence of zona glycoproteins [309], as well as, immuno-aggregation of seminolipid [32] also coincided with the acrosome reaction. Therefore, clustering of proteins or lipids by immuno-aggregation in vitro already coincides with acrosome reaction. Most likely, the zona pellucida induces the acrosome reaction in a comparable manner.

Aggregation of receptors is a common theme in signal transduction. Growth hormone for instance binds to two growth hormone receptors, inducing dimerization of these receptors. Dimerization results in activation of an associated tyrosine kinase, resulting in tyrosine phosphorylation of the receptor as well as the tyrosine kinase. Subsequently, a variety of signalling molecules are recruited and/or activated [310]. G-proteins have also been suggested to be implicated in receptor dimerization induced signal transduction [311]. Several models exist for signalling pathways including the involvement of G-protein receptors which could play an important role in ZP-induced acrosome reaction [312,313]. However, more research is needed to unravel the ZP-induced aggregation of sperm ZP receptors and the subsequent signal transduction.

3.3.2. Protein phosphorylation

Protein tyrosine phosphorylation is not only involved in capacitation (see section 3.1.2) but also in the acrosome reaction: mouse sperm p95 tyrosine phosphorylation is enhanced after ZPC binding (Fig. 6) [252], which is due to autophosphorylation induced by ZPC mediated p95 aggregation. Tyrosine kinase inhibitors blocked the ZP-induced tyrosine phosphorylation and the ZP-induced acrosome reaction [261]. Coupling of ZPC to p95 also stimulates tyrosine phosphorylation of PLCγ in capacitated mouse sperm cells (for PLC signalling see section 2.4.3) and this effect is abolished in the presence of tyrosine kinase inhibitors [128]. Sperm capacitation resulted in the translocation of PLCγ from the cytosol to the membrane which is believed to be a result of tyrosine phosphorylation [128]. Therefore, a biphasic activation of tyrosine kinase has been suggested (see Fig. 7). The ZP-induced acrosome reaction can be blocked by tyrosine kinase inhibitors but these inhibitors did not affect motility nor the ionophore induced acrosome reaction, indicating that capacitation was not affected [199]. The induction of Ca^{2+} entry into sperm cells challenged with solubilized ZP proteins is blocked by tyrosine kinase inhibitors [203]. Furthermore, Brewis et al. [255] showed that recombinant human ZPC-induced AR in capacitated human sperm cells that coincided with an increased tyrosine phosphorylation state of a 95 kDa protein. Progesterone, which is also able to induce the acrosome reaction (see section 3.3.5), induces a Ca^{2+} influx in human sperm cells. Tyrosine kinase inhibition reduces the plateau phase of the progesterone induced Ca^{2+} increase [198].

Other protein kinases may also be involved in the induction of the acrosome reaction. Staurosporine, an inhibitor of PKC, blocked ZP-induced acrosome reaction in sperm cells of human and various other mammalian species but did not affect motility [134,137,314-317] (Fig. 6). The activation of various types of PKC is detailed in section 2.4.3. Platelet activating factor induced acrosome reaction is affected by inhibitors for PKC, PTK, and PKA in human sperm cells [316], possibly because signalling cross-talk is involved in the activation of the three protein kinases. In fact cross-talk of PKA and PKC has been proposed to induce the human acrosome reaction [315].

3.3.3. G-proteins

Guanine nucleotide binding proteins (G-proteins) are involved in signal transduction in virtually all mammalian cells [318] and most likely are involved in the induction of the acrosome reaction. Mammalian sperm cells possess a subset of G-proteins distributed over different regions including the acrosome and the equatorial segment [319-321]. The ZP-induced acrosome reaction can be blocked by pertussis toxin (a G_i-proteins inhibitor) without affecting ZP binding itself [312,313,322-324]. G_i-proteins are directly activated by ZPC [312,325-327]. In fact, the non-physiological stimulation of G-proteins with GTPγS, GppNHp, mastoparan and AlF_4^- also induced the acrosome reaction [328]. The ZP-induced aggregation of β1,4-galactosyltransferase (see section 3.2.3) has been suggested to activate G-proteins in mouse sperm cells [329]. The activation of G-proteins by ZP binding seems to increase adenylate cyclase activity [330]. Furthermore, ZPC binding induces alkalinization via a G_i-protein and activates a poorly selective cation channel (pertussis toxin-insensitive) [331]. The concerted effects of depolarization and alkalinization appear to open T-type voltage-sensitive Ca^{2+} channels (Fig. 6) [331].

3.3.4. Ion channels

The acrosome reaction is a membrane fusion event requiring rather high (mM-range) cytosolic Ca^{2+} levels. In capacitated sperm cells the intracellular Ca^{2+} concentration is considerably lower (μM-range) and, as is mentioned before (section 3.1.2.), sperm cells do not possess an intracellular pool of mobilizable Ca^{2+}. Therefore, extracellular Ca^{2+} has to pass the plasma membrane prior to the initiation of the acrosome reaction. Significant influx of Ca^{2+} into sperm cells is accomplished by binding to the ZP [203,204]. Low voltage-activated Ca^{2+} channels are fully active after ZP interaction leading to Ca^{2+} influx necessary for the acrosome reaction [221]. ZPC binding to sperm cells induces a membrane depolarization, from approximately -60 mV to between -25 and -20 mV, via a poorly selective cation channel, and a G_i-protein dependent alkalinization which synergistically results in the opening of voltage dependent Ca^{2+} channels (Fig. 6) [331]. However, G_i-dependent opening of voltage dependent Ca^{2+} channels in human sperm cells seems to be independent of alkalinization [332]. The opening of T-type voltage dependent Ca^{2+} channels is believed to be responsible for the Ca^{2+} influx required for the acrosome reaction. Other types of voltage dependent Ca^{2+} channels (subunits) are present in the sperm cell at specific locations [333], however, a function for non-T-type voltage dependent Ca^{2+}-channels in the induction of the acrosome reaction is not evidenced (for review see [334]). Progesterone has been shown to induce the acrosome reaction in several mammalian species, via an increase in intracellular Ca^{2+}. Possibly progesterone directly activates Ca^{2+} channels [296] or indirectly via voltage dependent Ca^{2+} channels (see section 3.3.5).

Sodium channels may also be involved in the induction of the acrosome reaction. The sodium channel inhibitor amiloride inhibited the acrosome reaction, whereas ionophore monensin enhanced the acrosome reaction [335]. A role for Na^+/Ca^{2+} exchange was postulated, although others claim a role for Ca^{2+}-dependent potassium channels in the hamster acrosome reaction [336].

3.3.5. Progesterone

Sperm cells like other mammalian cells [337,338] possess progesterone receptors at the plasma membrane (non-genomic progesterone receptor) [226,228,339]. These receptors differ considerably from the genomic progesterone receptor located in the cytosol of somatic cells [340]. The sperm receptor has a rather low affinity for progesterone when compared to the genomic progesterone receptor in the cytosol of somatic cells (μM and pM respectively [228,341]). However, follicular fluid contains an enormous amount of progesterone (~6 μg/ml in follicular fluid vs ~10 ng/ml in serum [342]) and this fluid is released at ovulation (the zona pellucida is impregnated with high levels of progesterone as well). The calculated physiological doses of 0.3 μg/ml progesterone will surround the sperm cell in the oviduct just after the ovulation (i.e. at the fertilization period). These doses of progesterone induce the acrosome reaction in several mammalian species: [284,343] by rising the intracellular Ca^{2+} levels [344]. The signal transduction involved in the progesterone induced Ca^{2+} influx is uncertain (Fig. 6). Binding of progesterone to and stimulation of a γ-aminobutyric acid (GABA) receptor/Cl^- channel has been reported in brain [345]. The idea that progesterone acts via a $GABA_A$-receptor/Cl^- channel in sperm cells was strengthened by the finding that GABA binds to human sperm cells and that

progesterone stimulation induced a chloride efflux [227], whereas the effect was blocked by $GABA_A$-receptor/Cl^- channel antagonists [346]. Antagonists also inhibited the progesterone induced acrosome reaction [126]. Progesterone and ZP seem to induce the acrosome reaction in a synergistic and comparable way [126]. Progesterone primed calcium influx that was not enough to induce the human acrosome reaction required the addition of ZP [347]. Activation of the sperm $GABA_A$ receptor by progesterone or GABA resulted in elevated activity of PLC resulting in increased sperm diacylglycerol production [348] and induction of chloride efflux from sperm cells [349,350]. DAG probably induces Ca^{2+} release in sperm cells [351] and the chloride efflux is likely involved in membrane depolarization and subsequent Ca^{2+} influx through voltage dependent Ca^{2+} channels [332,352]. For more details on the regulations and signalling of PLC and DAG see section 2.4.3.

Progesterone mediated induction of the acrosome reaction can be blocked by tyrosine kinase inhibitors, whereas, progesterone itself stimulates protein tyrosine phosphorylation [198,227,353]. This suggests that progesterone mediated signalling is transduced via protein tyrosine phosphorylation. The protein tyrosine kinase substrates that become phosphorylated after progesterone remain to be investigated in order to get a better link between progesterone binding and the subsequent acrosome reaction. It is important to remind here, that tyrosine kinase inhibitors not only block progesterone effects, but also sperm capacitation, which physiologically has been completed before sperm cells are sensitized for progesterone [353] (see section 3.1.1). This is also demonstrated by the finding that progesterone induced acrosome reaction is inhibited by specific blocking of capacitation induced tyrosine phosphorylation of two kinases (ERK-1 and ERK-2) [354]. Similar arguments can be raised against the postulated role for A kinase anchoring proteins (APAKs) and PKA in the progesterone induced signal transduction pathway (inhibitors for anchoring and direct PKA inhibitors block capacitation as well as progesterone induced acrosome reaction [355]).

Progesterone exhibits two distinct binding sites on the human sperm membrane [356]. Isolation and identification of the plasma membrane receptor(s) for progesterone remain to be performed and probably is one of the major goals in further detailing signalling events leading to the acrosome reaction. It may prelude the discovery of a new family of hormone receptors oriented at the plasma membrane, with low affinity for steroids eliciting rapid non-genomic physiological changes.

4. The final goal: binding and fusion with the oolemma

By the time the sperm reaches the egg plasma membrane (oolemma) it has passed the processes described in section 2 and 3. Briefly, the sperm cell was capacitated in the female genital tract, it bound to the ZP and after the acrosome reaction it has successfully penetrated the ZP and reached the perivitelline space, where it now will meet the oolemma (Fig. 2). Therefore, the sperm cell is dramatically altered from its original ejaculated physiological state upon oolemma binding. It is not clear whether the ZP-induced alterations are required to achieve fertilization, since zona denuded oocytes can be fertilized by capacitated acrosome-intact sperm cells [357]. However, it should be noted that such an approach easily leads to polyspermy and that such sperm-oolemma fusions can be achieved with sperm from different mammalian species with

oocytes from heterologous mammals [357]. This is illustrated by the fact that nude hamster oocytes are used to test the fusion competence of human sperm cells in the IVF clinic [358]. Sperm binding to the oolemma is a multistep process which has been most extensively studied in rodents [359,360].

The sperm first seems to approach the oolemma by binding with its apical tip of the sperm head to the oolemma (Fig. 8) [361,362]. In golden hamsters, it was shown that this preliminary binding of sperm to the oolemma was maintained for an extended period of time in presence of protease inhibitors [363]. In this particular experiment, sperm cells remained attached with the apical tip to the oolemma. Binding of the lateral side of the sperm head to nor fusion with the oolemma could be observed [363]. Therefore, this preliminary binding to the oolemma seems to be important for positioning the sperm head to the oolemma. Most likely this preliminary binding involves species specific sperm oolemma binding components. Subsequently, proteases enable the sperm cell to go to the second stage of sperm binding. The preliminary binding sites are degraded and the sperm cell is now competent to turn parallel with the oolemma [363]. During this stage sperm cells lie flat on the surface of the oocyte; the equatorial region of the sperm head plasma membrane is attached to the oolemma while the sperm tail continues to beat vigorously. After a period of time, the two adhered membranes fuse, a process that coincides with a stiffening of the sperm tail [364]. Only the equatorial sperm head plasma membrane is competent to fuse with the oolemma [6,365].

In all three steps proteins may play a role that belong to the ADAM family of membrane proteins that contain A Disintegrin And Metalloprotease domain [366,367]. These are multidomain proteins (Fig. 8) of which the protease domain and the disintegrin domains are probably functional in sperm-oolemma interactions as will be detailed below.

4.1. Sperm tip binding to the oolemma

As mentioned above the zona penetrated sperm cell initially binds with the tip of its head to the oolemma. This attachment is an association between the inner acrosomal membrane or with an acrosomal matrix protein and the egg surface [368,369]. A candidate adhesion molecule for this binding step is the sperm protein cyritestin. Cyritestin is a membrane protein which was first identified as a testis specific mouse gene belonging to the ADAM family [370]. Incubating oocytes with peptides from the binding site of cyritestin blocked the tip binding as well as fusion of mouse sperm to the oolemma [371,372]. The protein has been reported to be associated with the inner acrosomal membrane of mouse sperm [368,369]. The tip location of cyritestin would indicate that it is in the correct position for the initial sperm binding to the oolemma. The apical tip binding has to be diminished in order to facilitate the equatorial binding (see section 4.2). Most likely the protease domain of cyritestin is involved in diminishing this binding. After flattening to the oolemma the inner acrosomal membrane is excluded from membrane fusion and is not incorporated into the membrane that surrounds the developing zygote. Instead the inner acrosomal membrane is incorporated into the egg cytoplasm by a process that resembles phagocytosis [374]. The cyritestin binding component on the oolemma has not been determined yet, but one likely candidate is one or more of the oolemma's integrins (see section 4.2).

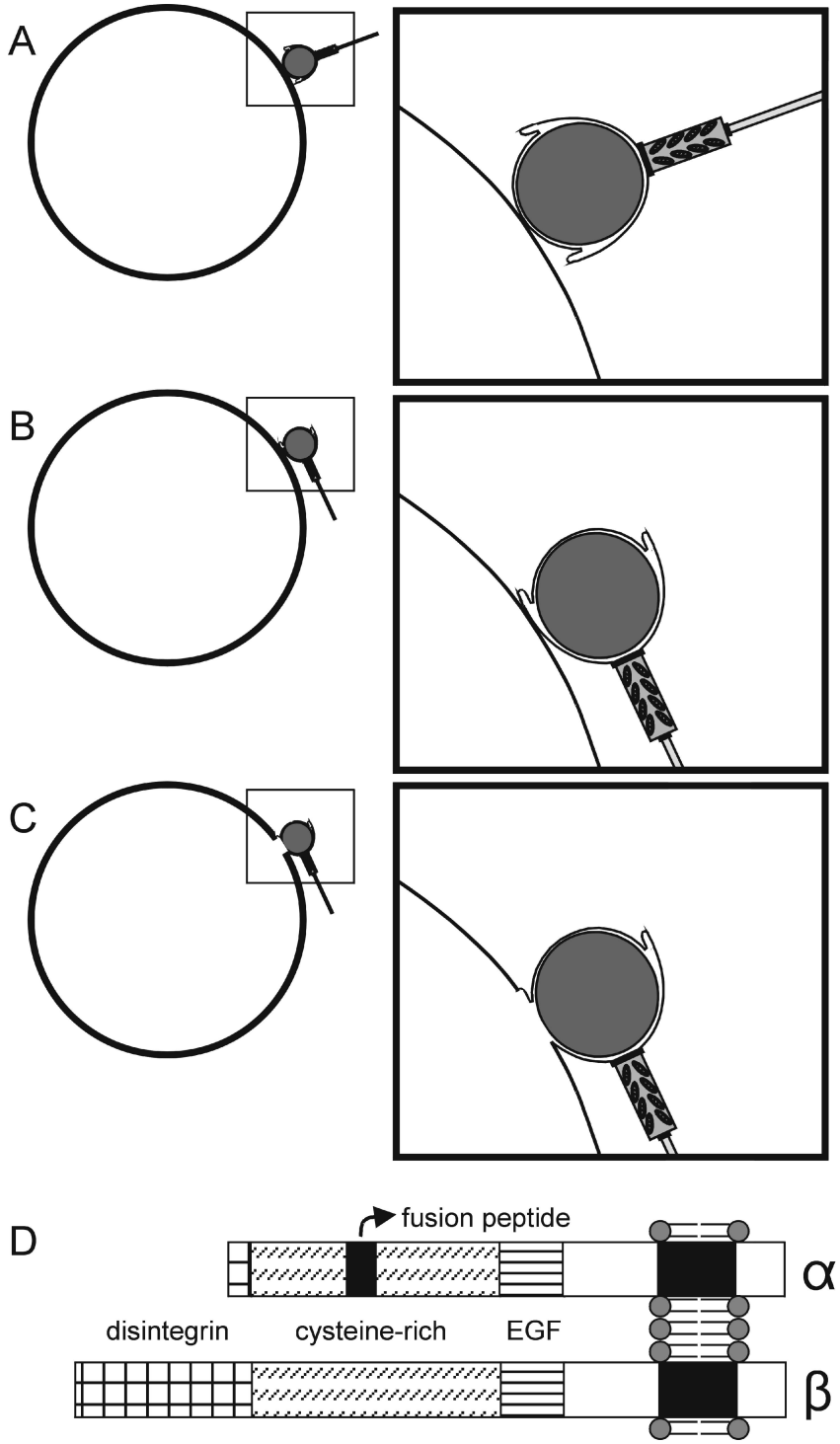

Figure 8. Sperm egg binding and subsequent fusion is a three-step event. A: Sperm initially binds with the apical tip of the inner acrosomal membrane to the oolemma. B: After proper positioning, the apical binding is diminished and has facilitated a lateral binding of the sperm cell to the oolemma. The equatorial region of the sperm head plasma membrane is involved in this process. C: The sperm cell is now capable to fuse its equatorial plasma membrane with the oolemma. The tail stops beating and stiffens after equatorial sperm binding. D: The multidomain organization (EGF: epidermal growth factor) of fertilin (heterodimer protein from the ADAM family) has been proposed to be directly involved in processes B and C. (A-C adapted from [6] and D from [373])

4.2. Equatorial sperm binding to the oolemma

After the relatively short time that sperm cells are attached to the oolemma with the tip, the sperm head binds laterally with its equatorial region (Fig. 8). However, this lateral binding is soon followed by the fertilization fusion which complicates the molecular dissectioning of these two processes. The most extensively studied protein from the sperm plasma membrane, with an apparent role in this binding and fusion, is fertilin. Fertilin also indicated as PH-30 is a heterodimer [375]. Both the α and β subunits are members of the ADAM family [376] and are non-covalently associated in guinea-pig sperm [375] and are also found in a variety of other species including the mouse [377]. The expression and processing of fertilin have been studied extensively (for review see [360]). Fertilin is localized at the apical head region of the guinea-pig [375] and on the equatorial region in mouse sperm [372]. In mouse sperm fertilin β is additionally observed on the inner acrosomal membrane [372], which may indicate that this fraction is involved in the initial sperm tip binding to the oolemma (see section 4.1). By sequence alignment of fertilin β with the related snake disintegrin a binding site had been predicted for integrins [378]. Homologous recombination experiments were designed to produce to generate mice lacking fertilin β [379]. Indeed sperm produced by mice lacking the fertilin β gene had severely reduced (eightfold) ability to bind to the oolemma in vitro. Like cyritestin, fertilin β has probably binding affinity for the oolemma's integrins [378]. It is not clear whether fertilin β and cyritestin bind to the same integrins as cyritestin or that they differ in integrin specificity. Several different integrins have been found on the oolemma of various mammalian species (for review see [380] and references therein). Consistent data are available for the presence of α5β1, α6β1 and αvβ3 integrins at the surface of human and hamster oocytes [381]. In in vitro assays on mouse oocytes it has been demonstrated that fertilin β has affinity for α6β1 [381]. However, it is likely that the other integrins are also involved in fertilin β binding (for review see [360]). A 94 kDa protein on the mouse oolemma protein has been shown to be involved in sperm-oocyte interaction [382], but it is not clear whether this protein has affinity for fertilin.

Recently, it has been demonstrated that the seminolipid immobilizing protein (SLIP see section 2.3.4) may be involved in sperm binding to the oolemma [383,384]. SLIP is probably a membrane bound isoforms of arylsulfatase A, which normally is a lysosomal enzyme involved in the desulfation of seminolipid [19,94]. SLIP has been detected on the oolemma and therefore may bind sperm cells by immobilizing seminolipid. In capacitated, but also in acrosome reacted viable sperm cells, seminolipid is predominantly localized at the equatorial region in the outer lipid leaflet of the sperm plasma membrane, which is the right position for lateral binding to the oolemma [31,32]. A particular molecule of interest is FertPlus®, which is a synthetic peptide derived from prosaposin (also called sulfated glycoprotein 1 or SGP-1) [385]. Because the peptide includes a region of saposin B (an activator protein for arylsulfatase), it may activate SLIP to desulfate seminolipid. If so, the interplay between SLIP and FertPlus® induces the formation of desulfoseminolipid, which due to its relatively small head group can induce inverted micelles that facilitate fusion [5].

4.3. Sperm-oolemma fusion

Currently, there are two basic models that explain how the fusion between the two gametes may occur. Basically, the first model describes this event in analogy with membrane fusion between membrane-enveloped viruses and host cell membranes [386,387], whereas the other is in analogy with the fusion that occurs between transport vesicles in the cytoplasm and target membranes [388]. The virus model predicts that a fusion peptide stretches from the virion to the host cell membrane, where it exposes a hydrophobic domain. This insertion enables conformational changes of membrane proteins in the host cell and the virion, destabilizes the membrane bilayer structure and generates the fusion pore. The fusion pore expands so that the viral and the host membranes become continuous. The transport vesicle model predicts that the two membranes contain two interacting proteins: For the transport vesicle v-snare and for the target membrane t-snare. After binding these two proteins assemble more complexed linkages between the two membranes in such a way that fusion has become energetically favourable. A clear understanding of the molecular nature of membrane fusion between sperm and egg plasma membranes is still lacking. Fertilin α has been proposed to play a role in this fusion event, since it contains a 22-amino acid sequence in the cysteine-rich domain with an alpha helix structure exposing its bulky hydrophobic residues at one face of the protein (Fig. 8) [376]. This may imply that fertilin is required for sperm oolemma binding (fertilin β) as well as fusion (fertilin α), which would make sense because after binding the hydrophobic domain of fertilin α is well oriented for inserting its putative hydrophobic region into the lipid moiety of the oolemma. Some support for this idea can be found in literature [360,376]. A synthetic peptide representing the hydrophobic region of fertilin α from guinea-pig can bind to vesicles and can induce fusion between large unilamellar vesicles [389], although such properties have not yet been described for the entire protein. However, bovine fertilin α contains a hydrophobic region at a different site [390] and rabbit fertilin contains alpha helix breaking amino acids [391,392]. Furthermore, mice lacking the fertilin β gene (see section 4.2) have a marked reduced amount of fertilin α on the sperm surface, but still (despite the absence of fertilin β) have a limited potential to fuse with the oolemma [379]. Both findings indicate that fertilin is not absolutely required for the fertilization fusion.

Only a limited number of studies have been dedicated to sperm fusion factors besides fertilin although unidentified proteins that facilitate porcine [393] hamster [394] and bovine [395] sperm-oolemma fusions have been reported.

Interestingly, capacitated human, bovine [396,397] and boar sperm cells [30] spontaneously fuse in a Ca^{2+}-dependent manner with fluorescently labelled liposomes. The fusion occurred in living capacitated sperm cells at the equatorial region of the sperm head at 38˚C. This is exactly the site of the sperm cell that is specified in the fusion with the oolemma and therefore we believe that with the fluorescent liposomes a new assay has been developed to monitor the fusability of the sperm cell with the oolemma. Since the liposomes did not contain any membrane proteins, these observations would be in favour for the idea that sperm contain a virus-like fusion protein. Indeed a sperm protein seems to be involved, since the liposome fusions were protease-sensitive [398].

5. Conclusions

An overview has been given on the dynamical aspects of the mammalian sperm plasma membrane in the process of fertilization. Special attention to the particular features of the sperm plasma membrane is of interest for reproductive biologists, but also for researchers that are more generally interested in biomembranes. The plasma membrane is highly polarized and dynamically reorganized during capacitation and other fertilization processes. Furthermore it is involved in various membrane adhesion and fusion processes.

Many conflicting studies have emerged on the presence and the involvement of surface components in sperm cells. Probably this is related to the fragility of the sperm plasma membrane after capacitation. Artifacts are easily introduced when primed cells are labelled or monitored under detrimental conditions (e.g. at temperatures lower then 30˚C), or at conditions where the incubation medium is not equilibrated with 5% CO_2 to maintain the appropriate bicarbonate concentrations for capacitation in vitro. Capacitation and acrosome reaction are two specific events that are required for fertilization. However, due to improper experimentations, in vitro capacitation itself has been reported to induce the acrosome reaction. At proper conditions the induction of the acrosome reaction only proceeds after ZP or progesterone mediated stimulation of capacitated sperm cells.

At least three intracellular signalling routes that have been described for sperm cells are relevant to be studied in more detail and also in other cell systems (Fig. 5): (i) Bicarbonate activates adenylate cyclase directly by binding 1:1 to this protein. The subsequent induction of PKA seems to cross-talk with tyrosine kinase and also is involved in the collapse of phospholipid asymmetry in the apical plasma membrane. The effects seem to be important for sperm binding to the ZP, but also for the acrosome reaction. (ii) Cholesterol efflux further activates membrane meditated signalling upon capacitation. The picture of the regulation of cholesterol efflux as well as the identification of proteins involved in this process is far from complete. (iii) Sperm cells contain a specific non-genomic progesterone receptor that enables extracellular Ca^{2+} to pass the sperm plasma membrane by opening voltage dependent Ca^{2+} channels (Fig. 6). The identification of this receptor may open a new research field of an entirely new family of steroid receptors.

Sperm binding to the ZP as well as the oolemma is divided into more than one phase and in each phase different proteins seem to be involved at different locations of the sperm plasma membrane (Fig. 8). The various lateral plasma membrane specializations and reorganizations thereof may not only be important for the regionalisation of gamete adhesion and fusion processes, but may also be functional as signalling compartments. Zona binding is initiated by the sperm plasma membrane proteins, whereas, secondary binding is mediated by acrosomal matrix proteins. Therefore, it is important to isolate the sperm plasma membrane from acrosomal components in order to study primary zona binding. Plasma membrane isolation might also be important in order to dissect the primary sperm tip binding to the oolemma, which is probably initiated by an inner acrosomal membrane protein, whereas, the secondary oolemma binding is mediated by a plasma membrane protein located in the equatorial sperm head. Furthermore, it seems to be important to study primary zona binding proteins from the sperm plasma membrane by using native ZP material rather then solubilized zona proteins.

The regulation of the sperm plasma membrane fusions with either the outer acrosomal membrane or the oolemma is far from understood. In fact no candidate fusion protein for the acrosome fusions has been described yet, whereas, the involvement of fertilin in oolemma fusion has only been evidenced by circumstantial data. Therefore, future research should be focussed on the components that induce fusions of the sperm plasma membrane with one of these two membranes.

Taken all these considerations in account, we believe that this review may help future membrane researchers as well as reproductive biologist to get introduced into the complex processes that prelude the fertilization of the oocyte. Basic understanding of the sperm plasma membrane organization and the relevance of its structural rearrangements in signalling events that leads to fertilization of the oocyte are of crucial importance. After passing all challenges described in this review the sperm cell may successfully activate the fertilized oocyte. New challenges in early life are now switched on and the processes that occur in the developing zygote are several orders of magnitudes more complex than the processes reviewed here.

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Chapter 2

The use of lectins to characterize plasma membrane preparations from boar spermatozoa: a novel technique to monitor membrane purity and quantity

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Abstract

The object of this study was to develop a method to quantify the amount of outer acrosomal membrane material in isolated plasma membranes from boar sperm cells. The cells were fractionated by nitrogen cavitation, and plasma membranes were isolated by subsequent differential centrifugation steps. Marker enzyme measurement showed that the plasma membrane isolates were enriched in plasma membrane markers and did not contain nuclei, inner acrosomal membranes or mitochondria. Since there is no marker enzyme known for the outer acrosomal membrane, lectins were used for the detection of this membrane.

The membrane specificity of a number of lectin-conjugates was tested with fluorescence microscopy and transmission electron microscopy. Membrane binding of these lectin-conjugates was quantified with flow cytometry and an enzyme linked lectin binding assay. Wheat germ agglutinin was specific for the plasma membrane while peanut agglutinin was specific for the outer acrosomal membrane. The use of these lectins made it possible for the first time to discriminate between these two membranes. The isolated plasma membrane fraction was enriched for more than 10 times (17 times after further purification by a sucrose gradient) in plasma membrane material over outer acrosomal membrane material.

Highly purified sperm plasma membranes should prove to be useful for the research on primary sperm-zona interactions.

Introduction

Mammalian fertilization is an extensively studied process but the exact molecular mechanism of the sperm-oocyte interaction remains to be established. The sperm cell initially adheres to the zona pellucida (the extracellular matrix of the oocyte) with the apical plasma membrane and therefore this site must contain zona binding molecules. The fusion of the sperm plasma membrane with the underlying outer acrosomal membrane (the acrosome reaction) is triggered after the initiation of zona binding [242]. The plasma membrane is also involved in the sperm cell binding and fusion to the oocyte [6]. Therefore, many attempts have been described to isolate the plasma membrane from sperm cells of several species [399-401]. However, only highly purified sperm plasma membranes are valuable for adhesion and fusion studies.

The purity of the plasma membrane has been expressed as enrichment in relative activities of plasma membrane marker enzymes [399,400,402-408]. Mostly these reports also have shown decreased activities of marker enzymes from other cellular membranes (i.e. mitochondria and acrosome). The acrosomal outer membrane is the most probable contaminant in plasma membrane isolates from sperm cells because it is very closely situated to the apical plasma membrane of the sperm head. However, marker enzymes for the outer acrosomal membrane have not been described and therefore the amount of outer acrosomal membrane contamination has never been determined.

In this study, the specificity of labelled lectins and marker enzymes for various sperm membranes were used to measure the purity of the isolated plasma membranes from boar sperm cells. A novel method to measure the purity and quantity of plasma membranes is described. With this novel assay we were able to discriminate between the plasma membrane and the outer

acrosomal membrane. The applicability of this new technique in fertilization research will be discussed.

Materials and methods

Semen preparation

Boar semen was obtained from the Cooperative Centre for Artificial Insemination in Pigs "Utrecht en de Hollanden" (Bunnik, The Netherlands). Semen was filtered through gauze to remove gelatinous material. All buffers and other solutions used were iso-osmotic (285-300 mOsm) and at room temperature.

Semen was allowed to cool to room temperature within 1.5 h after collection. Semen samples were diluted in fixative (0.5% w/v formaldehyde in saline) and the sperm concentration was estimated in a Bürker cell chamber under a phase contrast microscope (200x, Olympus, Tokyo, Japan). The ejaculate was diluted in Beltsville Thawing Solution (BTS: 0.2 M glucose, 20 mM sodium citrate, 15 mM $NaHCO_3$, 3.36 mM Na_2EDTA , 10 mM KCl, 20 mM Hepes, pH 7.4) to $1.5 \cdot 10^8$ cells/ml, and seminal plasma was removed by washing over a Percoll® (Pharmacia, Uppsala, Sweden) density gradient (35% - 70%). Percoll was removed by washing pelleted sperm 3 times in Tris Buffered Sucrose Solution (TBSS: 5 mM Tris, 0.25 M sucrose, pH 7.4).

Plasma membrane isolation

Plasma membranes from boar spermatozoa were isolated as described by Buhr et al. [409] with modifications. Briefly, washed sperm cells in TBSS ($\pm 4 \cdot 10^8$ cells/ml) were subjected to nitrogen cavitation (10 min, 45 bar) in a cell disruption bomb (Parr Instrument Company, Moline, IL). The cavitate was slowly extruded into a clean tube, and 0.2 mM PMSF (dissolved in dimethyl sulfoxide) was added to the cavitate, which was then gently rotated during 15 min. Subsequently the cavitate was centrifuged for 10 min at 1000 *g* (room temperature). The pellet was washed with 5 ml TBSS and centrifuged for 10 min at 1000 *g* (room temperature). Supernatants were combined and centrifuged for 10 min at 6000 *g* (room temperature). The 6000 *g* supernatant was then centrifuged during 70 min at 285.000 *g* (4˚C). The 285.000 *g* membrane pellet was washed in Hepes Buffered Saline (HBS: 5 mM Hepes, 2.7 mM KCl, 146 mM NaCl, pH 7.4) during 40 min at 285.000 *g* (4˚C). Samples were frozen in liquid nitrogen and kept at -20˚C prior to examination.

Plasma membrane isolates were also layered on a discontinuous sucrose gradient and centrifuged according to Gilles et al. [401]. Fractions of 1 ml were collected and protein content as well as lectin binding properties were determined.

Biochemical analysis

Alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatase were assayed according to Soucek and Vary [403]. β-N-acetylglucosaminidase (EC 3.2.1.30) activity was measured according to Khar and Anand [410]. 5'-nucleotidase (EC 3.1.3.5) activity was determined by incubation of samples in a buffer containing 0.1 M glycine, 10 mM $MgCl_2$ and 5 mM 5'-AMP (pH 8.5) during 30 min at 37˚C. The reaction was stopped by adding phosphate reagent and phosphate measurement was

done according to Rouser et al. [411]. Acrosin (EC 3.4.21.10) activity was determined by its esterolytic activity on N-α-benzoyl-*L*-arginine-*p*-nitroanilide (*L*-BAPNA, 4.0 mM , Sigma, St. Louis, MO). The formation of the product *p*-nitroaniline was measured continuously at 410 nm (ϵ_{410} = 9.9 $mM^{-1} \cdot cm^{-1}$ [412]) during the incubation in a buffer containing 0.1 M Tris and 67 mM NaCl (pH 8.0) at 25˚C. Succinate dehydrogenase (SDH, EC 1.3.99.1) was measured at 25˚C according to Van Hellemond et al. [413]. Lactate dehydrogenase (EC 1.1.1.27) activity was continuously measured in a spectrophotometer at 340 nm (ϵ_{340} = 6.6 $mM^{-1} \cdot cm^{-1}$). Samples were incubated at 25˚C in 0.1 M sodium phosphate buffer (pH 7.2) containing 1.1 mM pyruvate and 0.2 mM NADH.

Protein amount was determined by the method of Lowry [414] with a slight modification, using bovine serum albumin as the standard. Samples were boiled for 10 min in 30 mM SDS, 160 mM Na_2CO_3 and 12 mM KNa-tartrate in 80 mM NaOH. Samples were 10 min incubated in 1.5 mM $CuSO_4$, and the colour was enhanced with 0.1 M Folin-Ciocalteu's phenol reagent for 30 min.

DNA content was measured according to Young-Jo et al. [415] (DNA standard from Sigma, St. Louis, MO).

Detection of lectin binding

I Fluorescence microscopy

To visualize the sperm regions to which the FITC-lectins bind, samples were fixed in Karnovsky solution (2% paraformaldehyde, 2.5% glutaraldehyde, 80 mM Na-cacodylate, 250 μM $CaCl_2$, 500 μM $MgCl_2$, pH 7.4) or in pure methanol. Before the incubations with lectins, Karnovsky fixed samples were centrifuged (500 *g*, 10 min) and the pellets were recovered in PBS containing 50 mM glycine (pH 7.4). Fixed samples were dried at room temperature on slides, and then incubated in HBS containing 1 mM $CaCl_2$ and 2 or 10 μg/ml of one of the following Fluorescein isothiocyanate (FITC) conjugated lectins (EY Laboratories, San Mateo, CA): PNA (*Arachis hypogaea* (peanut) agglutinin), LEA (*Lycopersicon esculentum* agglutinin), UEA-I (*Ulex europeaeus* agglutinin), GS-I (*Griffonia simplicifolia* I agglutinin), GS-II (*Griffonia simplicifolia* II agglutinin), WGA (*Triticum vulgare* (wheat germ) agglutinin), ConA (*Canavalia ensiformis* agglutinin), MPA (*Maclura pomifera* agglutinin), AIA (*Artocarpus integrifolia* agglutinin), DBA (*Dolichos biflorus* agglutinin), BPA (*Bauhinia purpurea* agglutinin), SBA (*Glycine max* (soybean) agglutinin), LPA (*Limulus polyphemus* agglutinin). After incubation, slides were washed with 2 ml HBS containing 1 mM $CaCl_2$, counter-stained with 10 μg/ml propidium iodide and imbedded in 1 drop of Slowfade® (Molecular Probes, Eugene, OR). Cover slips were placed on top of the samples and were sealed with nail polish. The sperm cells were studied with a fluorescence microscope (BH-2, Olympus, Tokyo, Japan) equipped with differential interference contrast optics, a 100 W Hg lamp and a filter set (470 nm; 30 nm BP excitation filter, a 515 nm dichroic mirror and a 530 nm LP emission filter).

II Electron microscopy

Binding sites for FITC conjugated lectins were localized using immunogold-labelling combined with electron microscopy. Percoll washed spermatozoa were centrifuged and pelleted

spermatozoa were fixed in Hepes Buffered Sucrose Solution (HBSS: Tris was replaced by Hepes in TBSS) containing 2% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde) and stored overnight at 4˚C. Subsequently the sample was treated as described by Fazeli et al. [242].

III FACS analysis

Percoll washed and permeabilized sperm cells were incubated for 5 min in HBBS containing 1 μg/ml of the lectin-FITC conjugate of choice. The membrane impermeable vital stain, propidium iodide was added to counter-stain deteriorated cells (final concentration 10 μg/ml). The labelled sperm suspensions were analysed in a FACS-Scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 100 mW argon laser. Cell fluorescence was exited at 488 nm while the FITC- and the propidium iodide-emission intensity per cell was detected in the logarithmic mode of FL-1 (530/30 nm band pass filter) and FL-3 (620 long pass filter) respectively. Forward and sideward light scatter (FSC and SSC) data were collected in linear mode. At the light scatter settings for FSC (E00) and SSC (400 mV) the sperm specific events were recognizable as a typical L-shaped scattering profile. The non sperm specific events (mostly small particles, < 3% of total events) were gated out for further analysis. The flow cytometric data were stored in Becton Dickinson software and analysed on WinMDI version 2.1.4 (J.Trotter; free software, E-mail: trotter@scripps.edu). Regions were drawn on specific cell populations reflecting dead and life cells respectively, and mean fluorescence as well as proportions of the subpopulation were calculated.

IV Quantification of lectin binding

An enzyme linked lectin binding assay (ELLBA) was developed to quantify lectin binding of the cavitate and the membrane samples: ELISA plates were coated with samples (500 to 4 ng protein) in 0.1 M Na_2CO_3 (pH 9.6) for 8 h at room temperature. Plates were blocked for 10 min with 0.3% (v/v) Tween-20 in HBS and subsequently for 1 h with 0.05% (v/v) Tween-20 and 1% (w/v) casein in HBS. Biotin conjugated lectins (EY Laboratories, San Mateo, CA; 0.5 μg/ml) were allowed to bind to the coated plates for 2 h at room temperature in incubation buffer (0.05% (v/v) Tween-20, 0.1% (w/v) casein, 1 mM $CaCl_2$ in HBS). Free lectin was removed by washing the plates 5 times with wash buffer (10 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% (v/v) Tween-80, 0.0001% (w/v) benzalkonium chloride, pH 7.5). Horseradish peroxidase (HRP) conjugated to streptavidin (Sigma, St. Louis, MO; 0.1 μg/ml) was allowed to bind to the biotin-lectin conjugates for 1 h in incubation buffer. Free HRP-streptavidin conjugates were removed by washing 5 times with wash buffer. Bound enzyme activity was measured according to Bos et al. [416] and absorption was measured in a plate reader at 450 nm. Calculations were made with OD_{450} corrected for background absorption (same procedure but the biotin-lectin conjugate was omitted). The purification rate was calculated from the linear range as the increase or decrease in the amount of bound lectin upon differential centrifugation.

Western-blotting

To visualize proteins with WGA and PNA binding properties, samples containing 10 μg protein were separated on 12% gels (SDS-PAGE) and blotted on polyvinylidene fluoride membranes

(Millipore Corporation, Bedford, MO). Blots were blocked and incubated with PNA-biotin or WGA-biotin as described for the ELLBA, only casein was replaced by BSA and the blots were washed with HBS containing 0.05% (v/v) Tween-20, 1 mM $CaCl_2$ and 0.1% (w/v) BSA. Proteins that bound lectin were visualized with 1.7 mM diaminobenzidine in 50 mM Tris (pH 7.2) containing 1.3 μM $NiCl_2$ and 1.8 μM H_2O_2 [417].

Morphological Study

Percoll washed spermatozoa, nitrogen cavitated spermatozoa (1000 *g* and 6000 *g* pellets) and the isolated plasma membrane fraction were pelleted and fixed overnight at 4˚C in Karnovsky fixative. Pellets were washed with 0.1 M Na-cacodylate pH 7.4, post-fixed with 1% osmiumtetroxide in 0.1 M Na-cacodylate pH 7.4 for 1 hr, washed with distilled water and stained with 2% aqueous uranylacetate for 1 hr. Samples were dehydrated in graded series of acetone, and embedded in Durcupan ACM resin (Fluka, Bachs, Switzerland). Ultrathin sections (50 nm) were cut on a Reichert UltracutS (Leica Aktiengesellschaft, Vienna, Austria) and stained for 2 min with Reynolds' lead citrate [418]. Sections were observed and photographed in a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV.

Results

Marker enzyme analysis

Purification of the cavitated plasma membranes by differential centrifugation steps resulted in a membrane fraction that was enriched in relative enzyme activities for alkaline phosphatase, acid phosphatase and 5'-nucleotidase, three marker enzymes of the plasma membrane of boar spermatozoa [403]. The specific activities of these plasma membrane marker enzymes were respectively 9, 13, and 10 times higher in the membrane isolate than in the complete cavitate (Table 1). Contamination of marker enzymes from the inner acrosomal membrane (acrosin [419,420]) and the inner mitochondrial membrane (SDH [421]) was minimal in the plasma membrane fraction (below the detection limit, see Table 1). The commonly used acrosomal marker enzyme β-N-acetylglucosaminidase, however, had a higher specific enzyme activity in the plasma membrane fraction than in the complete cavitate (4 times, see Table 1). DNA was measured to detect nuclear contamination. Only a minor amount could be detected in the plasma membrane samples (< 0.1% of the total DNA amount in the cavitate).

Only a trace amount of the three analysed plasma membrane markers (< 1% of alkaline phosphatase, 11% of acid phosphatase and < 1% of 5'-nucleotidase) were confined to the sperm cell while the rest of the enzyme activity was recovered in seminal plasma (probably soluble enzymes, Table 1). Likewise, only 3% of the total β-N-acetylglucosaminidase activity was found in the sperm cells fraction, while 81% of this enzyme (previously designated as an acrosomal marker enzyme [402,408,422]) was also recovered as a soluble enzyme in the seminal plasma. In contrast to the above described marker enzymes, only minor amounts of the enzyme activities of succinate dehydrogenase, acrosin and lactate dehydrogenase could be detected in seminal plasma (< 2%, 8% and 18%, respectively, see Table 1).

Table 1. Markers in spermatozoa and purified sperm plasma membranes.

enzyme	specific activity[a]		purification[b]	distribution of activity[c]	
	sperm cells	membrane isolate		sperm cells	seminal plasma
plasma membrane marker					
alkaline phosphatase	7.5 ± 3.0	76.2 ± 38.5	8.8 ± 1.7	< 1%	88%
acid phosphatase	13.6 ± 1.5	169 ± 62	13.2 ± 3.1	11%	63%
5'-nucleotidase	3.3 ± 0.9	21.7 ± 9.7	10.1 ± 4.9	< 1%	84%
mitochondrial marker					
succinate dehydrogenase	14.3 ± 11.1	0.16 ± 0.10	0.01 ± 0.01	> 98%	< 2%
acrosomal marker					
acrosin	1010 ± 230	14 ± 10	0.01 ± 0.01	92%	8%
ß-N-acetylglucosaminidase	1.8 ± 0.7	7.0 ± 8.1	4.0 ± 2.5	3%	81%
cytosolic marker					
lactate dehydrogenase	103 ± 1	ND[d]	ND	77%	18%
nuclear marker					
DNA	246 ± 20[e]	12.3 ± 1.1	0.05 ± 0.03	ND	ND

[a] nmol·mg protein^{-1}·min^{-1} in Percoll washed sperm cells or in membrane isolate (n=5, ± SD)
[b] ratio of the specific activities in membrane isolate and cavitate (n=5, ± SD)
[c] total activity in spermatozoa (pellet) and in seminal plasma (supernatant) obtained after Percoll centrifugation as percentage of the total activity in the ejaculate (n=2)
[d] Not Determined
[e] µg·mg protein^{-1} in Percoll washed sperm cells (n=4, ± SD)

Localization of lectin binding-sites

Several lectins were tested on membrane specificity. Transmission electron microscopy revealed that FITC conjugated WGA, detected by anti-FITC and protein A-gold, bound primarily to the plasma membrane (Fig. 1A). LEA bound to the plasma membrane of fixed boar spermatozoa as is shown in Fig. 1B, but also to the inner acrosomal membrane and the acrosomal content. However LEA did not bind to the outer acrosomal membrane. The acrosomal marker lectin PNA [242] was used to detect the outer acrosomal membrane (Fig. 1C). Other tested lectins (GS-I, GS-II, AIA, DBA, ConA, UEA-I, MPA, BPA, SBA, LPA) did not show defined binding patterns.

Fluorescence microscopy revealed that WGA-FITC (green) stained the complete surface of Percoll washed spermatozoa, fixed with Karnovsky solution (Fig. 2A). Similar staining patterns were found after methanol fixation. Some spermatozoa (approximately 10%) showed a patchy WGA distribution (not shown). Most of the green (WGA) fluorescence on the sperm head disappeared after nitrogen cavitation except for the post equatorial subdomain (Fig. 2B). The tails and midpieces remained also fluorescent. LEA-FITC bound over the entire plasma membrane of the sperm head of Karnovsky fixed spermatozoa (Fig. 2C). Methanol fixed spermatozoa showed a slightly increased fluorescence at the acrosomal region (not shown). After nitrogen cavitation most of the LEA-FITC fluorescence had disappeared (Fig. 2D) from the sperm head, but the post equatorial region was still labelled. Like WGA, the tails and midpieces were still fluorescent after nitrogen cavitation. PNA-FITC exclusively bound to the acrosomal region in permeablized

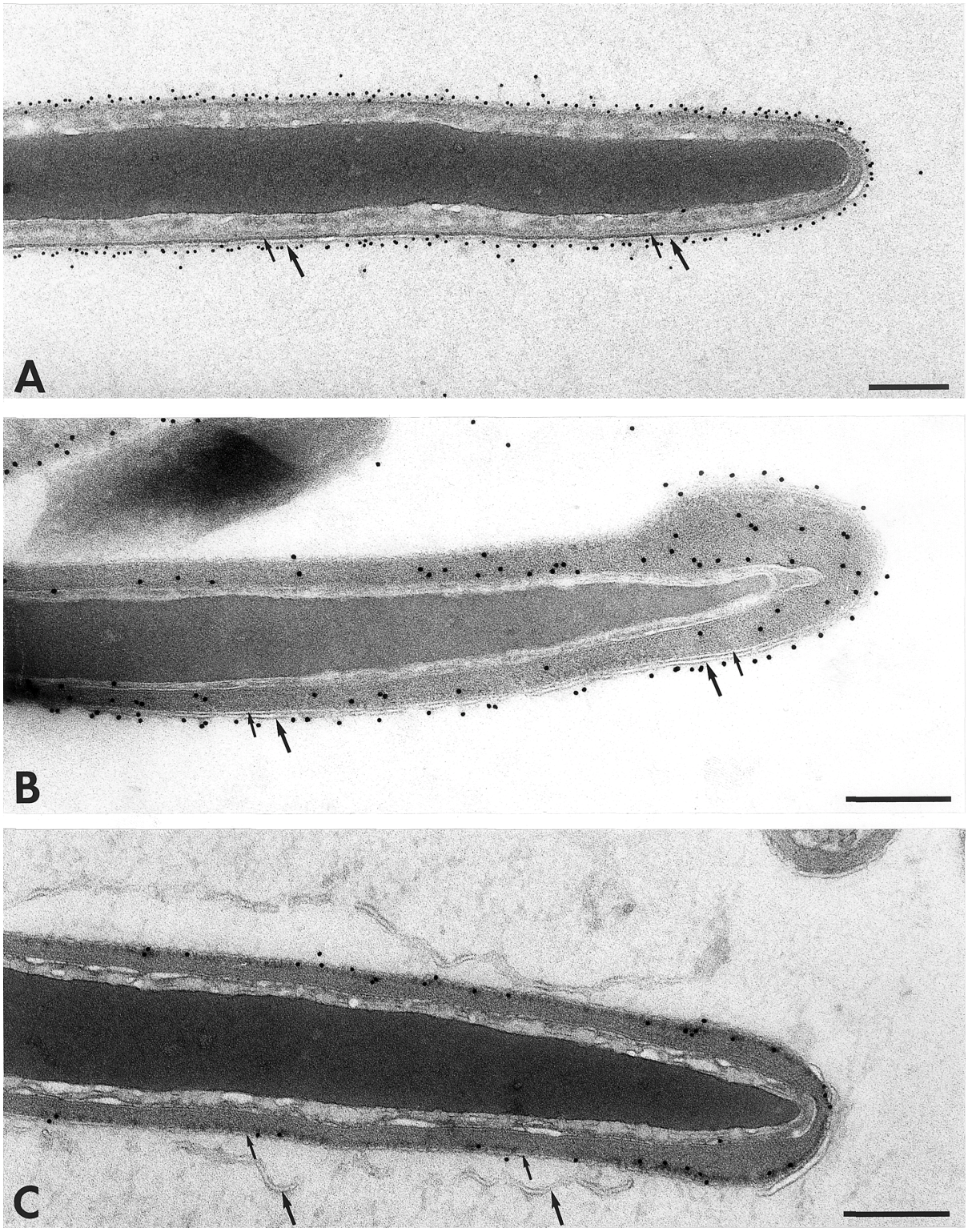

Figure 1. Ultrastructural localization of lectin binding sites in fixed boar spermatozoa on ultrathin cryo-sections. Cryo-sections were labelled with 0.5 µg/ml FITC conjugated lectin: panel A: WGA-FITC, panel B: LEA-FITC and panel C: PNA-FITC. The ultrastructural localization of FITC-conjugates was visualized after further incubation with a rabbit polyclonal antiserum against FITC followed by 10 nm protein A-gold. The plasma membrane is indicated with large arrows, the outer acrosomal membrane with small arrows. Bar represents 250 nm.

(methanol fixed) cells (not shown) and also to approximately 5% of the Karnovsky fixed spermatozoa (probably cells with deteriorated acrosomes, Fig. 2E). Nitrogen cavitation resulted in staining of most acrosomes by PNA-FITC (Fig. 2F). Although Karnovsky fixed cells were impermeable for lectin-conjugates, propidium iodide stained the nucleus of both Karnovsky fixed as well as methanol fixed spermatozoa.

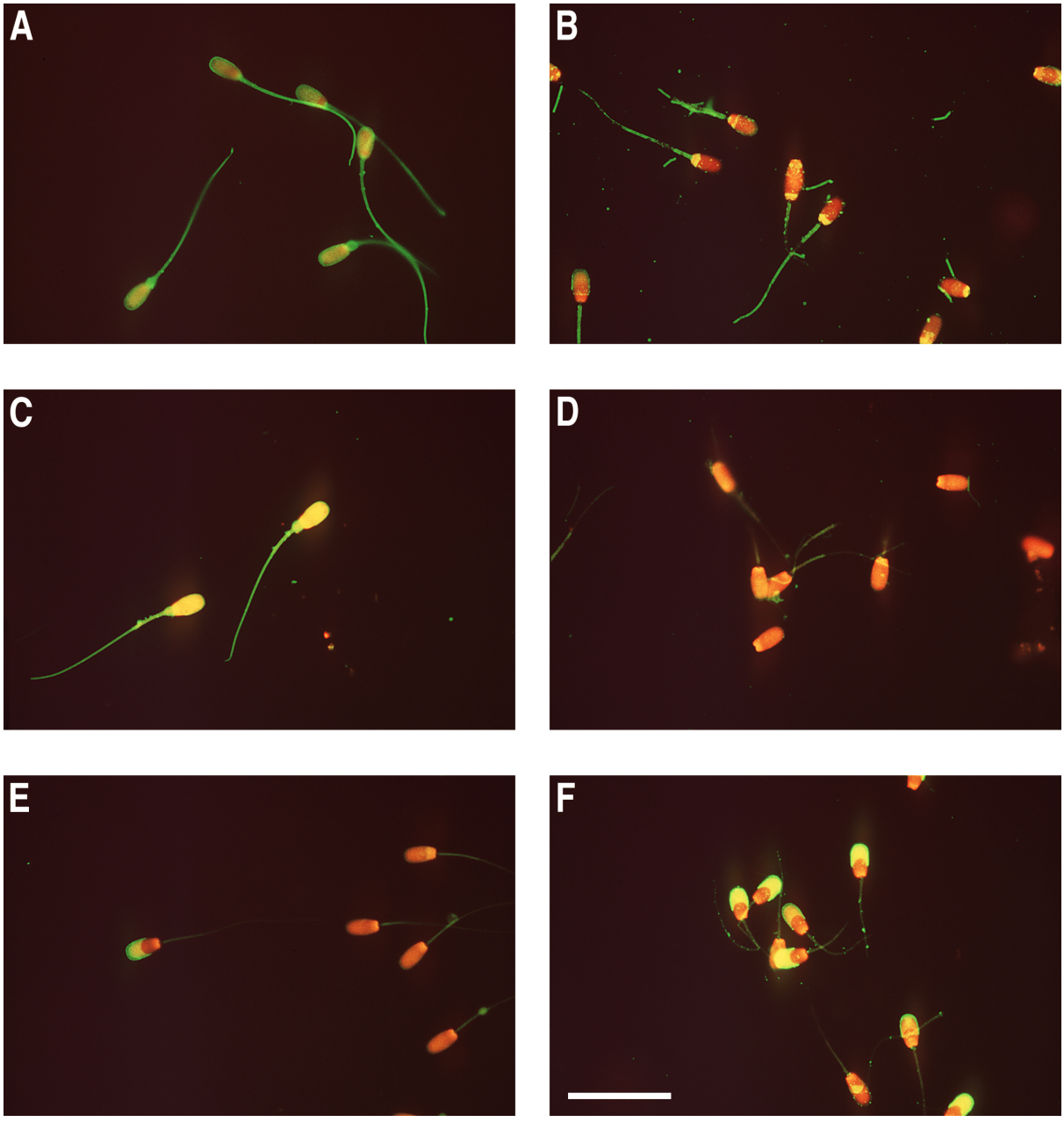

Figure 2. Fluorescent labelling patterns of boar sperm cells incubated with lectin-FITC conjugates. Percoll washed boar spermatozoa and nitrogen cavitated spermatozoa were fixed and labelled with FITC conjugated lectins (green) and counter stained with propidium iodide (10 µg/ml; red). Panels A and B: WGA-FITC (2 µg/ml). Panels C and D: LEA-FITC (10 µg/ml). Panels E and F: PNA-FITC (2 and 10 µg/ml respectively). Panels A, C and E: Percoll washed spermatozoa. Panels B, D and F: nitrogen cavitated spermatozoa. Bar represents 25 µm.

FACS analysis revealed that WGA-FITC was able to bind to living cells (Fig. 3A) and that permeabilization by Triton X-100 did not result in an increase of bound WGA-FITC (Fig. 3B). LEA-FITC bound also to living cells (Fig. 3C) and the intensity of labelling to permeabilized cells increased slightly (Fig. 3D). PNA-FITC bound much more intensive to dead cells (Fig 3E) compared to living cells (mean intensity for living and dead cells: 14 vs 123). Triton X-100 permeabilization resulted even in a higher intensity of FITC-PNA (mean intensity: 201, Fig. 3F).

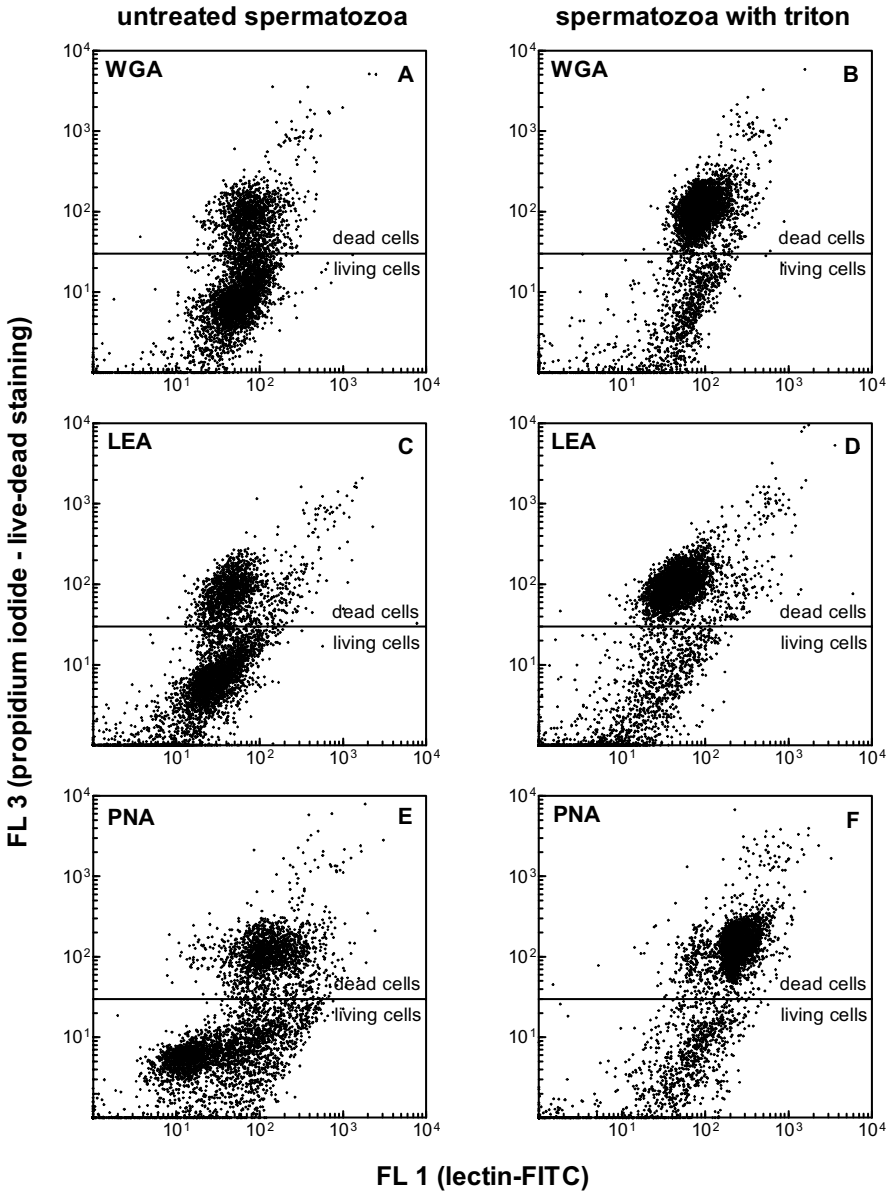

Figure 3. Flow cytometric detection of lectin-FITC binding to intact and permeabilized Percoll washed boar spermatozoa. Percoll washed spermatozoa were incubated either in absence or presence of Triton X-100 (0.05 %) with 1 µg/ml FITC-conjugated lectin and counter-stained with 10 µg/ml propidium iodide to detect deteriorated cells. Each panel represents the analysis of 5000 events. Panels A and B: WGA-FITC. Panels C and D: LEA-FITC. Panels E and F: PNA-FITC. Panels A, C and E: spermatozoa in absence of Triton X-100. Panels B, D and F: spermatozoa in presence of Triton X-100 (0.05 %).

Contamination by the outer acrosomal membrane

Biotin conjugated lectins were used in an ELLBA, in order to determine the contamination by outer acrosomal membranes in isolated sperm plasma membranes. Biotin conjugated lectin was allowed to bind to ELISA plates coated with isolated sperm plasma membrane or cavitate. The amount of lectin binding was determined after coupling of HRP-streptavidin to the immobilized biotin and detecting HRP activity normalized on the basis of coated protein. WGA binding to the plasma membrane increased on protein basis with a factor five by purification (Fig. 4), while LEA binding increased 2 times normalized on protein basis. PNA-binding to the isolated membrane fraction decreased by a factor 2 compared to the cavitate (Fig. 4). This implies that the membrane fraction is at least ten times enriched in plasma membrane material over outer acrosomal membrane material.

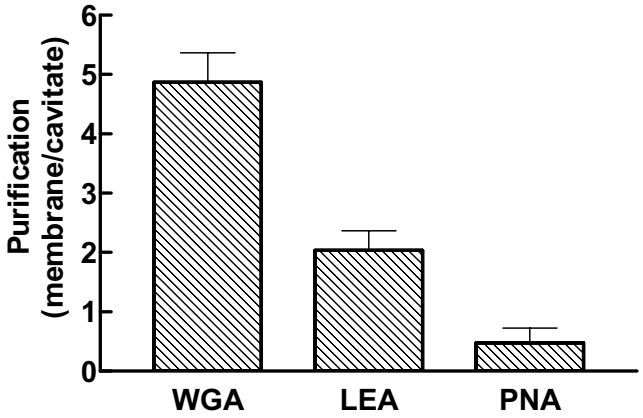

Figure 4. Relative purification of the isolated plasma membrane fraction after nitrogen cavitation and subsequent differential centrifugation of boar sperm cells. The isolated plasma membrane fraction as well as the complete cavitate were coated on 96 wells plates. The purification of the plasma membrane is calculated for the relative increase/decrease of specific lectin binding to the isolated plasma membrane compared to the corresponding cavitate based on protein amount.

Plasma membrane isolates were also further purified by centrifugation of the isolates layered on top of a sucrose density gradient. About 60% of the material (on protein base) recovered on top of the 1 M sucrose layer with a density of 1.13 mg/ml; typical for plasma membrane material [423]). The WGA/PNA affinity ration of this membrane subfraction was 170% higher than for the original membrane isolate placed on top of the sucrose density gradient (see Fig. 5).

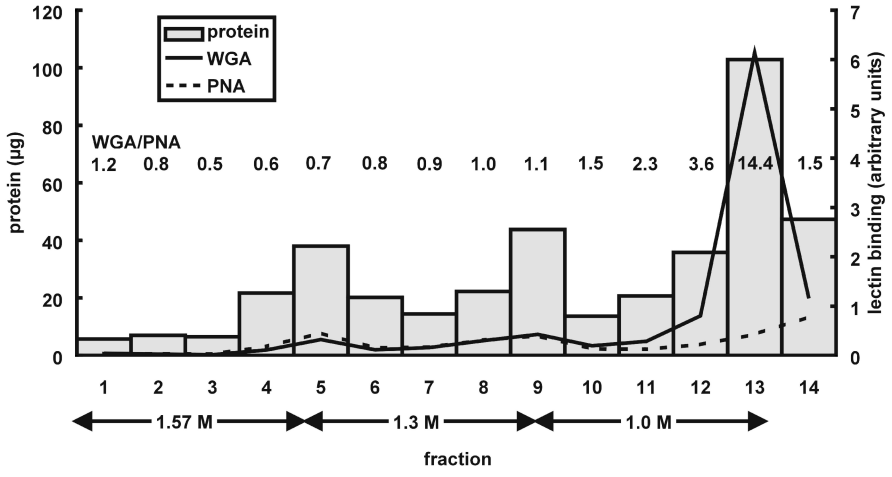

Figure 5. Profile of a sucrose density gradient loaded with a 1 ml sample of plasma membrane isolate. The amount of protein and WGA as well as PNA binding was measured for 1 ml fractions. The ratio of WGA/PNA binding is indicated for each fraction in the middle of the figure. The arrows with numbers under the graph indicate the sucrose concentration in the density gradient (in moles/l).

Protein samples from washed sperm cells, cell free seminal plasma and purified membranes were separated by SDS-PAGE and western blots were prepared in order to identify PNA and WGA binding sites (see Fig. 6). PNA binding sites were absent in seminal plasma and in the plasma membrane isolate, however, a 60 kDa band from washed sperm cells showed high affinity for PNA (Fig. 6). Contrarily the protein sample of washed sperm had only low affinity for WGA (plasma membrane material is highly diluted by intracellular membrane proteins within this sample) while a 18 kDa protein present in the plasma membrane but also in the seminal plasma showed high affinity for WGA (Fig. 6).

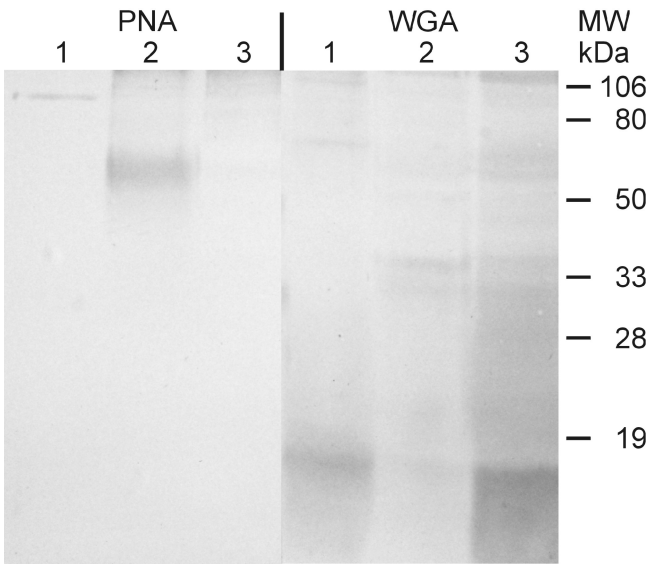

Figure 6. Western blot of boar semen protein samples (10 µg for each lane) separated by SDS-PAGE. The left lanes depict PNA binding sites, the right lanes WGA binding sites. Lanes 1: plasma membrane isolate, lanes 2: washed sperm cells, lanes 3: seminal plasma. Numbers on the right of the western blot indicate the molecular weight of markers.

Morphological examination of the cavitated spermatozoa by electron microscopy revealed that approximately 80-90% of the spermatozoa lost their plasma membrane at the peri acrosomal region (Fig. 7A). This finding is in agreement with the fluorescence microscopical observations. The 1000 *g* pellet mostly consisted of spermatozoa with released plasma membrane at the apical region (Fig. 7B) and unattached sperm heads. The 6000 *g* pellet contained tails and midpieces and also some vesicles were present (Fig. 7C). The membrane vesicles were recovered in the 285.000 *g* pellet and most of the membrane isolate was composed of unilamellar vesicles (Fig. 7D).

Discussion

The aim of this study was to obtain a biochemically well defined plasma membrane fraction of boar sperm cells with a unilamellar morphology and as much as possible devoid of outer acrosomal membrane contamination. Such a well defined membrane system can be used for the further analysis of adhesive and fusogenic properties of the sperm cell that are important during fertilization. The purity of the isolated plasma membranes (obtained by nitrogen cavitation and differential centrifugation procedures) has to be established before such studies can be performed.

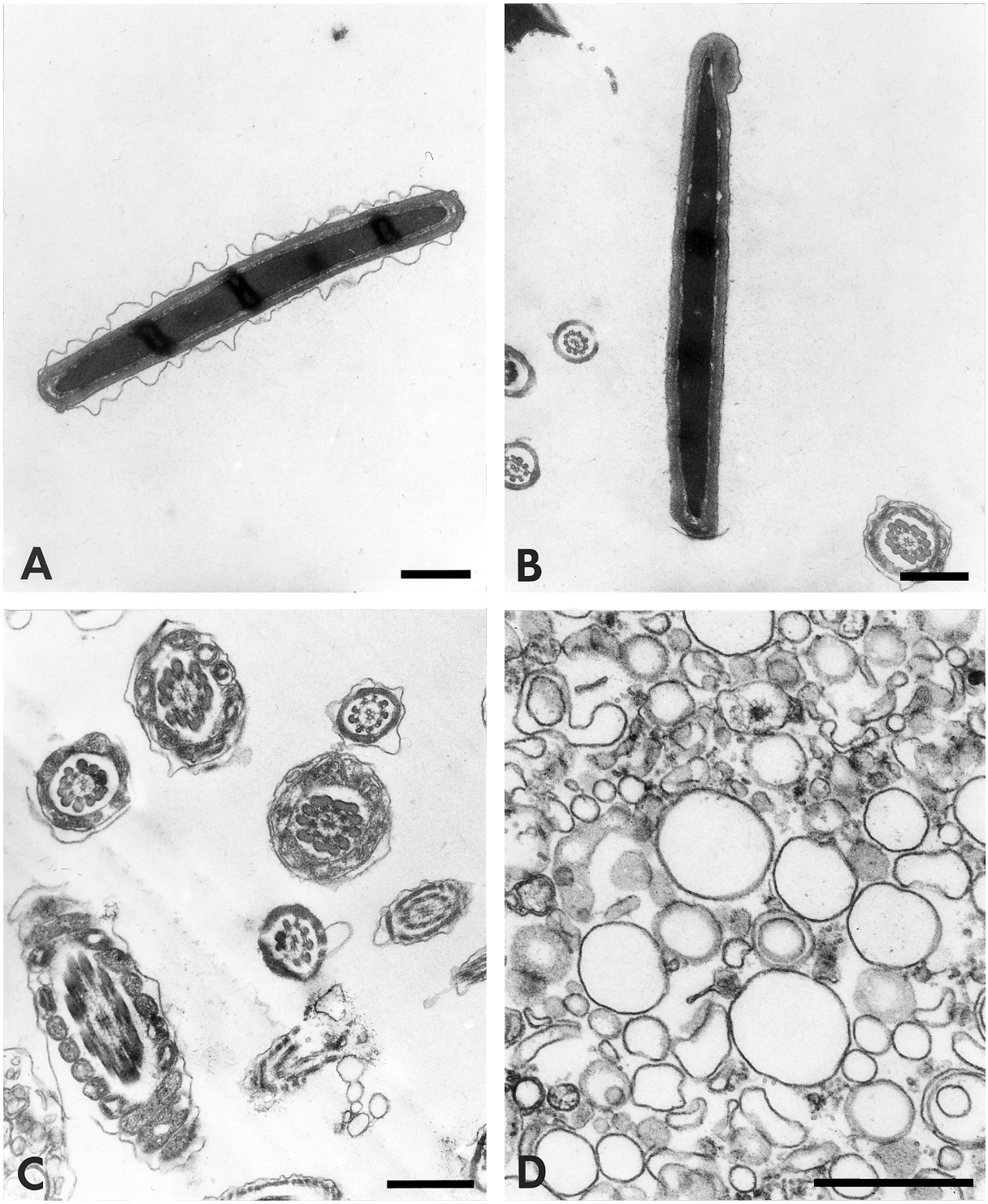

Figure 7. Ultrastructural morphology of intact and cavitated boar sperm cells and the isolated plasma membrane fraction. Panel A: Percoll washed spermatozoa. Panel B: nitrogen cavitated spermatozoa in the 1000 *g* pellet. Panel C: tails and midpieces from cavitated spermatozoa in the 6000 *g* pellet. Panel D unilamellar plasma membrane vesicles in the 285.000 *g* pellet. Bar represents 500 nm.

The isolation of the apical plasma membrane from boar sperm cells by nitrogen cavitation has been described before [401]. Although the isolation of plasma membranes was confirmed by marker enzyme analysis [401-403,424,425] and ultrastructural analysis [402,426], these studies have not addressed possible contamination of outer acrosomal membranes in the membrane preparations itself. However, this membrane is the most probable contaminant in the plasma membrane preparations. In this paper a novel method involving the use of conjugated lectins is described to assay the amount and purity of plasma membrane isolates, but also the amount of outer acrosomal membrane contamination in the membrane isolates itself. Our study shows that the lectin WGA can be used as a marker for the plasma membrane while the lectin PNA can be used as a marker for the outer acrosomal membrane.

Marker enzymes are commonly used to determine the purity of isolated plasma membranes of spermatozoa [399,400,402-408,425,427], in analogy to that of somatic cells [423]. However, in contrast to somatic cells [428-432], the ultrastructural localization of most marker enzymes is poorly described for mammalian sperm cells. In this paper we describe the presence of high activities of several putative plasma membrane (e.g. 5'-nucleotidase) and acrosomal marker enzymes in seminal plasma. This observation is supported by the literature [433,434] and in fact a specific extracellular soluble form (isoenzyme) of 5'-nucleotidase has been described [404]. The existence of an extracellular soluble form of a marker enzyme in the seminal plasma is not crucial for a plasma membrane marker. If the enzyme adhere to the plasma membrane and will be detached during the isolation procedure, the purification of this membrane will be only under estimated. More crucial is the adherence of a marker enzyme for an intracellular membrane to the plasma membrane, like the acrosomal marker β-N-acetylglucosaminidase [407,408,410,435]. Our results showed that this acrosomal enzyme was present in our plasma membrane isolates. Interestingly, a very high activity of the enzyme was recovered in the seminal plasma, but more important was that the majority of the enzyme activity was detected extracellular in Percoll washed sperm cells. We therefore believe that extracellular β-N-acetylglucosaminidase is adsorbed to the surface of sperm cells, which already has been proposed for human spermatozoa [433]. The use of membrane marker enzymes [401-403,425], which are abundant in seminal plasma, is unreliable for intracellular membrane structures. Therefore, the membrane characterization employing membrane marker lectins is superior over the old method using marker enzymes.

In contrast to the marker enzymes described above, other marker enzymes such as succinate dehydrogenase (inner mitochondrial membrane [421]) and acrosin (inner acrosomal membrane [419,420]) are virtually absent in seminal plasma of freshly ejaculated semen. Most likely the small amounts of enzyme activities detected in the seminal plasma are a result of enzyme leakage from deteriorated sperm cells. In fact, these enzymes are used in clinical assays as a parameter that reflects the relative integrity of sperm cells. Another intracellular marker for sperm cells that has been used in similar clinical assays is the cytosolic enzyme lactate dehydrogenase [436]. However, similar to others [437], we also found a rather high enzyme activity in the seminal plasma. Therefore succinate dehydrogenase is the preferred enzyme to check sperm membrane integrity and that acrosin is a reliable enzyme to check the integrity of the sperm acrosomes. The use of these marker enzymes show that the isolated plasma membrane fractions are free of inner

mitochondrial membrane and inner acrosomal membrane. These results are comparable to or lower than the activities reported in other studies [401,425,435]. DNA measurements show that only minor amounts of the nucleus were present in the isolated plasma membrane fractions, which is in agreement with previous reported data [435]. Taken together our data demonstrate that DNA and enzymes can only be used as markers for intracellular structures in sperm cells when they are not present in seminal plasma of freshly ejaculated semen.

Lectins are widely used in biochemistry and are known to bind to carbohydrates in a very specific manner [438]. In theory, the specific carbohydrate binding properties can make lectins useful to detect specific subcellular substances. With this respect a well known application in spermatology is the use of fluorescent conjugates of PNA [242], ConA [439,440] or PSA [441] to detect specific carbohydrates from the inner side of the acrosome. This property makes PNA, ConA and PSA useful for the detection of acrosome reacted spermatozoa [8,442]. Using ultra structural labelling techniques, Fierro et al. [443] showed that WGA bound to the surface of human spermatozoa. In this paper the use of PNA as a marker lectin for the outer acrosomal membrane and WGA as a marker for the sperm plasma membrane is described for the first time. Another lectin with affinity for the plasma membrane (LEA) was less suitable as a marker for this membrane because it also recognized an intra-acrosomal glycogonjugate. Fluorescence microscopy showed that WGA-FITC and LEA-FITC bound to the entire surface of the sperm cell. The observation that the sperm surface was exclusively labelled with WGA-FITC was supported by FACS analysis: in labelling experiments all sperm cells showed intensive staining with WGA-FITC (also observed for LEA-FITC), while the amount of staining was unaffected in sperm samples that had been permeabilized (a slight but significant increase of LEA-FITC staining was observed due to intra-acrosomal staining). Fluorescence microscopy and FACS analysis also supported the specificity of PNA binding to the acrosomal region of sperm cells with permeable membranes. An ELLBA has been developed to quantify lectin binding sites originating from the plasma membrane and the outer acrosomal membrane in order to qualify the progress of plasma membrane purity upon our isolation procedure. With the use of PNA-conjugates we were able to detect the amount of outer acrosomal contamination in sperm plasma membrane fractions. Obviously, it is important to follow especially the outer acrosomal membrane contamination in these fractions because this membrane is positioned closely to the apical sperm plasma membrane and it was found to be the primary source of membrane contamination in our plasma membrane isolates. In fact as evidenced in this paper with ultrastructural (see also [402,426]) and fluorescence microscopical data, indeed only the acrosome overlying plasma membrane is stripped off the sperm cells during the nitrogen cavitation procedure. This observation demonstrates even further the necessity to evaluate membrane isolates with a reliable marker for the plasma membrane but also for the outer acrosomal membrane.

We consider the isolation of sperm plasma membrane from the outer acrosomal membrane of great importance for molecular studies on (i) the adhesion of the sperm cell to the epithelial lining of the female genital tract and (ii) sperm cell to zona adhesion.

(i) Acrosome intact sperm cells have intimate contacts with the epithelial lining of the female genital tract [444]. During these contacts sperm cells may acquire capacitated properties[445] as hypermotility and a higher affinity for the zona pellucida [446]. Only the interaction of intact

sperm cells (i.e. by molecules on the plasma membrane) to the epithelial lining is of physiological interest since only these cells can fertilize the oocyte. The outer acrosomal membrane may contain other adhesive molecules that also recognize the female genital epithelia but those interactions may only serve to filter the deteriorated sperm cells from the fertile sperm subpopulation. Therefore it is very important to be sure about the quality of the plasma membrane isolates (i.e. the amount of acrosomal contamination).

(ii) A similar scenario is valid for sperm cell adhesion to the zona pellucida. Most likely this adhesion is initiated by one or more (peripheral or integral) glycoproteins of the apical sperm plasma membrane [251]. However, after this adhesion event the sperm cell is primed to undergo the acrosome reaction. The acrosomal membranes as well as the glycoprotein matrix in the lumen of the acrosome probably contain glycoproteins that adhere to the zona pellucida [6,272,447]). Only glycoproteins from the plasma membrane can serve as primary zona adhesion molecules and therefore acrosomal contamination should be eliminated from plasma membranes as far as possible in order to dissect between primary and secondary (e.g. acrosin [448,449], which is not present in the plasma membrane isolate) zona binding.

Interestingly, lectins can be employed for further purification of sperm membrane fractions by using immobilized marker lectins as solid state in affinity chromatography. In fact, the plasma membrane fraction can be isolated from a crude membrane isolate from hepatocytes with the use of WGA linked to dextran [450].

The morphology of the isolated plasma membranes was detected with transmission electron microscopical techniques. The majority of the pieces of isolated plasma membrane fraction were resealed as unilamellar vesicles. Such vesicles have a right side-outside orientation [424], which makes these vesicles potent tools for adhesive studies. The membrane proteins are in a more physiological condition in these vesicles than after solubilization from the membrane with detergents [423]. These vesicles can be used to determine fusion properties of the sperm plasma membrane (i.e. the acrosome reaction with inside-outside vesicles, and the fertilization fusion with right side-outside vesicles).

In conclusion, with the use of WGA and PNA it is now possible to assay cavitated plasma membrane isolates from sperm cells for the amount of plasma membrane material and outer acrosomal membrane material respectively. Further purification was obtained after centrifugation over a sucrose density gradient. In the future better results are expected with lectin affinity chromatography methods. Highly purified plasma membrane preparations, and more explicitly, almost free from outer acrosomal membrane contamination, are of great importance for biochemical characterization of plasma membrane specific molecules involved in sperm adhesion processes for fertilization.

Acknowledgments

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Chapter 3

Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane

Frits M. Flesch, Ben Colenbrander, Lambert M.G. van Golde, and Barend M. Gadella

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Abstract

Capacitation (activation) of mammalian spermatozoa is accompanied by protein phosphorylation, elevation of the intracellular calcium concentration and an increased plasma membrane fluidity. The subcellular localization of tyrosine phosphorylation during capacitation have not yet been elucidated. The aim of this study was to investigate whether boar sperm capacitation induces tyrosine phosphorylation of plasma membrane proteins.

Capacitation induced tyrosine phosphorylation of 3 proteins (27, 37 and 40 kDa), which coincided with an increase in the plasma membrane fluidity. The importance of the induced tyrosine phosphorylation in sperm binding to the zona pellucida and the induction of the acrosome reaction is discussed.

Introduction

Freshly ejaculated spermatozoa are not capable to fertilize the oocyte [6,173]. In order to acquire fertilizing ability, the spermatozoa have to undergo an activation process called capacitation. Although capacitation is of high importance, the biochemical understanding of this process is still far from complete. Especially the significance of capacitation induced changes for the primary binding of the sperm cell to the zona pellucida (the extracellular matrix of the oocyte) and for the subsequent acrosome reaction (fusion of the apical plasma membrane with the underlying acrosomal membrane) is still not clear. Capacitation normally occurs in the female genital tract [451], but for in vitro fertilization, the sperm cells are capacitated in chemically defined media [452]. Although minor variations exist between these media, depending on the mammalian species, most of these media contain bicarbonate, calcium and macromolecules (generally BSA) [154]. Bicarbonate activates a sperm specific adenylate cyclase and thereby induces increased cAMP levels in the sperm cell [453]. The subsequent activation of protein kinase A (PKA) [193] induces, via a yet unknown signalling mechanism, tyrosine phosphorylation of several proteins [194,259]. Tyrosine phosphorylation can induce conformational changes in proteins thus leading either to their activation or inactivation [454]. Studies on capacitation and tyrosine phosphorylation have focussed on the whole sperm cell in relation to (i) the binding of sperm cells to the zona pellucida and (ii) the acrosome reaction [180,194,199,260,314]. However, the sperm cell initially adheres to the zona pellucida only with its apical plasma membrane and therefore this site must contain primary zona binding molecules. This site of the sperm plasma membrane is also of significance for the acrosome reaction, since the acrosome reaction is a multiple fusion event of the apical plasma membrane with the outer acrosomal membrane [6]. However, sperm cells contain a considerable amount of tyrosine phosphorylated proteins that are not localized at the apical plasma membrane. For example, flagellar proteins that induce hypermotility after being tyrosine phosphorylated [260]. Taken together, it is of major interest to investigate protein phosphorylation during capacitation at the apical plasma membrane level rather than the entire sperm cell. This necessitates the isolation of the apical sperm plasma membrane proteins from other sperm proteins after in vitro capacitation.

Our major goal was to identify capacitation induced changes in the tyrosine phosphorylation state of proteins in the plasma membrane of boar spermatozoa. Therefore, we incubated washed spermatozoa under different conditions to induce capacitation and isolated the plasma membrane

of capacitated cells following a previously reported method for the isolation of plasma membranes from freshly ejaculated sperm cells [455]. The role of phosphorylation of plasma membrane proteins in the binding of the sperm cell to the zona pellucida and the acrosome reaction will be discussed.

Materials and methods

Semen preparation

Semen was obtained from fertile boars from the Faculty of Veterinary Medicine of Utrecht University and from the Cooperative Centre for Artificial Insemination in Pigs "Utrecht en de Hollanden" (Bunnik, The Netherlands). Freshly ejaculated semen was filtered through gauze to remove gelatinous material. All buffers and other solutions used were iso-osmotic (285-300 mOsm) and kept at room temperature unless stated otherwise.

Semen was allowed to cool to room temperature within 1.5 h after collection. Semen samples were diluted in fixative (0.5% w/v formaldehyde in PBS [456]) and the sperm concentration was estimated in a Bürker cell chamber under a phase contrast microscope (Olympus, Tokyo, Japan). The ejaculate was diluted in Beltsville Thawing Solution (BTS: based on [457]: 0.2 M glucose, 20 mM sodium citrate, 15 mM $NaHCO_3$, 3.36 mM Na_2EDTA , 10 mM KCl, 20 mM Hepes, and 100 µg/ml kanamycin, pH 7.4) to $1.5 \cdot 10^8$ cells/ml and layered on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient of 35% and 70% [176]. Seminal plasma was removed by subsequent centrifugation for 10 min at 300 *g* and for 20 min at 750 *g*.

Induction of capacitation and the acrosome reaction

In vitro capacitation was performed at 38˚C in a humidified incubator with 5% CO_2 in air. Sperm cells were incubated in Tyrode's medium [458] (100 mM NaCl, 21.7 mM lactate, 20 mM Hepes, 15 mM $NaHCO_3$, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, and 100 µg/ml kanamycin, pH 7.4). BSA was omitted to avoid disturbance of biochemical assays. Controls were incubated in Tyrode's medium without $NaHCO_3$ and/or $CaCl_2$ (NaCl was added to maintain osmolality at 300 mOsm) in the same incubator in air-tight sealed flasks. The gas phase in the air-tight sealed flasks was refreshed outside the incubator every 15 minutes.

To obtain mixed vesicles (plasma membrane fused with acrosomal membrane), we induced the acrosome reaction by calcium ionophore A23187 (Sigma, St. Louis, MO) as described previously [459] with modifications: Percoll washed sperm samples that were additionally three times washed in Tris Buffered Sucrose Solution (TBSS: 5 mM Tris, 0.25 M sucrose, pH 7.4) 10 min at 2500 *g*) were incubated for 1.5 h in TBSS supplemented with 5 mM glucose, 2 mM $CaCl_2$ and 2 µM calcium ionophore A23187 (dissolved in DMSO at 2 mM) at 38˚C. Incubated spermatozoa were subjected to nitrogen cavitation and subsequent membrane isolation.

Detection of membrane fluidity

To determine the effect of the addition of bicarbonate, calcium, and BSA to Tyrode's medium, a flow cytometric assay was performed using merocyanine-540 (Molecular Probes, Eugene, OR)

as a membrane fluidity probe and Yo-Pro 1 (Molecular Probes, Eugene, OR) as a viability stain [46]. Tubes containing medium with bicarbonate were bubbled through with 5% CO_2 in air. Sperm samples were incubated at 38˚C in a water bath. After opening of the tubes that contained bicarbonate in the medium, the air phases were flushed with 5% CO_2 in air, and the tubes were air-tight resealed. Sperm samples (500 µl) were incubated for 1.5 min at 38˚C with 0.8 µM merocyanine-540 and 60 nM Yo-Pro 1. Flow cytometry was performed as described by Harrison et al. [46]. Sperm samples from three different boars were analysed. The amount of capacitated sperm cells was expressed as the percentage (mean ± SEM) of living cells (no Yo-Pro 1 fluorescence) with increased membrane fluidity (high merocyanine-540 fluorescence) of the total recorded sperm specific events (10.000). Statistical analysis including the factor time was done using two-way ANOVA, whereas statistical analysis at a certain time point was done using the Student's-t Test.

Plasma membrane isolation

Sperm samples were washed twice (2500 *g*, 10 min, room temp) in TBSS except for the induced acrosome reacted spermatozoa to avoid removal of mixed vesicles that resulted from the plasma membrane fusions with outer acrosomal membrane. Plasma membrane isolation and characterization was performed as described before for fresh ejaculated sperm cells [455]. Briefly, spermatozoa suspensions in TBSS were subjected to nitrogen cavitation (10 min, 45 bar) in a cell disruption bomb (Type 4639: Parr Instrument Company, Moline, IL) at room temperature. The spermatozoa suspension was slowly extruded in a clean tube and 0.2 mM PMSF was added. The plasma membrane was purified by differential centrifugation and the final pellet (285.000 *g*) contained the plasma membrane vesicles. The purity of the plasma membrane isolates was established by marker enzyme analysis (succinate dehydrogenase (SDH), acrosin, alkaline phosphatase and 5'-nucleotidase) and by the quantification of epitopes for WGA (*Triticum vulgare* (wheat germ) agglutinin; specific for the plasma membrane) and epitopes for PNA (*Arachis hypogaea* (peanut) agglutinin; specific for the outer acrosomal membrane) with an enzyme linked lectin binding assay (ELLBA [455]). Purification rates were calculated (mean ± SEM)

Western-blotting

In order to visualize proteins with phosphorylated tyrosine residues, samples containing 10 µg protein were separated on 12% gels (SDS-PAGE) and blotted on polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MO). Blots were first blocked for 10 min with 0.3% (v/v) Tween-20 in TBS (20 mM Tris, 100 mM NaCl, pH 7.4) and further blocked for 1 h with 1% (w/v) BSA in TBS containing 0.05% (v/v) Tween-20. Subsequently, the blots were incubated with 0.5 µg/ml anti-phospho tyrosine antibody conjugated to horseradish peroxidase (PY-20, Calbiochem-Novabiochem Corporation, La Jolla, CA) in TBS containing 0.05% (v/v) Tween-20 and 0.1% (w/v) BSA. The proteins that bound the antibody were visualized, after extensive washing, with 1.7 mM diaminobenzidine in 50 mM Tris (pH 7.2) containing 1.3 µM $NiCl_2$ and 1.8 µM H_2O_2 [417].

Results

Isolation of plasma membrane from capacitated spermatozoa

Plasma membranes of capacitated and control incubated sperm cells were isolated as described before for freshly ejaculated sperm cells [455]. The purity of plasma membrane isolates was evaluated by measuring a number of marker enzymes (Table 1). Plasma membrane isolates were almost free of detectable amounts of mitochondrial (SDH) or inner acrosomal membranes (acrosin). The relative specific enzyme activities of alkaline phosphatase and 5'-nucleotidase (both plasma membrane markers) were markedly increased in all plasma membrane isolates compared to the crude homogenate (Table 1). The increase in plasma membrane preparations of alkaline phosphatase activity in the capacitated sperm preparations (6 times) was not as high as in both freshly ejaculated and control incubated sperm samples (8 times), although these differences were not significant. The increase of 5'-nucleotidase activity in plasma membrane preparations compared to the sperm cell homogenate was 10, 13, and 11 times in the fresh, control, and capacitated sperm samples, respectively (Table 1).

In addition, an enzyme linked lectin binding assay (ELLBA) was performed on the sperm cell homogenate and the plasma membrane isolates to quantify the amount of outer acrosomal membrane in the isolated plasma membrane. Plasma membrane isolates from capacitated sperm cells were 2 times increased in plasma membrane epitope recognized by WGA compared to the PNA epitope on the outer acrosomal membrane (Table 1). So the ratio plasma membrane vs outer acrosomal membrane is 2:1, likely due to increased amount of acrosome reacted sperm cells during the in vitro capacitation. As expected, fresh and control sperm cells showed a much higher amount of plasma membrane to outer acrosomal membrane ratio in the isolates (4:1 and 5:1 respectively; Table 1).

Table 1. Isolation of plasma membranes from capacitated and control sperm cells. The specific activities of marker enzymes or marker lectins were measured in plasma membrane isolates (PM) and whole sperm cell homogenate (WS), and expressed as ratio (PM/WS, mean ± SEM, n=4).

enzyme or lectin rate	freshly ejaculated sperm cells[e]	control incubated sperm cells[e]	capacitated sperm cells[f]
SDH[a]	<0.05	<0.05	<0.05
acrosin[b]	<0.01	0.02 ± 0.01	0.05 ± 0.04
alkaline phosphatase[c]	8.1 ± 3.3	8.0 ± 1.8	6.0 ± 1.9
5'-nucleotidase[c]	10.4 ± 2.6	12.8 ± 2.6	10.8 ± 2.1
WGA/PNA[d]	4.0 ± 1.0	4.8 ± 1.5	2.0 ± 0.7

[a] succinate dehydrogenase: marker enzyme for the mitochondria
[b] marker enzyme for the inner acrosomal membrane
[c] marker enzyme for the plasma membrane
[d] WGA/PNA: the WGA and PNA binding for homogenate and plasma membrane isolates was detected by ELLBA, the WGA/PNA ratio in homogenate and isolates was calculated where after the increase in rate was calculated [15]
[e] control sperm cells before and after 2 h incubation at 38˚C in Tyrode's medium without bicarbonate
[f] capacitated sperm cells incubated for 2 h at 38˚C in Tyrode's medium with bicarbonate

Tyrosine phosphorylated proteins in sperm cells and isolated plasma membranes

An antibody raised against phosphorylated tyrosine residues (PY-20) was used to detect tyrosine phosphorylated proteins on immunoblots of sperm protein samples. Interestingly, isolated plasma membranes of capacitated sperm cells contained three tyrosine phosphorylated proteins (27, 37 and 40 kDa; Fig.1: lane 6), whereas, these proteins were not phosphorylated prior to capacitation or after induced acrosome reaction (Fig. 1: lanes 2 and 4). The tyrosine phosphorylation of these proteins was already detectable after 30 min incubation in capacitation medium (data not shown). Control incubations (omission of bicarbonate, 2 h, 38˚C) sometimes led to similar but much less intensive tyrosine phosphorylation of the sperm plasma membrane proteins (Fig. 1: lane 8). Besides the three clearly stained protein bands in plasma membrane isolates from capacitated sperm cells, the Western blot analysis revealed three other plasma membrane proteins (34, 47 and 55 kDa) that were tyrosine phosphorylated, however, these proteins were less intensively stained. Western blot analysis of crude sperm homogenates revealed that a different panel of proteins are tyrosine phosphorylated (31, 34, 41, 44, 46 and 48 kDa; Fig. 1: uneven lanes). Induction of in vitro capacitation and acrosome reaction led to an increase in tyrosine phosphorylation of a 34 kDa protein (Fig. 1: lanes 1 and 5), whereas the tyrosine phosphorylation of a 48 kDa sperm protein was significantly reduced after both treatments (Fig. 1: lanes 1 and 5) compared to the freshly ejaculated sperm cells (Fig. 1: lane 3).

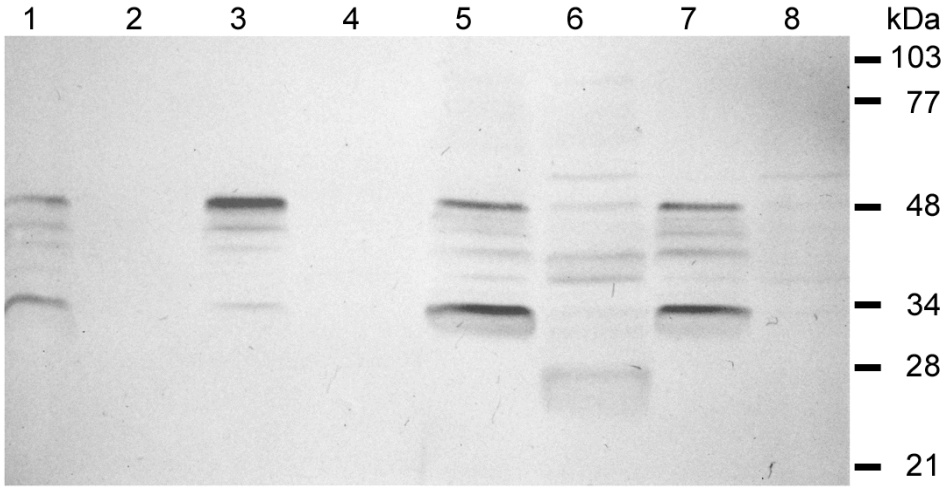

Figure 1. Immuno-detection of tyrosine phosphorylation in boar sperm proteins samples. Solubilized proteins samples (10 µg) were loaded and separated on 12% slab gels as described in materials and methods. After blotting on polyvinylidene fluoride membranes phosphorylated tyrosine residues were detected by PY-20 conjugated to horseradish peroxidase. Uneven lanes: solubilized proteins from whole sperm cells. Even lanes: solubilized proteins samples from corresponding isolated plasma membranes. Lanes 1, 2: calcium ionophore treated samples, lanes 3, 4: Percoll washed, freshly ejaculated samples, lanes 5, 6: capacitated samples (2 h incubated), lanes 7, 8 : control samples (2 h incubated).

Detection of capacitation in boar sperm cells

Although capacitation is defined as the ability of a sperm cell to fertilize a metaphase II arrested oocyte [6], we used a more simple in vitro assay to assess the membrane fluidity, that is known to be increased in capacitated sperm cells [46]. We analysed the capacitated living cells; i.e. those sperm cells that showed high merocyanine-540 fluorescence (increased membrane fluidity), in combination with the exclusion of Yo-Pro 1 (a membrane impermeable DNA stain). Bicarbonate was the essential compound to induce capacitation in the sperm samples (Fig. 2, $p<0.0001$). If bicarbonate was in the medium, 33.1% (± 3.4) of the sperm cells showed an increased membrane fluidity after 2 h, compared to 7.3% (± 1.2) in the groups without bicarbonate (in all tested conditions). Furthermore, calcium accelerated the induction of increased membrane fluidity in a large part of the incubated sperm cells, both in the case BSA was in the medium (Fig. 2B) as well as BSA was omitted (Fig. 2A, $p<0.0001$). The addition of BSA to the medium had no significant effect on the membrane fluidity in the first 30 min of the experiment. Although the percentage of capacitated sperm cells at the end of the experiment differed (without BSA: 37.6% ± 4.2; with BSA: 28.6% ± 2.8), these differences were not significant. It should be noted, however, that incubations with BSA in combination with bicarbonate induced some cell death (Fig. 2B, note the asterisks), while incubations without BSA retained over 90% viable cells during the 2 h incubation period.

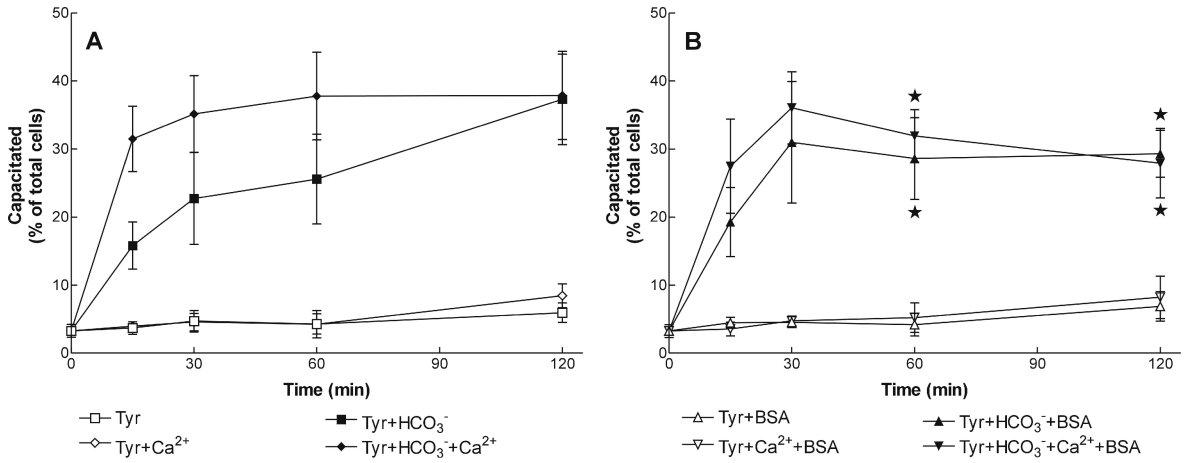

Figure 2. Effect of medium composition on capacitation of boar sperm cells. Percoll washed sperm cells were incubated at 38˚C in Tyrode's medium (additives: 15 mM $NaHCO_3$; 2 mM $CaCl_2$; 3 mg/ml BSA: for symbols see legends, Fig. 2A: without BSA, Fig. 2B: with BSA) and samples were analysed on a flow cytometer after the addition of merocyanine-540 (0.8 μM; membrane fluidity probe) and Yo-Pro 1 (60 nM; viability probe) [21]. Only viable (low Yo-Pro 1 fluorescence) sperm cells with a high membrane fluidity (high merocyanine-540 fluorescence) were scored as capacitated and expressed as percentage of the total analysed cells (10.000). Over 90% of the cells were viable except for the time points marked with an asterisk (Fig. 2B). Ejaculates from three different boars were used to obtain data.

Discussion

The involvement and importance of protein tyrosine phosphorylation in (in vitro) sperm capacitation has recently been reviewed [154]. Nevertheless, no attempts have been made to characterize capacitation induced tyrosine phosphorylation of proteins localized specifically in

the sperm plasma membrane. However, the tyrosine phosphorylation of plasma membrane proteins is of special interest, since these specific proteins are believed to initiate binding to the zona pellucida and induce acrosome reactions [199,260]. Kalab et al. [194] performed studies with crude boar sperm homogenates and reported several proteins, that were tyrosine phosphorylated (p34, p38, p40, p44) or became (more intensely) tyrosine phosphorylated upon bicarbonate induced capacitation (p20, p55, p93 p175, p220/230). In this manuscript we report for the first time a successful isolation of the apical plasma membrane of capacitated sperm samples. The tyrosine phosphorylation status of proteins from this membrane isolate were compared to plasma membrane isolates from control and calcium ionophore treated sperm samples. Three plasma membrane proteins (27, 37 and 40 kDa: p27, p37 and p40) were tyrosine phosphorylated after in vitro capacitation, but not after control or calcium ionophore treatments. It seems that at least one plasma membrane protein (p27) had not been detected by Kalab et al. [194], probably due to the fact that their experiments had been performed with whole sperm cells rather than with plasma membrane isolates.

Incubation of boar spermatozoa in Tyrode's medium (with our without bicarbonate) at 38˚C during 2 h, and subsequent analysis of the proteins in the sperm homogenate, resulted in our experiments in the increase of tyrosine phosphorylation of a 33 kDa protein, probably p34 and in a decrease in tyrosine phosphorylation of a 48 kDa protein, probably p44 or p55. In contrast with our results, Kalab et al.[194] did not observe changes in tyrosine phosphorylation induced by in vitro capacitation of the 33 and 48 kDa non-plasma membrane proteins. Differences in the detection method of the tyrosine phosphorylated proteins could contribute to this discrepancy: (i) different antibodies (4G10 and PT66 with a secondary antibody linked to horseradish peroxidase vs. PY20 directly linked to horseradish peroxidase) and (ii) different visualization methods (chemiluminescence detection kit vs. diaminobenzidine staining). Incubation of spermatozoa with calcium ionophore and calcium in the medium resulted in a decreased tyrosine phosphorylation signal, which agrees with results obtained by Kalab et al.[194]. This may indicate that the tyrosine phosphorylation is inhibited by an increased concentration of intracellular calcium or that the increased intracellular calcium concentration without bicarbonate is not sufficient to phosphorylate the proteins.

Capacitation-induced protein tyrosine phosphorylation has never been studied specifically for the sperm plasma membrane. In fact this manuscript describes for the first time a successful attempt to isolate and characterize apical plasma membranes from in vitro capacitated sperm cells using a method previously described by us to isolate plasma membranes from freshly ejaculated spermatozoa [455]. The clear-cut discrepancies in tyrosine phosphorylation patterns between total and apical plasma membrane proteins described in this paper show the necessity to separate the apical plasma membrane from the other sperm structures. We showed that the obtained apical plasma membrane preparations are useful to study capacitation induced changes at the plasma membrane level. In order to study plasma membrane proteins derived from capacitated spermatozoa, it is of major importance to check the purity of the plasma membrane isolates. All plasma membrane isolates were virtually free of inner acrosomal and mitochondrial membranes. The plasma membrane isolates derived from capacitated sperm samples were a little bit contaminated with outer acrosomal membrane material when compared to freshly ejaculated and

control incubated spermatozoa. This increase in outer acrosomal membrane material in the plasma membrane preparations is likely due to spontaneous acrosome reactions (data not shown and [460]).

We investigated which capacitation factors were required for the activation of tyrosine kinases. For this purpose we incubated sperm cells with media containing either bicarbonate, calcium or combinations and followed the changes in tyrosine phosphorylation of apical plasma membrane proteins. In addition, the increase in membrane fluidity (which is indicative for in vitro capacitation) was followed using a merocyanine-540 flow cytometric assay [46]. The induction of tyrosine phosphorylation was strictly dependent on bicarbonate. Omission of calcium resulted in comparable levels of tyrosine phosphorylation as in complete capacitation medium. A similar scenario was observed for the increase in membrane fluidity. As previously described, the increase in merocyanine-540 fluorescence in living sperm populations was strictly dependent on bicarbonate [46]. Addition of BSA to the Tyrode's medium supplemented with bicarbonate seemed to accelerate the membrane fluidity, although the differences were not significant. Addition of calcium to the Tyrode's medium supplemented with bicarbonate did accelerate the response to bicarbonate, although the responses were nearly identical after prolonged incubations (i.e. 2 h). It should be noted that prolonged incubations in Tyrode's medium supplemented with bicarbonate and BSA (with or without calcium) resulted in 10-20% cell death (see asterisks in Fig. 2, panel B). To show capacitation, we made use of a physiological relevant consequence of capacitation: capacitated cells have an increased plasma membrane fluidity [46]. Despite the fact that capacitation is often determined with the CTC stain [460], the physiological background of this stain is unclear and assessments can not be done in a simple flow cytometer. The power of the flow cytometer, however, is that it can detect multiple fluorescence signals of thousands of cells within a few seconds and that the staining protocols are not labourious [186]. Therefore, we preferred to use flow cytometric analysis with merocyanine-540 and Yo-Pro 1 to detect the plasma membrane fluidity and viability status of sperm cells as a measure for capacitation [46]. Nevertheless, on less accurate basis similar bicarbonate dependent responses were detected using the CTC stain (data not shown). Altogether these results indicate that our treatments indeed induced a bicarbonate dependent increase in plasma membrane fluidity. Although from these results it can be concluded that the changes in tyrosine phosphorylation on the plasma membrane and the remaining part of the sperm cell are linked to the bicarbonate dependent in vitro capacitation process, it is not clear how this is coupled with the increased membrane fluidity.

Several proteins with zona pellucida binding properties have been identified in human spermatozoa of which three had phosphorylated tyrosine residues present (14-18, 51 and 95 kDa) [260]. Also for mouse spermatozoa a 95 kDa has been proposed to be a zona pellucida receptor with phosphorylated tyrosine residues [252]. Aggregation of the 95 kDa receptor in mouse spermatozoa has been postulated as an important preparative step in the acrosome reaction [307]. However, tyrosine phosphorylation of a 95 kDa protein in boar sperm or in boar sperm plasma membranes was not observed. This probably implies that boar sperm can not be compared to human or mouse sperm with respect to the 95 kDa protein. On the other hand, Kalab et al [194] reported a 93 kDa boar sperm protein with phosphorylated tyrosine residues and it was also

reported that this protein is insoluble for the detergent Triton X-100. This finding in combination with our results showed that the 93 kDa protein is most likely not a plasma membrane protein.

In conclusion, it could be demonstrated that isolated plasma membranes from capacitated boar spermatozoa are an ideal object to study plasma membrane specific changes induced by capacitation. The apical plasma membrane is the specific site of the sperm cells that contain the proteins involved in primary zona pellucida binding as well as proteins involved in the induction of the subsequent acrosome reaction. Therefore it is of major interest to further investigate the function of the three plasma membrane proteins that become tyrosine phosphorylated (p27, p37 and p40) upon capacitation.

Acknowledgments

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Chapter 4

Capacitation dependent tyrosine phosphorylation of sperm head plasma membrane proteins is involved in primary zona pellucida binding

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submitted for publication

Abstract
The recognition and binding of sperm cells to the zona pellucida (the extracellular matrix of the oocyte) are essential for fertilization and are believed to be species specific. Freshly ejaculated sperm cells do not bind to the zona pellucida. Physiologically this interaction is initiated after sperm activation in the female genital tract (capacitation) via a yet unknown mechanism, resulting in the binding of a receptor in the apical sperm plasma membrane to the zona pellucida. In order to mimic this biochemically we isolated zona pellucida fragments to prepare an affinity column with the intact zona pellucida structure and loaded this column with solubilized apical plasma membranes of sperm cells before and after in vitro capacitation. With this technique we demonstrated that two plasma membrane proteins of capacitated boar sperm cells showed high affinity for zona pellucida fragments. Further analysis showed that these proteins were tyrosine phosphorylated. Plasma membrane proteins from freshly ejaculated sperm cells did not exhibit any zona pellucida binding proteins, likely because these proteins were not tyrosine phosphorylated.

Introduction
The first contact between the oocyte and the sperm cell is the binding of the sperm cell to the zona pellucida (the extracellular matrix of the oocyte, ZP) and this is a species specific event [6]. At the ultra-structural level the apical sperm plasma membrane will make the first contact with the ZP and thus the proteins that adhere and bind to the ZP, have to be situated in the sperm plasma membrane or its extracellular coating [461]. After binding of ZP proteins to the sperm-ZP receptor(s), the extracellular signal has to be transduced to initiate the acrosome reaction [462], thus most likely a transmembrane protein is involved in the sperm-oocyte interaction. Before a freshly ejaculated sperm cell can fertilize the oocyte, it has to be activated in the female genital tract [463]. In fact this activation (capacitation) is probably a key regulatory event for the sperm-ZP interaction [174]. The sperm-ZP affinity increases during capacitation by uncovering of putative ZP receptors [464,465] or by changes in the binding properties of receptors for the ZP [466]. The binding properties of a receptor could be changed by conformational changes (e.g. by phosphorylation [247]), dimerization or a combination of these. Indeed it has been described before that several sperm proteins become tyrosine phosphorylated upon in vitro capacitation [180,467]. Isolation of plasma membranes from intact sperm cells showed that in vitro capacitation induced also tyrosine phosphorylation of proteins in the boar sperm plasma membrane [175]. Aggregation in combination with tyrosine phosphorylation of putative ZP receptors has also been reported for mouse sperm cells [307].

Identification and characterization of sperm proteins with ZP affinity are of major interest, since the mechanism of sperm-oocyte interaction is still unclear. Scientifically this is of interest not only to gain understanding of the appropriate interaction that results in fertilization, but also for immuno-contraception in which the sperm-ZP receptor is a putative target for vaccines [468]. Several candidates for the primary ZP receptor have been postulated for spermatozoa from different mammalian species (for review see [243,251,469]). However, there is still no consensus about the binding mechanism and the proteins involved in the ZP binding. The goal of this study was to investigate the sperm-ZP interaction in an in vitro system in which the sperm-ZP binding

proteins and the ZP proteins were allowed to interact in a bioaffinity assay that resembles as far as possible the physiological situation: (i) instead of using solubilized ZP proteins [470-472] we used ZP fragments (this ensures a correct three-dimensional and native protein structure); (ii) instead of using total sperm protein samples [245] we used apical plasma membranes (the known site for sperm-ZP interaction) isolated from capacitated boar sperm cells [175]. Thus the two sites involved in the primary sperm-ZP interaction were isolated and characterized, and a ZP affinity column was prepared to obtain the sperm proteins that are most likely to be involved in this interaction.

Materials and methods

Capacitation and plasma membrane isolation

Semen was obtained from fertile boars from the Faculty of Veterinary Medicine of Utrecht University and from the Cooperative Centre for Artificial Insemination in Pigs "Utrecht en de Hollanden" (Bunnik, The Netherlands). Freshly ejaculated semen was filtered through gauze and subsequently washed through a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient as described before [176,455]. Capacitation was induced by incubation of spermatozoa in Tyrode's medium [458] without BSA for 2 h at 38˚C [176], and plasma membranes were isolated by nitrogen cavitation and differential centrifugation as previously described [175]. Briefly, after capacitation sperm cell suspensions were washed twice (5 mM Tris, 0.25 M sucrose, pH 7.4) at room temperature, 1000 *g*, 10 min) and subjected to nitrogen cavitation (10 min, 45 bar) in a cell disruption bomb (Parr Instrument Company, Moline, IL) at room temperature. The sperm cell suspension was slowly extruded in a clean tube and 0.2 mM PMSF was added. The plasma membrane was purified by differential centrifugation and the final pellet (285.000 *g*) contained the plasma membrane vesicles. The purity of the isolated plasma membranes was checked by marker enzymes and marker lectins as described before [455].

Zona pellucida isolation

Ovaries were isolated from cooled (4˚C) slaughterhouse material (kindly provided by "Hendrix' vlees Druten BV", Druten, The Netherlands) within a few hours after slaughtering of the gilts. Ovaries were stored directly after isolation in ovary buffer [473] on ice and transported to the laboratory. Ovaries were washed and stored at -20˚C until further use. Zonae pellucidae were isolated from thawed ovaries as described before [473]. Briefly, ovaries were ruptured by a series of ganged razor blades and oocytes were squeezed out and collected. Oocytes were isolated and washed on a series of sieves (450, 150 and 75 μm) and collected from the 75 μm sieve. Oocytes were further purified by centrifugation (45 min, 30000 *g*, 4˚C) after the addition of Percoll up to 15% to remove redundant cumulus cells (oocytes collected form the interface) [474]. Oocytes were homogenized in a Potter-Elvehjem tube and ZP fragments were washed and isolated on a 45 μm sieve. Samples obtained during the procedure to check purity and morphology were stored at -20˚C prior to examination.

The purity of the isolated ZP fragments was checked by two-dimensional SDS-PAGE. Therefore, ZP fragments were solubilized (50 mM NH_4HCO_3, pH 7.2 with acetic acid, 30 min, 70˚C) and concentrated on a microcon-30 microconcentrator (Amicon, Beverly, MA).

Concentrated ZP proteins (~25 μg) were separated on a two-dimensional SDS-PAGE and subsequently stained with silver [475].

To measure the quantity of oocyte remnants in the ZP fragments, an enzyme linked lectin binding assay (ELLBA) was developed [455]. For this purpose, the affinity of several lectins for oocyte structures was visualized by labelling oocytes with FITC conjugated lectins. Cryosections (12 μm) were made from fixed oocyte material, placed on a microscopic slide and dried at room temperature. Sections were washed with HBS (Hepes buffered saline, pH 7.4) containing 50 mM glycine for 10 min and subsequently with HBS containing 1% (w/v) BSA. The samples were incubated for 1 h with HBS containing 1% (w/v) BSA, 1 mM $CaCl_2$ and 10 μg/ml of one of the following FITC conjugated lectins (EY Laboratories, San Mateo, CA): PNA (*Arachis hypogaea* (peanut) agglutinin), LEA (*Lycopersicon esculentum* agglutinin), UEA-I (*Ulex europeaeus* agglutinin), GS-I (*Griffonia simplicifolia* I agglutinin), GS-II (*Griffonia simplicifolia* II agglutinin), WGA (*Triticum vulgare* (wheat germ) agglutinin), ConA (*Canavalia ensiformis* agglutinin), MPA (*Maclura pomifera* agglutinin), AIA (*Artocarpus integrifolia* agglutinin), DBA (*Dolichos biflorus* agglutinin), BPA (*Bauhinia purpurea* agglutinin), SBA (*Glycine max* (soybean) agglutinin), LPA (*Limulus polyphemus* agglutinin). After incubation samples were washed 3 times with HBS, and imbedded in 1 drop of Slowfade® (Molecular Probes, Eugene, OR). Subsequently a coverslip was placed on top and sealed with nail polish. Slides were examined under a fluorescence microscope as described before [455]. WGA (binds to ZP material) and LPA (binds to oocyte-material) conjugated to biotin were used in the ELLBA [455].

Since the ZP isolation procedure possibly causes zona hardening resulting in the loss of sperm binding and the subsequent induced acrosome reaction, the bioactivity of the ZP fragments was established. The potency of ZP fragments to bind sperm cells and to induce the acrosome reaction was visualized. Therefore a slide was coated with ZP fragments and subsequently spermatozoa were allowed to bind for 20 min to the coated fragments. The acrosome reaction was shown by staining the acrosomal outer membrane with PNA-FITC (5 μg/ml, 15 min) after chemical fixation and propidium iodide (10 μg/ml) was used as a counter staining [455]. As a control Percoll washed spermatozoa were stained without binding to ZP fragments.

Zona pellucida affinity

The affinity of biotinylated ZP proteins for immobilized sperm (plasma membrane) proteins was measured in analogy with an ELISA-like assay described before [455]. Briefly, either sperm homogenate or plasma membrane proteins were overnight coated in 0.1 M Na_2CO_3 (pH 9.6) on a 96-wells plate and after blocking of the 96 wells plate, solubilized ZP proteins conjugated to biotin (kindly provided by E. Topper, ID-DLO Lelystad, The Netherlands) were allowed to bind for 1 h. Bound ZP-biotin was measured after coupling of streptavidin conjugated horseradish peroxidase and expressed as enzyme activity (absorbance at 450 nm).

ZP fragments (3.5 mg protein) were coupled to 500 mg activated CH Sepharose 4B (Pharmacia Co., Uppsala, Sweden) according to the manufacturers manual. By this method ZP fragments were coupled to the beads as was confirmed by phase contrast microscopy and only the proteins of the ZP fragments that were directly coupled to the beads were chemically modified (covalent amide linkage to the beads) whereas the remaining proteins of the coupled ZP fragments

were unaltered. Isolated plasma membrane proteins (250 μg) form boar spermatozoa were solubilized using 0.05% (v/v) Tween-20 and subsequently applied on the column. All elution buffers contained 0.05% (v/v) Tween-20. Non-binding proteins were eluted with HBS, whereas proteins with ZP affinity were eluted with HBS adjusted to 1 M NaCl. Fractions were collected and stored at -20˚C until further use. Eluted proteins were detected continuously at 275 nm or afterwards in the collected fractions by measuring the protein concentration after trichloroacetic acid precipitation [476].

Proteins in the non-binding peak (directly after loading of the column) and binding peak (the first 3 ml directly after elution with 1 M NaCl) were concentrated by precipitation with trichloroacetic acid (10%), subsequently solubilized with 2% ammonia and dried in a Speedvac concentrator (Savant, Farmingdale, NY, USA). Molecular mass was determined by SDS-PAGE on a 12% non-reducing gel and subsequent silver staining (Biorad Laboratories, Richmond, CA, USA). Tyrosine phosphorylated proteins were visualized after SDS-PAGE on a 12% non-reducing gel and subsequent Western blotting as described before [175] using an antibody raised against phosphorylated tyrosine residues (PY-20, Pierce, Calbiochem-Novabiochem Corporation, La Jolla, CA, USA).

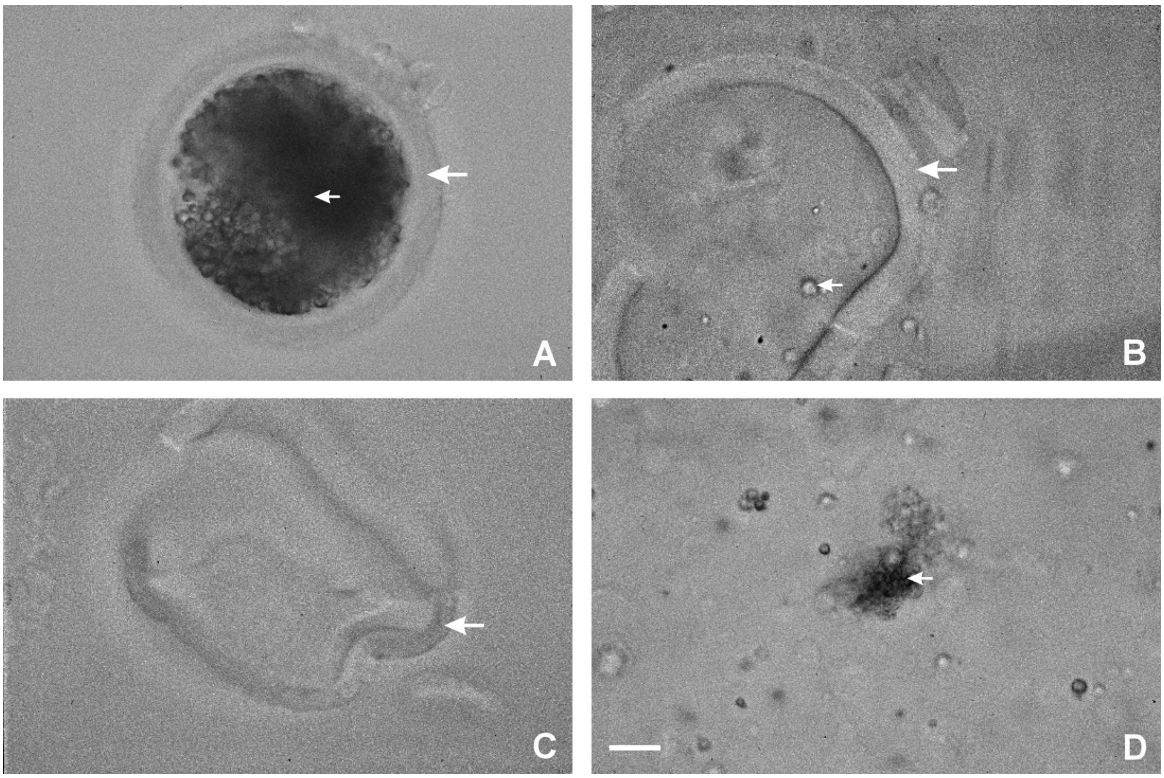

Figure 1. Phase contrast micrographs obtained during the isolation of oocytes and the subsequent preparation of ZP fragments. Oocytes were squeezed out of ovaries and washed through a series of sieves (450, 150 and 75 μm) and collected from the 75 μm sieve. Oocytes were freed from cumulus cells by an additional 15% Percoll wash step (A). Fragmentation of oocytes was done in a Potter-Elvjehem homogenizer (B), and subsequent sieving on a 45 μm sieve resulted in ZP fragments on the sieve (C) and oocytes remnants washed through the sieve (D). Large arrows indicate the zona pellucida (fragments) and small arrows indicate oocyte content (remnants). Bar represents 25 μm.

Results

Zona pellucida isolation

Mature oocytes with a diameter of 75 μm or larger were isolated from ovaries according to Dunbar et al.[473]. Centrifugation of oocytes collected from the 75 μm sieve with 15% Percoll resulted in oocytes with intact zonae pellucidae that were free of cumulus cells as judged from phase contrast micrographs (Fig. 1A). The cumulus free oocytes were homogenized in a Potter-Elvehjem tube and disrupted into ZP fragments and cellular remnants (Fig. 1B). Subsequent sieving (45 μm) resulted in ZP fragments collected from the sieve (Fig. 1C) while cellular remnants passed this sieve (Fig. 1D).

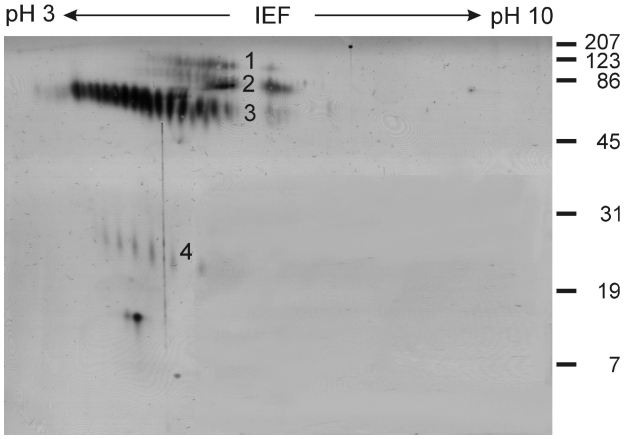

Figure 2. Two-dimensional protein analysis of solubilized zonae pellucidae isolated from pig ovaries. Approximately 25 μg ZP proteins were first separated on an immobilized pH gradient gel (IEF, pH 3-10) and subsequently separated on a 7.5 % SDS-PAGE gel (molecular mass (kDa) is indicated to the left). Proteins were visualized with silver staining. Numbers in the gel refer to groups of porcine ZP proteins originated from unfertilized eggs as described before [474,477].

The isolated ZP fragments were partly solubilized and subjected to two-dimensional SDS-PAGE and subsequently silver stained. The isolated ZP fragments contained the previously described 4 groups of porcine ZP proteins [474,477], whereas only minor amounts of other proteins were detected by silver staining (Fig. 2). Fluorescence microscopy showed that WGA-FITC exclusively stained the ZP but no other oocyte structures (Fig. 3A). In contrast, LPA-FITC stained the cytoplasm of oocytes and cumulus cells (Fig. 3B), but did not stain the ZP. Therefore, these two lectins were used in an ELLBA to quantify the amount of ZP material and contamination in our ZP fragments, homogenates and oocyte remnants. The ZP fragments bound based on protein basis 1.5 ± 0.3 more WGA compared with the homogenate, whereas the oocyte remnants bound less WGA compared with the homogenate (0.2 ± 0.1). In contrast, purified ZP bound no detectable amounts of LPA, whereas the oocyte remnants bound more LPA compared with the homogenate (1.2 ± 0.1). Taken together these results indicated that the isolated ZP fragments were highly enriched in ZP material (two-dimensional SDS-PAGE and WGA binding sites) and virtually free of other oocyte structures (no LPA binding sites).

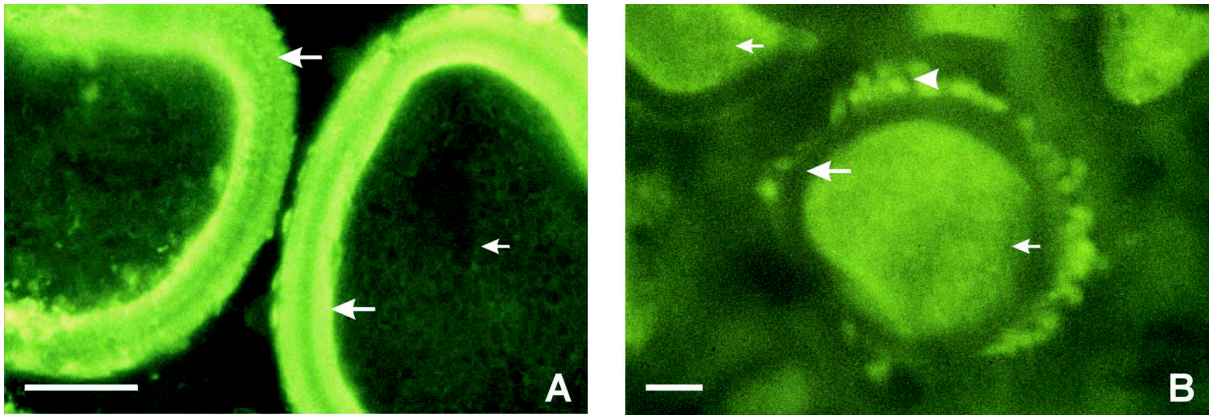

Figure 3. Lectin affinity for the zona pellucida (large arrow) and the oocyte content (small arrow). FITC conjugated lectins (10 µg/ml) were applied to cryosections of isolated oocytes on microscopic slides for 1 h. Subsequently sections were washed, imbedded with antifade and examined under a fluorescence microscope. WGA (A) bound only to the zona pellucida, whereas, LPA (B) bound to the oocyte content as well as to cumulus cells (arrow head). Bar represents 25 µm.

The isolated ZP fragments were bioactive, since the acrosome reaction was induced in sperm cells that bound to the ZP fragments. The Percoll washed sperm sample consisted mainly (>95%) of acrosome intact sperm cells (Fig. 4A; intact acrosome thus PNA-FITC cannot enter the acrosome resulting in the absence of FITC fluorescence). However, if the sperm sample was allowed to bind to the zona pellucida fragments, virtually all sperm cells were acrosome reacting after 20 min (Fig. 4B; PNA-FITC is able to enter the acrosome in acrosome reacting sperm cells resulting in FITC fluorescence).

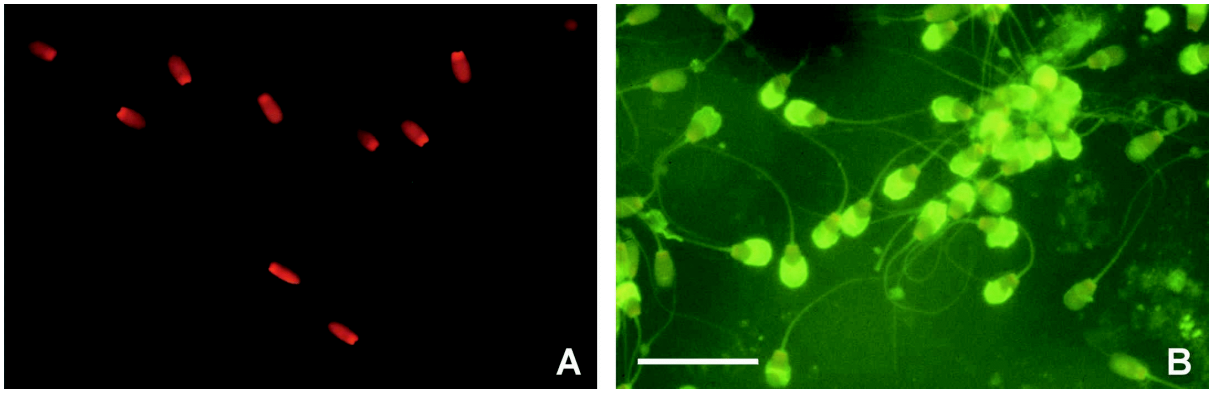

Figure 4. Isolated ZP fragments induce acrosome reaction in sperm cells. Sperm cells were labelled for 15 min with 5 µg/ml PNA-FITC (green) and counter stained post fixation with 10 µg/ml propidium iodide (red) to stain all sperm nuclei. PNA-FITC will not stain acrosomes of sperm cells with intact acrosomes, since PNA-FITC is not able to enter the acrosome. PNA-FITC will enter and stain the acrosome of sperm cells that are acrosome reacting. Sperm cells were stained without ZP fragments as a control and applied on a slide (A) or after binding for 20 min to a slide coated with ZP fragments (B; note the acrosome intact sperm cells in the upper left corner). After staining and washing, a drop of antifade was applied on the samples and the slides were examined under a fluorescence microscope. Bar represents 25 µm.

Zona pellucida affinity

Sperm protein samples were immobilized and the affinity for ZP proteins was detected in an ELISA-like assay (see materials and methods). Plasma membrane proteins from in vitro capacitated sperm cells bound 4 times more ZP proteins than plasma membrane proteins from control incubated sperm cells (Fig. 5, dotted lines). Plates coated with proteins from capacitated and control total sperm homogenates (both primary as well as secondary ZP binding proteins) showed only slight differences in ZP binding (Fig. 5, solid lines). The amount of ZP proteins bound to the immobilized sperm proteins was both in homogenate and in the case of plasma membranes dose dependently correlated with the amount of immobilized sperm protein (Fig. 5). The ZP affinity of the homogenate decreased if more than 500 ng sperm protein (total homogenate) was applied to the 96-wells plates, probably due to steric hindrance. The experiments shown in Fig. 5 were done in the presence of 1 mM EDTA. Similar results were obtained in presence of 1 mM $CaCl_2$ (data not shown) indicating that primary ZP affinity was not calcium dependent in vitro. A dramatic decrease (less then two times the background) in ZP affinity was obtained if 1 M NaCl was added to the binding buffer instead of 0.15 M NaCl, indicating that the binding is dependent on the ionic strength of the binding buffer.

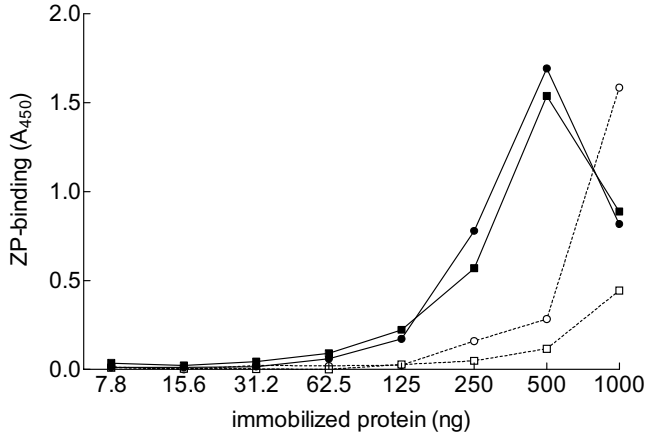

Figure 5. Binding of biotin conjugated ZP proteins to wells coated with proteins from sperm cells (solid lines) or sperm plasma membrane isolates (dotted lines). Sperm cells were either preincubated for 2 h at 38˚C in the presence of 15 mM bicarbonate and 2 mM calcium (capacitated; circles) or in the absence of both components (control; squares). Plasma membranes were subsequently isolated as described in materials and methods. Protein samples (sperm cells or plasma membranes) were overnight coated on 96-wells plates in 0.1 M Na_2CO_3 (pH 9.6). ZP-biotin was detected by an ELISA-like system with streptavidin conjugated to horseradish peroxidase as described before [455] and expressed as the absorbance at 450 nm (A_{450}). Total sperm homogenate showed higher affinity for ZP proteins due to the massive amount of secondary binding proteins at the inner acrosomal membrane.

Sperm plasma membrane proteins with ZP affinity were separated from other proteins by immobilization of ZP fragments (intact three-dimensional structure) on column material and subsequent affinity chromatography of solubilized plasma membranes. The size of the isolated ZP fragments and sepharose beads were approximately equal as estimated by phase contrast microscopy. Only a minor part of the total surface of the ZP fragments was attached to the beads.

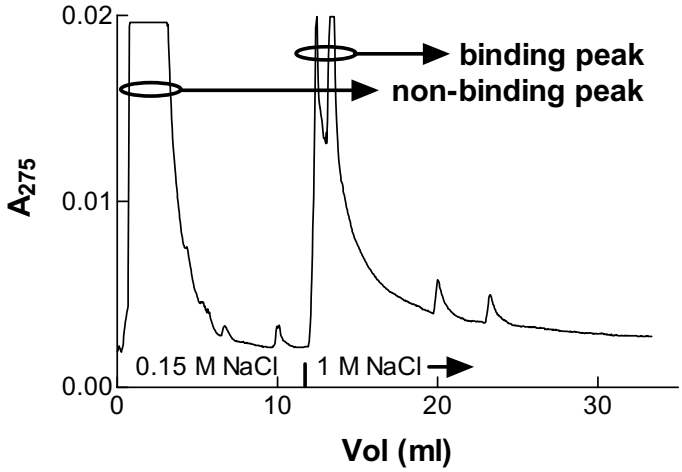

Figure 6. ZP binding proteins isolated from plasma membrane proteins derived from capacitated spermatozoa. Plasma membrane proteins (250 µg) were solubilized with 0.05 % Tween-20 and subsequently applied on an affinity column prepared from ZP fragments. Elution was performed with HBS (0.15 M NaCl) followed with HBS adjusted to 1 M NaCl, resulting in release of the ZP binding proteins.

In vitro capacitated sperm cells contained non-binding plasma membrane proteins that eluted from the column using the 'low-salt' buffer (0.15 M NaCl) and ZP binding proteins that eluted with the 'high salt' buffer containing 1 M NaCl (Fig. 6). Further characterization of this 'high salt' peak on SDS-PAGE showed that it consisted mainly of two proteins of 35 and 46 kDa (Fig 7A, lane 4). When plasma membranes isolated from freshly ejaculated sperm cells were applied on the ZP affinity column, only an elution peak of non-binding proteins emerged directly after loading of the column. Elevation of the NaCl concentration to 1 M did not result in an additional elution peak with ZP binding proteins (data not shown), and SDS-PAGE of the eluate did not reveal any protein bands (Fig. 7, lane 2). The plasma membranes from sperm cells incubated for 2 h at 38˚C but in absence of bicarbonate and calcium (i.e. at non-capacitative conditions) in one case contained some of these two ZP binding proteins. However, when we repeated this experiment twice we failed to detect these ZP binding proteins. Nevertheless, plasma membrane proteins from capacitated sperm cells always showed a peak with ZP binding proteins (n=3). Characterization of the ZP binding proteins on Western blots with PY-20 as a probe for tyrosine phosphorylated proteins, showed that both the 35 and 46 kDa ZP binding proteins were tyrosine phosphorylated (Fig. 7B, lane 4).

Discussion

Since the sperm cell adheres to the zona pellucida with its plasma membrane [461], it seems obviously that the molecules involved in ZP recognition are situated in the sperm plasma membrane. This paper describes the isolation of two ZP binding proteins (35 and 46 kDa) from the plasma membrane derived from capacitated boar sperm cells. Interestingly, the ZP binding properties were absent in plasma membranes derived from freshly ejaculated sperm cells and control incubated (without bicarbonate) sperm cells. Since the two proteins were tyrosine phosphorylated in capacitated sperm cells, but not in freshly ejaculated sperm cells, we propose

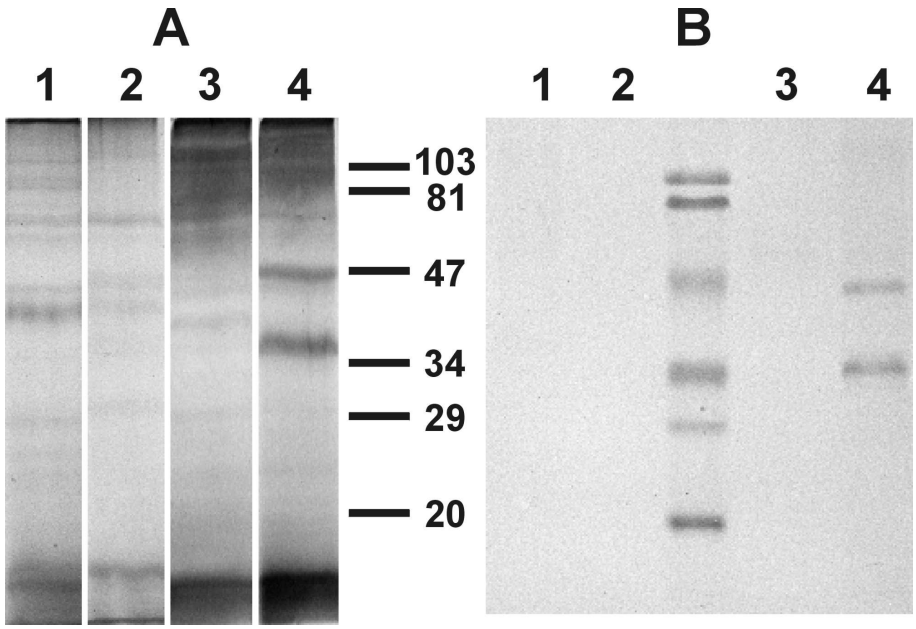

Figure 7. ZP binding proteins isolated from plasma membranes derived from capacitated boar sperm cells consists mainly of two tyrosine phosphorylated proteins. Plasma membrane isolates were solubilized (0.05% Tween-20) and applied on a ZP affinity column. Both the non-binding protein peaks (eluted with 0.15 M NaCl) as wells as the binding protein peaks (the first 3 ml after elution with 1 M NaCl) were collected and proteins in these fractions were TCA-precipitated, subsequently separated on a 12% non-reducing gel, and proteins were stained with silver (A). Similar protein gels were not silver stained but blotted and tyrosine phosphorylated proteins were visualized with horseradish peroxidase conjugated to a specific antibody (PY-20) raised against phosphorylated tyrosine residues (B). Even lanes: binding peak; uneven lanes: non-binding peaks. Lanes loaded with plasma membranes isolated from: 1,2: freshly ejaculated sperm cells; 3,4: capacitated sperm cells. Protein markers are depicted between lanes 2 and 3 and molecular mass (kDa) is indicated to the left. Experiments shown were repeated three times with similar results.

that tyrosine phosphorylation leads to activation of these two ZP binding proteins. The use of ZP fragments instead of solubilized ZP proteins is superior to previously described experiments, since the native three-dimensional structure is better preserved in our experiments. It is known that cell-cell interactions depend on receptor-ligand binding, in which the three-dimensional structure is very important as has previously been described for the zona pellucida binding [237] and the subsequent induction of the acrosome reaction [478]. Previous reports underestimate this important characteristic of cell-cell interaction and describe affinity columns prepared from solubilized ZP proteins [470-472] or Western blot techniques [185,470]. These techniques are often useful, although the interaction of receptor and ligand could be abolished due to treatments that interfere with the native state of the receptor and/or ligand. The molecular mechanism of the sperm-ZP interaction and the following acrosome reaction is still unclear, probably because there is no consensus about the sperm-ZP receptor. We used ZP fragments as the immobilized ligand to ensure that the receptor would face the ZP in its native state. In order to isolate putative ZP receptors, we first capacitated the sperm cells, so putative ZP receptors would be activated [174].

Subsequently the plasma membrane was isolated to discriminate between primary binding and secondary binding [271,273,479] and our approach resulted in the isolation of two proteins

(35 and 46 kDa). Hardy and Garbers [245] used a similar approach to isolate ZP binding proteins from boar sperm, although a different plasma membrane isolation was used and the proteins were subsequently biotinylated. Their experimental design resulted in the isolation of two protein complexes (p105/45 and p56-62) with species specific ZP affinity. Some proteins were visible in the high molecular mass region of our non-reducing SDS-PAGE analysis, although not as clear as the 35 and 46 kDa proteins. The discrepancy between our results and the results described by Hardy and Garbers could be due to differences in the native structure of the plasma membrane proteins (biotinylation by Hardy and Garbers) or differences in the purity of the plasma membrane isolates. The purity of the membranes isolated by Hardy and Garbers were not checked, so probably their membrane isolates contained both primary and secondary ZP binding molecules. Furthermore one of the isolated proteins with ZP affinity was proacrosin/acrosin that is known to be situated in the inner acrosomal membrane [419,420]; thus probably p105/45 and p56-62 are involved in secondary binding. In contrast our plasma membrane isolates were free of acrosin [175], hence our technique can discriminate primary from secondary ZP binding proteins. The ELISA-like binding assay described in this paper showed that proteins from the total sperm cell homogenates (both primary and secondary ZP binding proteins) bound far more ZP proteins compared with proteins from the plasma membrane (only primary ZP binding proteins). Another drawback of a total homogenate was the fact that capacitation did not induce increased ZP affinity, whereas this was clearly the case for plasma membrane isolates (no binding before capacitation).

Although capacitation is a prerequisite for the sperm-ZP interaction [174], the mechanism by which the increased ZP affinity is achieved is not clear. Dimerization as well as phosphorylation of putative ZP receptors have been postulated as the activation process [247,307]. The two proteins described in the present paper are both phosphorylated at tyrosine residues. The phosphorylation is most likely induced by in vitro capacitation and not induced by interactions with the ZP column, since substrates for the phosphorylation of proteins like ATP are not present in sufficient amounts during ZP affinity chromatography with capacitated plasma membrane isolates. It is not clear whether bicarbonate and/or calcium (capacitation factors [46,175]) are required for this tyrosine phosphorylation in Percoll washed sperm cells incubated in Tyrode's medium. Control incubations of Percoll washed sperm cells without bicarbonate and calcium for 2 h at 38˚C resulted twice in the absence of a ZP binding peak. However, in one experiment with plasma membranes derived from control incubated sperm cells a ZP binding peak with the same tyrosine phosphorylated proteins was observed (data not shown). We previously showed that capacitation induced tyrosine phosphorylation of three plasma membrane proteins (27, 37 and 40 kDa), and to a lesser extent the tyrosine phosphorylation of plasma membrane proteins with a molecular mass of 34, 47 and 55 kDa [175]. Most likely, the 34 and 47 kDa proteins are the two isolated ZP binding proteins described in this article. Incubation of sperm cells for 2 h at 38˚C can have a partial capacitative effect despite the absence of bicarbonate and calcium since slightly increased plasma membrane fluidity and weak tyrosine phosphorylation of plasma membrane proteins was observed [175]. It is obvious that the process of capacitation is not a black and white picture, since a population of sperm cells reacts heterogeneously upon a capacitation environment, as well as diversity exists in between ejaculates

and boars [186]. This phenomenon could contribute to the discrepancies between individual experiments.

The increased affinity of a receptor for its ligand can besides phosphorylation be achieved by dimerization. Dimerization of ZP receptors has been postulated in the mouse system [307]. As is known for growth factor receptors, not only homodimers but also heterodimers can be formed to achieve higher affinity for the receptor's ligand [480]. It is possible that capacitation induces heterodimerization of the two described plasma membrane proteins to form a protein complex with high affinity for the ZP. Likely the capacitation induced tyrosine phosphorylation is involved in this putative dimerization process.

The affinity of ZP proteins for sperm proteins in the in vitro system described here is not dependent on calcium ions in the binding buffer, since equal amounts of ZP proteins bound the immobilized sperm proteins (homogenate and plasma membrane) in the presence of EDTA or calcium. Gamete interaction in pig seems to be calcium dependent, since sp38, a ZP binding protein isolated from detergent extracts from porcine epididymal sperm, bound in a calcium dependent manner to ZP [275]. However, further analysis showed that this sp38 was an acrosomal enzyme and thus could not be involved in primary sperm-ZP interaction [276]. Previous reports described that calcium is important for gamete binding in mouse using intact epididymal sperm cells and oocytes [481]. However, the increased binding of sperm cells to oocytes seemed to be based on the preincubation of sperm cells instead of the binding itself. Discrepancies between the study of Saling et al. [481] (zona binding of living mouse sperm cells) and results described in this paper (binding of isolated porcine sperm proteins to zona pellucida proteins) could - besides species differences - be due to the excess of both primary and secondary ZP binding proteins in our assay in the whole sperm cell homogenates. The binding of putative ZP receptors in the sperm cell to the ZP is dependent on the ionic strength as could be shown in our in vitro assay and by others [471,472].

Our experimental design enabled the isolation of two plasma membrane proteins derived from capacitated boar sperm cells that bind to ZP fragments. The two proteins are tyrosine phosphorylated, which is most likely induced by capacitation since freshly ejaculated sperm cells do not exhibit the ZP binding properties, and lack tyrosine phosphorylation of plasma membrane proteins. Further characterization of these plasma membrane proteins is necessary to reveal the binding mechanism to the ZP and their enhanced role in the subsequent induction of the acrosome reaction. With this respect we are currently producing monoclonal antibodies in order to localize the sperm surface proteins and block its zona binding properties.

Acknowledgments

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Chapter 5

The role of cholesterol redistribution and efflux in sperm activation

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submitted for publication

Abstract
Mammalian sperm cells are activated prior to fertilization by high bicarbonate levels in the oviduct, which facilitates lipoprotein-mediated cholesterol efflux. The role of bicarbonate and cholesterol acceptors on the cholesterol organization in the sperm plasma membrane was tested. Bicarbonate induced an albumin-independent change in lipid architecture which was detectable by an increase in merocyanine staining (due to protein kinase A mediated phospholipid scrambling). The response was limited to a subpopulation of viable sperm cells that were sorted from the non-responding subpopulation by flow cytometry. The responding cells had reduced cholesterol levels (18% reduction) compared to non-responding cells. The subpopulation differences were caused by variable efficiencies in epididymal maturation as judged by cell morphology. Membrane cholesterol organization was observed with filipin, which labelled the entire sperm surface of non-stimulated and non-responding cells, but labelled only the apical surface area of bicarbonate responding cells. Addition of albumin caused cholesterol efflux, but only in bicarbonate responding cells that exhibited virtually no filipin labelling in the sperm head area. Albumin had no effect on other lipid components, and no affinity for cholesterol in absence of bicarbonate. Therefore, bicarbonate induces first a lateral redistribution in the low cholesterol containing spermatozoa, which in turn facilitates cholesterol extraction by albumin. The role of cholesterol as a signalling modulator in cellular membranes is discussed.

Introduction
Mammalian sperm cells released into the lumen of the seminiferous tubules in the testis are immotile and incompetent to interact with the oocyte and its extracellular vestment, the zona pellucida [482]. The sperm cell requires two physiological maturation phases in order to acquire optimal fertilization properties: (i) epididymal maturation, a process in which severe surface protein and lipid modifications take place [435,482,483], which results in the generation of sperm cell motility [484]; (ii) further surface modifications and activation in the female genital tract (especially in the lumen of the oviduct) [6]. Activation in the female genital tract triggers diverse signalling pathways such as cAMP dependent protein kinase (PKA) and induced protein tyrosine phosphorylation [180,193] and leads ultimately to the generation of sperm cells with high binding affinity for the zona pellucida. The sperm activation processes are collectively termed capacitation [6].

The amount and distribution of cholesterol in the sperm plasma membrane alter upon capacitation. These cholesterol alterations are believed to play a role in modulating signalling pathways in sperm cells [66,67]. One of the key events in sperm capacitation is the activation of adenylate cyclase by high levels of bicarbonate that are present either in IVF media or in upper parts of the female genital tract (i.e. in the lumen of the oviduct), but virtually absent in epididymal and ejaculated sperm [186]. Increased cAMP levels activate cAMP dependent protein kinases (PKA's) and indirectly induce protein tyrosine phosphorylation by a yet unknown signalling pathway. Bicarbonate also induces PKA dependent changes in the lipid architecture of the sperm plasma membrane [47], due to phospholipid scrambling [29]. These membrane lipid changes can be monitored by merocyanine-540 (M540), which has been used as a probe to monitor bicarbonate activation in individual cells using flow cytometry, and only a subpopulation

of sperm cells appeared to be activated [46,175]. The role of cholesterol in activation of sperm cells has recently been studied using cyclodextrin, an agent that extracts cholesterol from membranes. It has been demonstrated that sperm cells incubated with cyclodextrin [66,69] had markedly activated PKA and enhanced tyrosine phosphorylation levels in absence of bicarbonate. Moreover, cholesterol efflux has been demonstrated during in vitro capacitation by albumin [67,485] as well as by lipoproteins that originate from oviductal and follicular fluids (under fertilization conditions in vivo the sperm will encounter both components in the oviduct) [61,486]. The bicarbonate- and albumin-mediated lipid changes seem to be related with the initiation of the acrosome reaction (a Ca^{2+}-dependent exocytotic multiple fusion event between the apical sperm plasma membrane and the underlying outer acrosomal membrane [487]).

Filipin complexes cholesterol into clusters that can be visualized using freeze fracture techniques and electron microscopy at the level of the sperm surface. Due to its intrinsic UV fluorescent properties, the lateral distribution can also be followed using fluorescence microscopy. In this study we analysed the effects of the capacitation factors bicarbonate, calcium and albumin on the lateral organization of cholesterol in sperm cells using filipin as a microscopical marker for cholesterol. We also tested their inducing effects on reduction of cellular cholesterol levels by determining the molecular composition of lipid extracts from washed sperm cell pellets after various incubations. Sperm suspensions were also activated by bicarbonate, and non-responding cells were sorted from responding cells that acquired high M540 fluorescence. The lipid composition and filipin labelling was determined in both cell subpopulations.

The cholesterol organization in ejaculated sperm cells is heterogeneous due to variations in the extent of epididymal maturation between individual cells. Sperm cells with low levels of cholesterol are activated by bicarbonate (monitored by M540 fluorescence), which causes a lateral redistribution of cholesterol in these cells. The redistributed cholesterol then becomes available for extraction by albumin. The consequences of this biphasic change in cholesterol organization for sperm cell signalling are discussed.

Materials and methods

Materials

Merocyanine-540 (M540), propidium iodide and Yo-Pro 1 were purchased from Molecular Probes, Inc. (Eugene, OR), PNA-FITC from EY-laboratories, Inc. (San Mateo, CA) and Filipin from Sigma (St. Louis, MO). All organic solvents used for lipid analyses were obtained from Labscan (Dublin, Ireland) and were of HPLC grade. Lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL).

Semen preparation

Semen was obtained from the Cooperative Centre for Artificial Insemination in Pigs "Utrecht en de Hollanden" (Bunnik, The Netherlands). Freshly ejaculated semen was filtered through gauze to remove gelatinous material and diluted, washed and stored in Beltsville Thawing Solution as described previously [30]. All buffers and other solutions used were iso-osmotic (285-300 mOsm) and kept at room temperature unless stated otherwise. Sperm cells were washed through a

discontinuous Percoll® (Pharmacia, Uppsala, Sweden) density gradient as described before [176,455].

Incubation media

The investigations centred on sperm behaviour during incubation in one of two media: (i) The 'control' medium, Hepes buffered Tyrode's (HBT: 120 mM NaCl, 21.7 mM lactate, 20 mM Hepes, 5 mM glucose, 3.1 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM pyruvate, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, and 100 μg/ml kanamycin; 300 mOsm/kg, pH 7.4) and (ii) the 'capacitating' medium, HBT-Bic, i.e. HBT containing 15 mM $NaHCO_3$ in equilibrium with 5% CO_2 in humidified atmosphere (the bicarbonate replaced a molar equivalent of NaCl so that osmolality was maintained). HBT, though relatively physiological, does not induce capacitative changes, whereas HBT-Bic induces capacitative changes (see [29], and references therein). The two media were supplemented with one, or a combination of the following components (i) 2 mM $CaCl_2$ or 1 mM EGTA, (ii) 0.3% (w/v) BSA (defatted fraction V, Boehringer Mannheim, Almere, The Netherlands) or 0.5 mg/ml polyvinyl alcohol and 0.5 mg/ml polyvinylpyrrolidone. Sperm suspensions were incubated at 38.5˚C in a cell incubator with humidified air containing 5% CO_2. Sperm suspensions in HBT were placed in air tight sealed tubes during incubation, whereas suspensions in HBT-Bic were placed in the opened tubes in the cell incubator.

Flow cytometry

For flow cytometric purposes, sperm cells were also capacitated in HBT-Bic containing 2.7 μM M540 (a reporter probe for phospholipid scrambling; [29]), and 25 nM Yo-Pro 1 (a membrane impermeable nucleic acid stain; [46]) and 0.5 mg/ml polyvinyl alcohol and 0.5 mg/ml polyvinylpyrrolidone. In vitro capacitation was performed in airtight sealed 5 ml flow cytometer tubes (Becton Dickinson, San Jose, CA, USA) containing 3 ml medium, which were flushed with air containing 5% carbon dioxide before closing. Capacitation was performed for approximately half an hour in a shaking waterbath at 38.5˚C before flow cytometric analysis and sorting.

Sperm cell sorting and analysis were performed on a FACS Vantage SE (Becton Dickinson, San Jose, CA, USA). The system was triggered on the forward light scatter signal (FSC). Yo-Pro 1 and M540 were both excited by an argon ion laser (Coherent Innova, Palo Alto, CA) with 200 mW laser power at 488 nm. Yo-Pro 1 was measured through a 500 nm long pass filter. M540 emission was deflected with a 560 short pass dichroic mirror in the emission pathway and measured through a 575/26 band pass filter. Sperm cells were analysed at a rate between 8.000 and 10.000 per second. For each file 10.000 events were stored in the computer for further analysis with Cell-Quest software (Becton Dickinson, San Jose, CA) or WinMDI 2.8 (http://facs.scripps.edu/). FSC and sideward light scatter (SSC) were recorded and only sperm cell-specific events, which appeared in a typically L-shape scatter profile [46], were positively gated for further analysis. During sorting the sample-input tube on the FACS Vantage SE was kept at 38.5˚C and 5% CO_2 to maintain constant incubation conditions during the complete sorting procedure using a controlled temperature bath/circulator. Sperm cells were run through the machine using PBS as a sheath fluid. Two subpopulations were sorted: (i) sperm cell events that were not stained with Yo-Pro 1 (viable) showing low M540 fluorescence (non-responding

cell subpopulation) and (ii) viable cells with high M540 fluorescence (responding cells). Sorted cells were collected in precooled 50 ml tubes that were placed in a tube holder which was kept at -20˚C. Sorted sperm cell events were also collected immediately on microscopic slides and subsequently examined with a spectral confocal microscope as stated below. Alternatively, sorted sperm cells were collected at room temperature in tubes that were half filled with Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 80 mM Na-cacodylate, 500 µM $MgCl_2$, 250 µM $CaCl_2$, pH 7.4) until tubes were filled for 3/4 and further processed to visualize the surface cholesterol organization with filipin. In order to analyse the efficiency of cell sorting, sperm cells were collected in flow cytometer tubes and rerun within 10 min.

The acrosomal status was checked routinely by staining the same incubated sperm samples that were used for sperm sorting as described above with 5 µg/ml fluorescein conjugated peanut agglutinin (PNA-FITC as a marker probe for acrosomal leakage; [455]) and 1 µm propidium iodide (as marker probe for cell deterioration; [488]), and subsequent analysis on a FACScan (Becton Dickinson, San Jose, CA) as described before [10]. Alternatively 10 µl of the labelled sperm suspension was used to make microscopic slides [10] and 200 cells were counted in triplicate for each treatment on damage of the acrosome or the plasma membrane.

Visualization of M540 fluorescence

Sperm cells were incubated in various media for 2 hours at 38.5˚C in a humidified air of 5% CO_2 and stained with 2.7 µM M540 and 25 nM Yo-Pro 1 for 10 minutes. Aliquots of 250 µl sperm suspension (containing approximately 1 million sperm cells) were placed into a life chamber (37˚C, 5% CO_2 in humidified atmosphere) and placed on an epifluorescence microscope (Leica DMRE, Leica GmbH, Germany) equipped with a Hg lamp (100 mW) and a filter block (480 nm excitation filter, 500 nm dichroic mirror and a 520 nm long pass emission filter) in order to simultaneously assess M540 fluorescence (red) and Yo-Pro 1 fluorescence (green). Of each sperm sample 200 cells were counted in triplicate. For visualization (sorted) sperm samples were placed under a spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany) and excited with the 488 nm Argon laser line. Yo-Pro 1 fluorescence was detected with photomultiplier tube 1 (emission selected in the wavelength range of 500-550 nm) and M540 fluorescence with photomultiplier tube 2 (580-620 nm). Single scans were made to capture labelling patterns of (hyper) motile sperm cells.

Lipid analysis

Sperm cell suspensions that were incubated for 2 hours in HBT or HBT-Bic were further subjected to lipid extraction according to Bligh and Dyer [489]. All sperm suspensions were washed through a 30% Percoll cushion prior to lipid extraction because this procedure was required to separate BSA from the sperm cells (10 min 700 x *g*). Alternatively sperm cells that were sorted for low and high M540 fluorescence and collected in tubes at -20˚C were centrifuged (285.000 *g*, 70 min, 2˚C) and supernatant was discarded and the lipids from the resulting cell pellets were extracted. The composition of lipid classes of total sperm populations was detected by high performance liquid chromatography (HPLC) that consisted of an LKB low pressure mixer, a model 2248 pump (Pharmacia, Uppsala, Sweden), and a Rheodyne injector. Lipid classes

of sorted sperm cell populations were separated on a Lichrospher DIOL-100 column (250 x 3.2 mm, 5 μm particle size) obtained from Alltech Applied Sciences (Breda, The Netherlands). Elution was performed at 40˚C using a method adapted from Silversand and Haux [490]. In brief, lipid classes were eluted with a ternary gradient using the solvents hexane/acetone 99/1 v/v (A), hexane/2-propanol/acetone 82/17/1 v/v/v (B), and 2-propanol/water/acetone 85/14/1 v/v/v (C). The gradient was developed as follows: (time in min, %A, %B, %C), (0, 90, 10, 0), (10, 57, 43, 0), (11, 20, 70, 10), (15, 0, 80, 20), (38, 0, 60, 40), (40, 0, 60, 40), (45, 0, 100, 0), (49, 90, 10, 0), (55, 90, 10, 0). Lipids were detected using a Varex MKIII light scattering detector obtained from Alltech (Deerfield, IL). The detector was calibrated with lipid standards at a drift tube temperature of 90˚C and a gas flow of 1.8 l/min [16]. Lipid classes and molecular species composition were determined by on-line electrospray ionization mass spectrometry on a Sciex API-365+ triple quadrupole mass spectrometer (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Visualization of cholesterol distribution by filipin

Sperm suspensions were immediately fixed after capacitation incubations by diluting 1:1 with 4% glutaraldehyde, 150 mM Na-cacodylate buffer pH 7.4. Sperm samples were fixed for 30 minutes under gentle shaking. Before staining, cell suspensions were washed twice (500 *g*, 15 min) in 0.15 M Na-cacodylate buffer (pH 7.4). After washing sperm cells were resuspended in 0.15 M Na-cacodylate buffer containing 25 μM filipin (an antibiotic capable to complexes unesterified cholesterol [491]). Filipin was dissolved in the buffer from a 10 mM DMSO stock solution. Blanc samples were treated with similar DMSO concentrations without filipin. Tubes were wrapped in aluminum foil to keep the fluid in the dark and labelling was performed for 30 min under gentle shaking. Subsequently, tubes were centrifuged (500 *g*, 15 min) and washed in 0.15 M Na-cacodylate buffer. Cell pellets were mixed with 30 vol % EM-grade glycerol 70 vol % 0.15 M Na-cacodylate buffer for 1 hour under gentle shaking. Ultrastructural localization of filipin labelling was evaluated by electron microscopy. For this purpose 1 μl aliquots of sperm suspensions were pipetted on golden heads and were frozen in a mixture of liquid and solid nitrogen. The cells were stored in liquid nitrogen until further processing. Cells were freeze fractured in a Balzers BAF-300 device at -105˚C and a vacuum of 10^{-7} Torr. A coat of 200 nm platinum/carbon was evaporated under an angle of 45˚ followed by a second coat of carbon at 90˚. The cells were digested overnight in house hold bleach and the replicas were examined in a Philipis CM 10 electron microscope (Philips, Eindhoven, The Netherlands). The freeze fracture procedure was further performed as described before [54].

The fluorescent properties of filipin immobilized to the sperm cells were analysed in an LS50 luminescence spectrophotometer (Perkin Elmer Ltd., Beaconsfield, Bucks, UK). Emission scans were made at 357 nm excitation, in the range of 400-600 nm. Excitation scans were made at 480 nm, in the range of 275-400 nm (in all cases 5 nm slid width settings were used). Filipin fluorescence was also observed on a fluorescence microscope (Leica DMRE , Leica GmbH, Germany) equipped with a Hg lamp (100 mW) and a UV filter block (340-380 nm excitation filter, 400 nm dichroic mirror and a 425 nm long pass emission filter). Specimens were mounted and coverslips were sealed with nail polish for fluorescence microscopical inspection. Fluorescence patterns of filipin cholesterol complexes were observed in three different sets of

boar sperm samples that were incubated for 2 hours at 38.5˚C in humidified air: (i) in HBT with and without 0.3% (w/v) BSA (ii) in HBT-Bic with and without 0.3% (w/v) BSA, and (iii) in HBT-Bic, subsequent sorted for low and high M540 fluorescence, and collected in fixative. Of each sample 200 cells were counted in triplicate.

Detection of sperm morphology

Sperm suspensions from incubated specimens (total cell population as well as the low and high M540 sorted subpopulations) were diluted to a concentration of 1 million cells per ml, fixed as described above, and 200 cells were counted in triplicate from each preparation for three cell morphology types: (i) normal well-matured sperm cell without cytoplasmic remnants, (ii) normal but poorly matured sperm cell containing visible cytoplasmic droplets (iii) deteriorated sperm cell or sperm cell exhibiting abnormal morphology. Sperm morphology was scored under an Olympus 209376 Phase Contrast microscope (100 x objective, 10 x ocular; Olympus, Tokyo, Japan). Sperm suspensions were routinely assessed for acrosomal integrity as described before [175].

Statistics

Ejaculates of three different boars were examined three times after incubation at 38.5˚C for 2 hours. The effect of medium composition on lipid composition, morphology, acrosomal status, and capacitation was analysed using ANOVA in combination with Bonferroni's multiple comparison test. Lipid compositions of sorted sperm cells were statistically compared using a paired Student's t-test.

Results

Sorting of bicarbonate responding from non-responding viable sperm cells

Sperm cells were incubated in HBT-Bic in presence of M540 (to detect bicarbonate mediated scrambling of phospholipids; [29]) and Yo-Pro 1 (to distinguish viable from deteriorated cells; [46]) in a flow cytometer tube (38.5˚C) at the sample-input of the FACS Vantage SE flow cytometer. Sperm cells were continuously run through the flow cytometer. The subpopulation of viable sperm specific events that acquired high M540 fluorescence and the subpopulation that remained low fluorescent for M540 were sorted into two collection tubes (Fig. 1 A). Rerun of these two viable sperm cell subpopulations through the flow cytometer within a time period of 10 minutes revealed that the cells remained viable and did not change M540 fluorescence characteristics (Fig. 1 B and C). The efficiency of sorting was >96%. Labelling patterns of M540 were established within 15 min and these labelling patterns did not change during the coarse of the sorting experiments.

The viable sperm events sorted for low and high M540 fluorescence were immediately analysed under a confocal microscope (Fig. 1 D-F). Unsorted cells were either low or high fluorescent for M540 (for the sperm sample depicted in Fig. 1 D approx. 54% of the viable cells had high M540 fluorescence, whereas, >98% of the cells sorted for low M540 fluorescence indeed had a M540 labelling pattern of Fig. 1 E and >98% of te cells sorted for high M540 fluorescence had a M540 labelling pattern of Fig. 1 F. During the sorting experiments the sperm

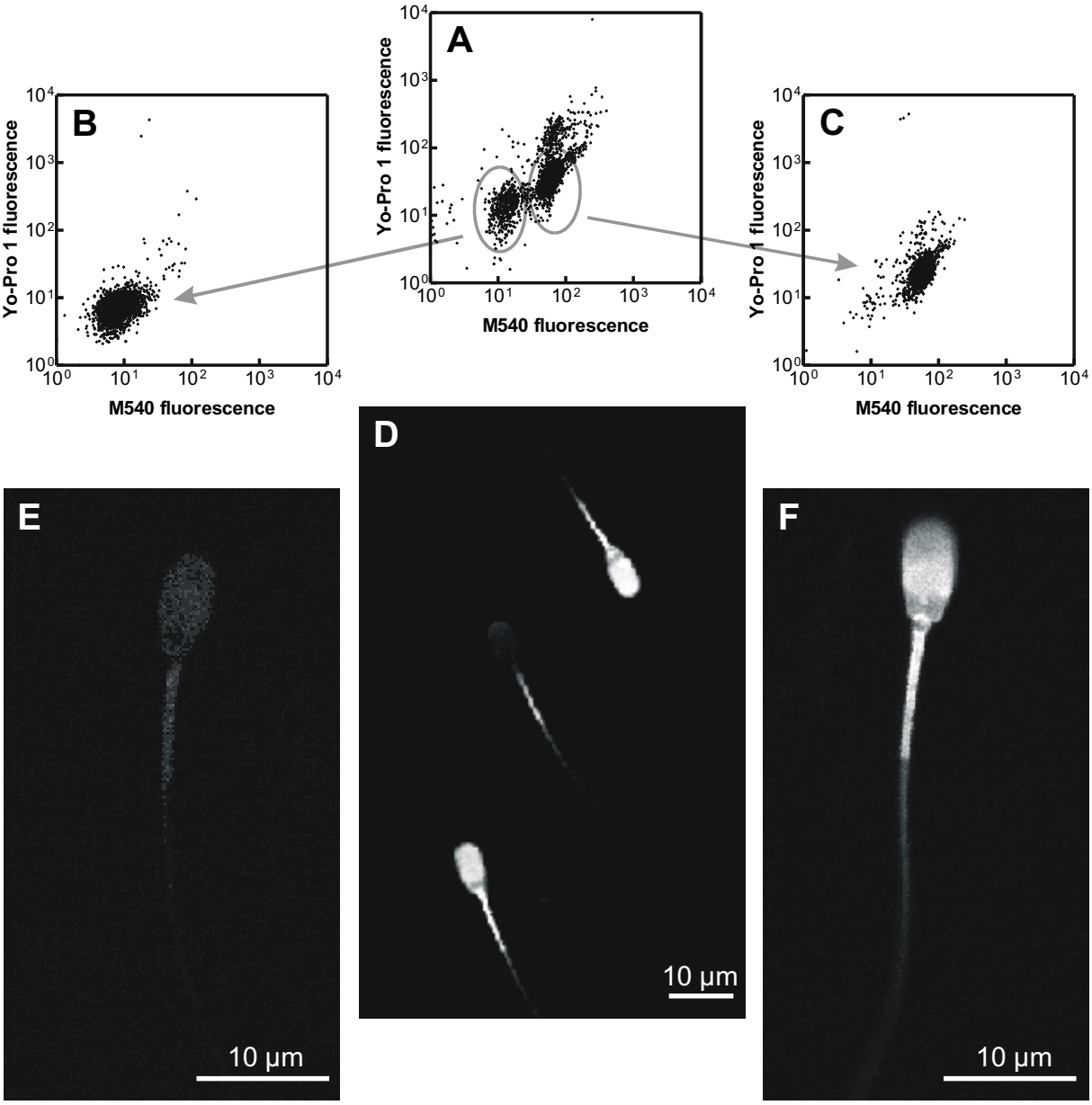

Figure 1. Flow cytometric analysis and sorting of low and high M540 fluorescent viable sperm cell subpopulations. Sperm cells were incubated, analysed and sorted using a FACS Vantage SE as described in materials and methods. Sperm specific events with fluorescent properties in M540 fluorescence (membrane fluidity) and Yo-Pro 1 fluorescence (viability) were continuously recorded and sperm cells in specified regions (gray circles) were sorted and collected at room temperature (A). Within 10 minutes, the sorted and collected sperm cells were re-analysed for low M540 fluorescence (B) as well as for high M540 fluorescence (C) demonstrating that cells remained viable and did not alter M540 fluorescent properties. The sorting efficiency was >99% for the low M540 and >95% for the high M540 fluorescent sperm subpopulations, respectively. Under a confocal microscope the unsorted sperm cells contained low and high M540 fluorescent cells (D), whereas, the subpopulation sorted for low M540 fluorescence indeed showed very dim fluorescence (E), and the subpopulation sorted for high M540 fluorescence showed bright fluorescence (F).

cells remained acrosome intact and viable as was detected by staining the cells with PNA-FITC in combination with propidium iodide (incidence of damaged acrosomes and/or cell deterioration was <10%).

Lipid composition of sperm cells

Lipids were extracted from viable sperm cell subpopulations with either low or high M540 fluorescence. Total lipid extracts were analysed after a single HPLC run using a light scattering detector (Fig. 2 A). The lipid composition of the sperm cell membranes is given in table 1. Although appreciable differences in lipid composition were observed between boars, a paired

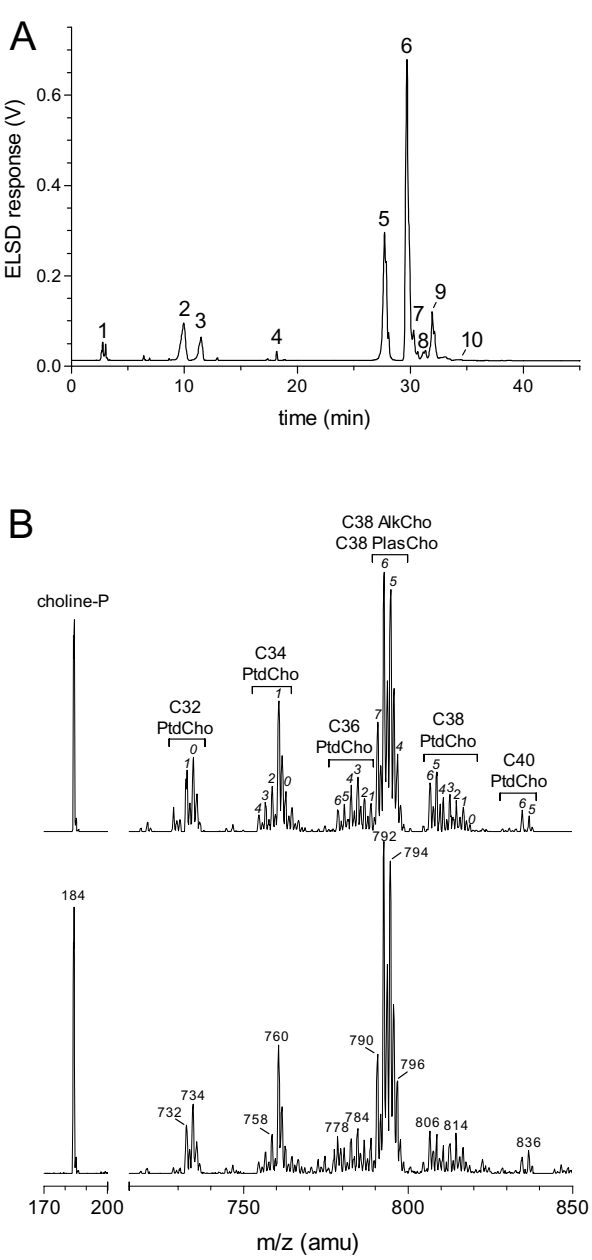

Figure 2. Composition of lipid classes in viable sperm cell subpopulations with low or high M540 fluorescence. (A) Separation of lipid classes on HPLC as detected by evaporative light scattering detection. Lipid classes were identified by comparison with lipid standards and on-line electrospray ionization mass spectrometry as triacylglycerols and cholesterol esters (1), cholesterol (2), diacylglycerol (3), ceramides (4), PE (5), PC (6), PS (7), SM (8), SGG and PI (9), lysoPC (10). (B) On-line identification of individual molecular species during the elution of PC. The distribution across the mass spectrum results from the variety in fatty radyl chain length, the degree of unsaturation and the type of linkage at the sn-1 position (ester versus ether) of the glycerol backbone. Peaks are labelled with their nominal masses (bottom) or the total number of carbon atoms in the radyl groups and the type of sn-1 linkage (top). PtdCho: 1,2 diacyl phosphatidylcholine, AlkCho: 1-alkyl 2-acyl phosphatidylcholine, PlasCho: 1-alk-1'-enyl 2-acyl phosphatidylcholine (Plasmalogen PC). The italic numbers indicate the total number of unsaturations (double bonds) in the fatty radyl chains. The top line was recorded during elution of lipids from low M540 fluorescent cells, the bottom line during elution of lipids from high M540 fluorescent cells. Note the occurrence of isotope peaks at odd m/z ratios, due to the natural occurrence of approximately 1% [^{13}C]. Experiments were performed 3 times with similar results.

t-test revealed no significant difference in the composition of lipid classes between cells with high and low membrane fluidity from the same boar ($p>0.05$) except for the amount of cholesterol, where the difference was highly significant ($p<0.01$). To investigate whether the observed differences in membrane fluidity were related to differences in the fatty radyl moieties of the phospholipids, on-line electrospray mass spectrometry was performed on the lipid classes as they eluted from the column (depicted for PC species in Fig. 2 B). However, no differences in the molecular species composition were observed between cells with high and low M540 fluorescence (Fig. 2 B).

Table 1. Viable boar sperm cells with high plasma membrane fluidity (capacitated) have low amounts of cholesterol compared with viable boar sperm cells with low plasma membrane fluidity. Dead cells were detected with Yo-Pro 1 (cell impermeable DNA stain) and plasma membrane fluidity was monitored with M540 during sperm sorting. Lipids from sorted sperm cells were extracted and lipid classes were analysed using HPLC and quantified with evaporative light scattering detection (see materials and methods).

lipid class*	low membrane fluidity	high membrane fluidity
cholesterol	38.9 ± 3.4	31.9 ± 2.7
phosphatidylethanolamine	8.6 ± 5.2	11.0 ± 8.0
phosphatidylcholine	31.0 ± 4.8	34.4 ± 3.7
phosphatidylserine	7.6 ± 3.6	7.4 ± 2.4
sphingomyelin	3.2 ± 1.4	3.6 ± 1.0
sulfogalactosyl glycerol	9.0 ± 4.8	10.3 ± 3.6

* lipid classes are expressed as percentage of total molar lipid amount (n=3, average ± SD).

Lipids were also extracted from entire sperm populations after a 2 hour incubation period in HBT and HBT-Bic media. The lipid composition (including the cholesterol concentration) remained unaltered in HBT-Bic treated sperm cells when compared to the HBT treated sperm cells (Fig. 3 A). Therefore, the intrinsic low cholesterol containing sperm subpopulation appeared to be selectively sensible for the bicarbonate meditated M540 response. Addition of 0.3% (w/v) albumin to HBT-Bic media resulted in a marked decrease in cellular levels of cholesterol and this effect was calcium independent (Fig. 3 A). The albumin-mediated extraction of cholesterol was dependent on bicarbonate because the drop in cellular cholesterol levels was nihil in HBT treated cells in absence of bicarbonate (Fig. 3 A). Albumin specifically extracted cholesterol from the bicarbonate activated sperm cells since no changes in other lipid classes were detected (Fig. 3 B). In a parallel experiment, sperm cells were first incubated for 2 hours in HBT, or HBT-Bic in absence or presence of albumin and further processed to isolate plasma membranes as described before [175,455]. The results clearly demonstrate that the albumin/bicarbonate mediated extraction of cholesterol was mediated at the level of the sperm plasma membrane and the effect on other lipid classes was nihil (Fig. 3 C). The relative proportion of the other lipid classes increased somewhat due to the lower amount of cholesterol.

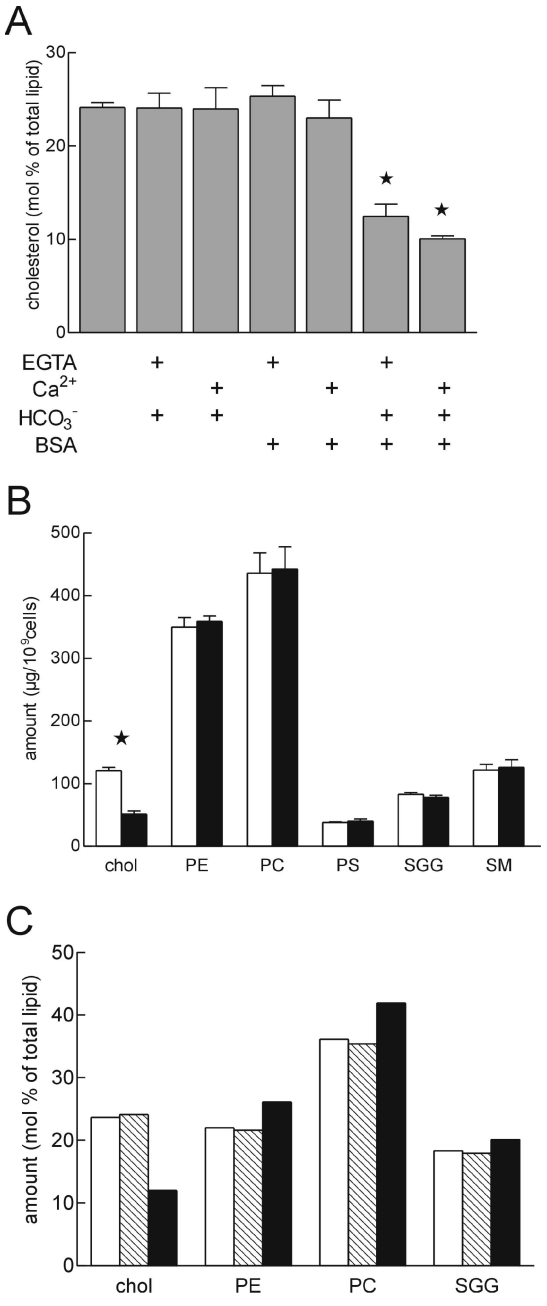

Figure 3. Effects of bicarbonate, albumin, and extracellular calcium on cholesterol efflux in boar sperm cells incubated for 2 hours in HBT. (A) The relative amount of cholesterol (mol %) is expressed of sperm samples that has been subjected to incubation in HBT containing indicated additions (1 mM EGTA, 2 mM $CaCl_2$, 15 mM $NaHCO_3$, 0.3% (w/v) BSA; sperm samples were washed through Percoll prior to lipid extraction). Mean values of 3 independent experiments (measured in triplicate) are indicated (error bars represent SD values, ★: $p<0.001$). (B) Absolute amounts (µg/10^9 cells) of the major lipid classes in the corresponding samples that were incubated in HBT (open bars) and HBT-Bic supplemented with 0.3% (w/v) albumin (filled bars). Major lipid classes: cholesterol (chol), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), sulfogalactosyl glycerol (SGG), sphingomyelin (SM). Mean values of 3 independent experiments (measured in triplicate) are indicated (error bars represent SD values, ★: $p<0.001$). (C) Relative composition (mol %) of the most abundant lipid classes that were extracted from plasma membrane fractions derived from sperm suspensions incubated in HBT (open bars), HBT-Bic (hatched bars) and HBT-Bic supplemented with 0.3% (w/v) albumin (filled bars). Mean values of one experiment measured in triplicate.

Cholesterol localization

Sperm cells were labelled with filipin in order to reveal the lateral organization of cholesterol and other free sterols at the sperm surface [491]. Filipin aggregates sterols into complexes that can be distinguished on freeze fracture replicas of the sperm plasma membrane (Fig. 4 A and B), whereas, unlabelled cells were devoid of such complexes (Fig. 4 C; the small dots represent trans membrane proteins). Two different types of filipin labelling were observed (Fig. 4 A and B; labelling type A and B respectively). However, due to the fact that the fracture plane only rarely runs through extended areas of the sperm head plasma membrane this very labourious technique

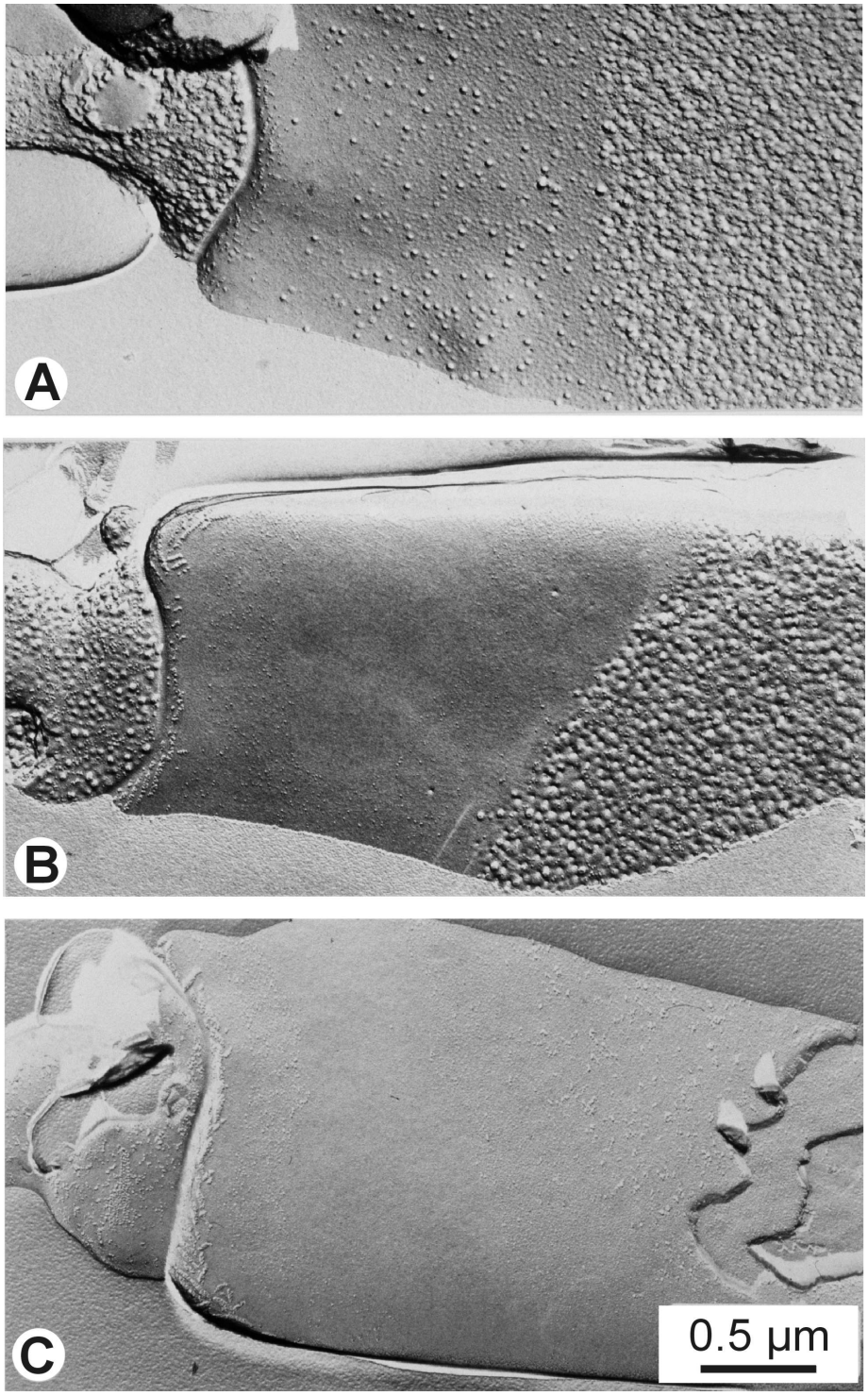

Figure 4. Ultrastructural localization of filipin sterol complexes on freeze fracture replicas of the head plasma membrane of fixed boar sperm cells. (A) Freeze fracture replica of a large proportion of the plasma membrane of a boar sperm head with filipin labelling type A (see also Fig. 7 A). (B) Idem with filipin labelling type B (see also Fig. 7 B). The particles on this replica represent filipin cholesterol complexes. (C) Freeze-fracture replica of a non-labelled sperm cell from a DMSO control experiment showing the absence of the complexes, the small particles represent membrane associated proteins.

cannot be used to assess the relative frequencies of cells with one of each filipin labelling pattern. Filipin only formed complexes with sterols at the sperm surface as is depicted in Fig. 5.

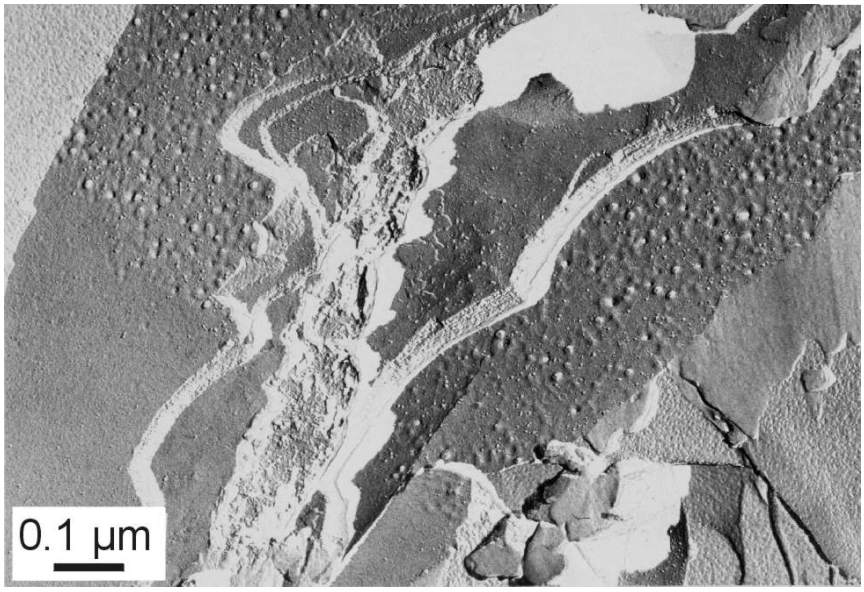

Figure 5. Ultrastructural localization of filipin sterol complexes on freeze fracture replicas of the head plasma membrane of fixed boar sperm cells. Freeze-fracture replica of a sperm cell in which the fracture plane is running cross through the sperm head. Filipin/sterol complexes are only visible at sites where the plasma membrane is in the fracture plane whereas, acrosomal and nuclear membranes as well as the protamine condensed DNA (organized into multilammelar sheets; [492]) are devoid of these particles.

Filipin contains a set of 5 conjugated trans double bonds (-CH=CH- see inset of Fig. 6) and therefore can be used as a UV fluorescent probe. The excitation and emission properties of filipin immobilized to sperm cells are depicted in Fig. 6. The excitation peak of 357 nm in combination with the emission peak at 480 nm makes this probe suitable for UV fluorescence detection. In fact UV fluorescence detection of filipin sterol complexes on sperm cells revealed similar surface

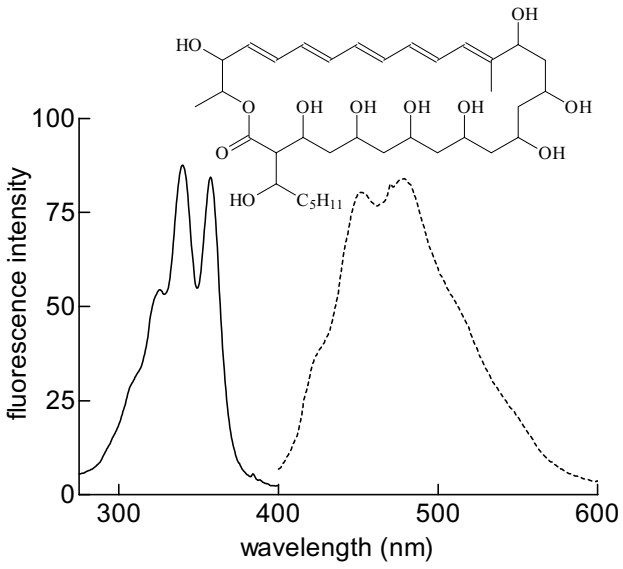

Figure 6. Excitation and emission scans of filipin complexed to boar sperm cells. Sperm cells were labelled with filipin (see materials and methods) and suspensions of 10^6 labelled cells/ml were pipetted in quarts cuvettes and placed in a fluorimeter. The excitation of complexed filipin was detected at an emission wavelength of 480 nm over the range of 275-400 nm (solid line). The emission of complexed filipin was detected at 357 nm excitation (one of the excitation peaks) at a range of 400-600 nm (broken line).

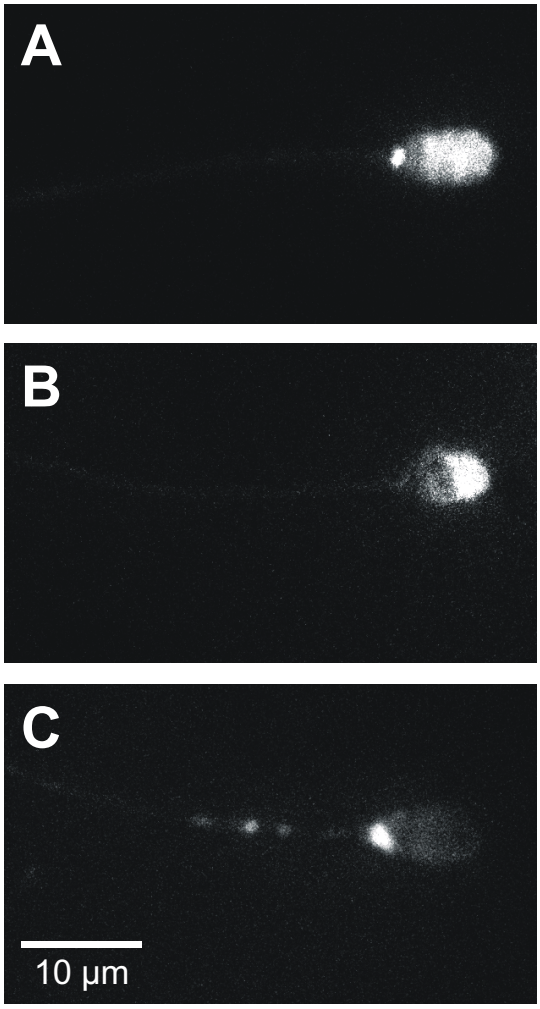

Figure 7. Fluorescent labelling of filipin sterol complexes on fixed boar sperm cells. Filipin labelled cells were mounted in coverslips and sealed with nail polish. The filipin fluorescence was observed under 340-380 nm excitation and fluorescence signals >425 nm were selected by the emission filter. (A) Filipin labelling of a sperm cell from the low M540 fluorescent sperm subpopulation (identical to pattern A, Fig. 4 A). (B) Idem but from the high M540 fluorescent subpopulation (identical to pattern B, Fig. 4 B). (C) Filipin labelling was absent in the sperm head of cells that were capacitated in HBT-Bic supplemented 0.3% (w/v) albumin (pattern C).

labelling patterns as detected with electron microscopy on freeze fracture replicas (compare pattern A depicted in Fig. 4 A with Fig. 7 A and pattern B depicted in Fig. 4 B with Fig. 7 B). When sperm samples were treated with HBT-Bic in combination of 0.3% (w/v) albumin a subpopulation of cells also showed negative filipin labelling (Fig. 7 C, pattern C). The filipin pattern of Fig. 7 C is difficult to examine using electron microscopy on freeze fracture replicas because albumin further destabilizes the sperm plasma membrane which hampers fracture planes to run through extended plasma membrane areas of the sperm head. The relative frequencies of the three types of filipin labelling were scored by UV fluorescence microscopy for three individual sperm ejaculates. The transition from filipin pattern A to B reflects the cholesterol redistribution (Fig. 8, dotted line). Clearly, bicarbonate was necessary to induce cholesterol redistribution, whereas, the inclusion of BSA did not affect cholesterol redistribution. On the other hand, bicarbonate and BSA both were necessary to induce cholesterol efflux as reflected by pattern C (Fig. 8, solid line). Note that the degree of response to albumin and/or bicarbonate varied between the ejaculates examined.

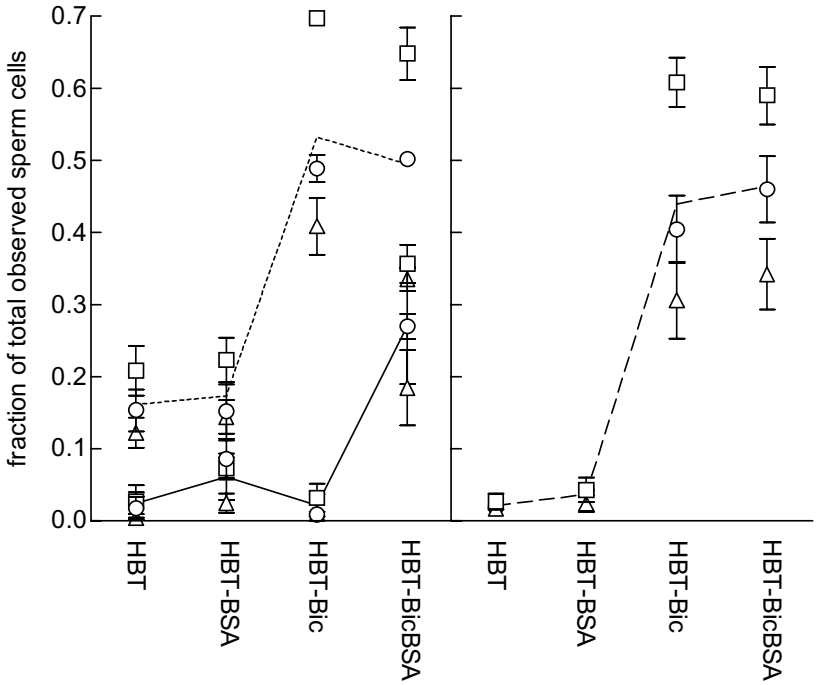

Figure 8. The effect of bicarbonate and albumin on cholesterol and phospholipid scrambling in incubated boar sperm samples. Ejaculates from three different boars (boar I: circle; boar II square; boar III: triangle) were incubated in HBT, HBT supplemented with 0.3% (w/v) albumin (HBT-BSA), HBT-Bic or HBT-Bic supplemented with 0.3% (w/v) albumin (HBT-BicBSA) for a period of 2 hours. Cholesterol was fluorescently visualized by filipin and phospholipid scrambling by M540 (see materials and methods). Sperm cells showing filipin pattern as depicted in Fig. 7 C were scored as cells featuring cholesterol efflux (solid line), whereas filipin pattern 7 B and C were scored as cells featuring cholesterol redistribution (dotted line). Sperm cells showing high M540 fluorescence (Fig. 1 F) were scored as cells that underwent phospholipid scrambling (broken line). After each incubation condition 200 cells were counted in triplicate and mean values with SD values are expressed. Lines represent means of three boars.

Phospholipid scrambling was followed, by staining the same sperm samples with M540 [29] and relative frequencies of low and high fluorescent M540 cells were scored. Sperm suspensions incubated in HBT almost exclusively expressed low M540 fluorescence, whereas, a variable, but a considerable shift to high M540 fluorescence was observed in sperm suspensions treated with HBT-Bic (Fig. 8, broken line). It should be noted that inclusion of 0.3% (w/v) albumin did not change this distribution in labelling pattern frequencies and therefore did not affect phospholipid scrambling [175]. Moreover, viable sperm subpopulations that were sorted for low and high M540 fluorescence showed the corresponding filipin labelling: >95% of the low M540 fluorescent sorted cells showed filipin labelling pattern A, whereas, >90% of the high M540 fluorescent sorted cells showed filipin labelling pattern B. Collectively these data indicate that the membrane architectural changes picked up by M540 probe allow albumin mediated cholesterol efflux.

Sperm morphology

Cell morphology of the same sperm samples was scored under a phase contrast microscope. The sperm morphology (cytoplasmic droplet content and acrosome morphology) did not change under the different HBT and HBT-Bic incubations tested (data not shown). It was noted that two boars (I and II; Fig. 9) contained sperm cells with very good morphology, whereas, the third boar (III; Fig. 9) contained a rather large proportion of sperm cells with cytoplasmic droplets, which is an indication for sub-optimal epididymal maturation [493]. Interestingly, the bicarbonate induced M540 response as well as the shift from filipin labelling pattern A to B (and in presence of albumin to pattern C, Fig. 9) was poor in boar III, whereas, boars I and especially II had much higher responses (compare Figs. 8 and 9). Therefore, variations in bicarbonate induced effects in the ejaculated sperm samples may well relate to differences in epididymal maturation efficiencies.

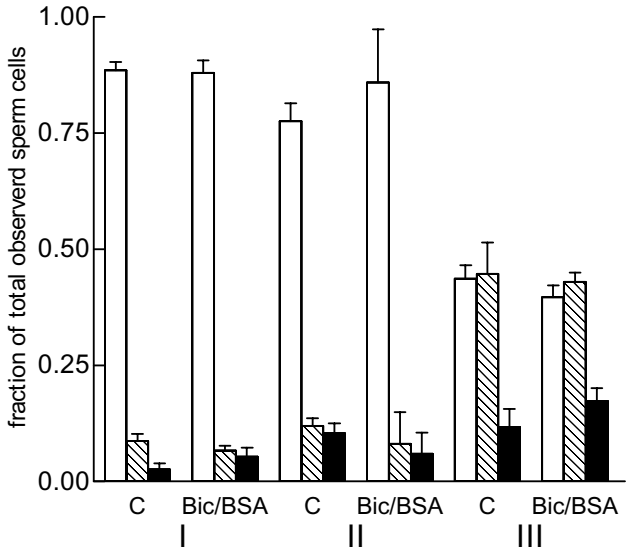

Figure 9. Morphology scores of the boar samples after a two hour incubation in HBT (modified) media. Sperm cell samples from the three corresponding ejaculates of the boars examined in Fig. 8 were used for morphological examination. Sperm cells were diluted, fixed and examined under a phase contrast microscope. The relative amount of sperm cells with a normal mature morphology are expressed with open bars, the relative proportion of cells normal but immature morphology (i.e. with cytoplasmic remnants at the head, midpiece or tail) are expressed with hatched bars, whereas the proportion with abnormal morphology are expressed with solid bars. 200 cells were counted in triplicate and mean values with SD values are expressed.

Discussion

In this paper the dynamics of cholesterol organization in the plasma membrane of bicarbonate activated sperm cells was assessed. Filipin labelling studies revealed that bicarbonate induced a redistribution of cholesterol in the sperm head from a more general distribution (Fig. 7 A) to the apical site (Fig 7 B). Furthermore, cholesterol was extracted from sperm cells incubated with bicarbonate if an acceptor (e.g. BSA) was present (Fig. 7 C). Bicarbonate plays a major role in the activation of sperm cells [180,182,193,494,495]. In pigs, bicarbonate brings about modifications in sperm surface coating [488], a PKA mediated scrambling of phospholipids [29],

induction of protein tyrosine phosphorylation [175] and an increase in the ability of viable acrosome intact cells to bind to zona pellucida components [174]. Of particular interest has been the observation that bicarbonate-induced scrambling of phospholipids in boar sperm cells can be monitored with M540 [29]. In other cell types, an increase in M540 has also been interpreted as indicative for a loss of transverse phospholipid asymmetry in the plasma membrane [496,497]. In this paper the effect of bicarbonate on the organization of cholesterol was measured because several studies have indicated that the lateral distribution of cholesterol [491] and lipoprotein cholesterol removal from the plasma membrane [61,67] are important events in sperm capacitation and modulate cell signalling molecules [66,67].

Percoll washed sperm suspensions were incubated with HBT-Bic and stained with M540 and viable cells were sorted for high and low M540 fluorescence (Fig. 1). The phospholipid composition of the high and low M540 sperm subpopulations was nearly identical in the two sperm subpopulations, whereas, the high M540 subpopulation had 18% reduced cholesterol levels when compared to the low M540 subpopulation (Table 1). In absence of albumin, bicarbonate did not affect the lipid composition and cholesterol levels in complete sperm suspensions (Fig. 3). Therefore, it can be concluded that bicarbonate did not induce a reduction in cholesterol in the high M540 cells (the sorting experiments were performed in absence of albumin). This implies that individual ejaculated sperm cells contain variable amounts of cholesterol and that the cells with low cholesterol levels were primed by bicarbonate (as detected with M540). It should be noted that the high and low cholesterol containing cells (i.e. the M540 responding and non-responding cells respectively) contained an identical phospholipid composition and unsaturation degree in fatty acids attached to phospholipids (Fig. 2). Therefore, the bicarbonate induced M540 response depended only on the intrinsic level of cholesterol in each individual cell at ejaculation. With this respect it is interesting to note that the M540 response reflects the scrambling of phospholipids in the sperm plasma membrane [29]. Bicarbonate appears to stimulate directly a sperm-specific adenylate cyclase [192], which results in increased cAMP that activates PKA to initiate one or more as yet unidentified protein phosphorylation cascades resulting in the phosphorylation of protein tyrosine residues [154,175]. Our results imply that only low cholesterol levels in sperm cells allow all these bicarbonate triggered events. The variety of cholesterol levels is probably a result of differential efficiencies in epididymal maturation. (i) severe modifications in the lipid composition take place during this process including a decrease in cholesterol [483,498] and (ii) sperm cells with uncomplete matured morphology (i.e. with cytoplasmic remnants; [493]) never acquired high M540 fluorescence after incubation in HBT-Bic. In fact, the relative number of incomplete matured sperm cells (as scored on cytoplasmic remnants) correlates very well with the amount of cells that did not show high M540 fluorescence after incubation in HBT-Bic (Figs. 8 and 9). Furthermore, the sorted high M540 fluorescent sperm cell subpopulation did not contain cells with incompletely matured morphology. Taken together, these data indicate that differences in epididymal maturation are responsible for the variations in cholesterol levels in individual ejaculated sperm cells. Only the cells with relatively low cholesterol levels are primed by bicarbonate (resulting in high M540 fluorescence). The boar variation in M540 response to bicarbonate [46] most likely can be explained by boar to boar variation in epididymal sperm maturation.

Cholesterol is distributed non-randomly in and between biological membranes and can form transbilayer domains in the plasma membrane in a variety of cell types (for review see [499]) including sperm cells [491]. It is believed that the dynamics in these cholesterol domains play a role in receptor effector coupling, membrane ion transport and membrane mediated cell signalling [500]. In sperm cells, cholesterol seems to modulate PKA activity and protein tyrosine phosphorylation [67]. Obviously, it was of interest to detect the membrane organization of cholesterol in capacitating sperm cells. For this purpose, sperm cells were labelled with filipin, a polyene antibiotic which forms 25-30 nm complexes with 3-β-hydroxysterols (including cholesterol), which are visible in freeze-fractured membranes [501] (Fig. 5). The lateral distribution of filipin-sterol complexes on sperm cells could be alternatively visualized by UV fluorescence due to the intrinsic fluorescent properties of filipin (Fig. 6). Both visualization techniques gave similar labelling results (compare Fig. 4 A and B with Fig. 7 A and B). Sperm cells incubated in HBT showed predominantly labelling pattern A, whereas incubation in HBT-Bic caused a shift of a subpopulation to labelling pattern B (Fig. 8). Interestingly, sperm cells incubated in HBT-Bic, that were sorted for high M540 fluorescence, showed almost exclusively filipin labelling pattern B, whereas, cells that were sorted for low M540 fluorescence showed labelling pattern A. This indicates that the bicarbonate mediated M540 response that occurred only in sperm cells with relatively low cholesterol levels (Figs 1 and 2), induced a reordering of cholesterol in these cells (in absence of bicarbonate only a few cells appeared with labelling type B). The M540 response closely relates to the induction of phospholipid scrambling [29] and therefore topological redistribution of cholesterol may relate to lateral as well as transverse membrane cholesterol redistributions. It should be noted that cholesterol redistributions may occur after fixation. Therefore, the lateral distribution of filipin may not reflect the natural cholesterol organization in living cells. However, one can conclude from the labelling patterns of sperm cells incubated in HBT compared to HBT-Bic, that bicarbonate induces rearrangement in cholesterol ordering.

The changes in cholesterol organization upon bicarbonate activation of sperm cells enable albumin mediated cholesterol extraction. Sperm cells incubated in HBT supplemented with albumin showed no alterations in cholesterol distribution (Fig. 8) nor a reduction in cholesterol levels or other modifications in lipid composition (Fig. 3). However, when sperm cells were stimulated in HBT-Bic supplemented with albumin, a marked reduction in cholesterol levels was noted (Fig. 8). Moreover, a subpopulation of sperm cells with filipin labelling pattern B shifted to very low filipin labelling (pattern C, see Fig. 7 C). These data collectively indicate that cholesterol is extracted from the bicarbonate stimulated sperm subpopulation only. Albumin mediated the cholesterol efflux at the cell surface (see Fig. 3 C). The importance of lipoprotein mediated cholesterol extraction in mammalian fertilization has been widely studied since the mid 80s (for review see [65]). In fact incubation media used to capacitate sperm cells in vitro (IVF media) or in vivo (oviduct fluid) must contain cholesterol acceptor proteins for optimal fertility results [6,61,486]. More recently, the modulating effect of cholesterol efflux in signal transduction in rodent sperm has been reported [66,67].

In summary bicarbonate showed to have a biphasic effect on cholesterol organization in sperm cells: (i) filipin labelling pattern changed from a homogeneous to an apical distribution

pattern in the subpopulation of sperm cells with low cholesterol levels and (ii) only the bicarbonate responsive cells were susceptible to lipoprotein mediated cholesterol extraction, leading to sperm cells with very low filipin staining. It should be noted that rodent sperm cells are collected from the cauda epididymides, a site where sperm cells are stored and undergo final maturation changes [493]. During collection of rodent ejaculates, sperm cells are mixed with the spermicidal coagulation plug [4]. This makes rodents a less suitable model system for monitoring the biphasic effects of bicarbonate on cholesterol organization and cell signalling. Non-rodents are more favourable species for such studies. For instance ejaculated porcine sperm can be stored for up to a week in dilution buffers and can easily be worked up for capacitation in vitro [186]. The bicarbonate mediated changes in cholesterol topology coincide with a partial scrambling in phospholipid asymmetry (this manuscript and [29]), which is driven by the activation of PKA [47]. Interestingly, there is considerable evidence that maximal activated adenylate cyclase activity may require an optimal transbilayer fluidity gradient [500], which may explain why only sperm cells with low cholesterol (i.e. with higher membrane fluidity) were activated by bicarbonate (which directly activates adenylate cyclase [191,192]). The data indicate that the membrane changes detected by M540 (i.e. phospholipid scrambling and cholesterol redistribution) are required for albumin mediated cholesterol extraction. The role of cholesterol as a cell signalling modulator appears to be an important general cell biological phenomenon [499]. More specifically, the described changes in cholesterol organization are probably required for final and functional preparing sperm cells to interact and fertilize the female gamete.

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Chapter 6

Summarizing discussion

Frits M. Flesch

Relevance of fertility research

Mammalian fertilization is achieved by the combination of the hereditary properties of father and mother. Therefore, the two gametes containing a haploid nucleus that possess all the information to develop a new individual, fuse to form a new diploid nucleus in the zygote. Subsequently, the zygote divides to become the embryo, and further cell proliferation and differentiation continue to end in a newborn. The information to build a new individual is present in the two gametes: oocyte and sperm cell. Couples that have a wish to have children do not always succeed to fulfil this wish. Sperm-oocyte fusion has happened long before a child is born, however, in some cases natural mating will not lead to fertilization. In human medicine, couples that seem to be infertile can sometimes benefit from in vitro fertilization (IVF) techniques, although the molecular background of the fertilization defect is often not known. One of the reasons that fertilization defects cannot be detected, is that the process of fertilization is not completely understood yet. Therefore, it is of great importance to unravel the mechanism of sperm-oocyte interaction.

Fundamental research is needed to get insight in the process of fertilization. Many molecules play important roles in sperm-oocyte interaction or the activation of sperm cells, however, most of them have not yet been identified. A better understanding of the fertilization process could improve IVF in order to overcome yet unresolved fertility defects. Extending our knowledge about the molecular background of fertilization could also improve anti-conception. Nowadays, hormones are often administered to prevent pregnancy. These hormones can cause site effects and the administration has to be done accurately and often daily. Discovery of sperm receptors for the egg extracellular matrix (i.e. zona pellucida, ZP) could make it possible to design anti-conception methods based on antibodies against these sperm ZP receptors. Such method or the use of immuno-castration could be useful to control wildlife populations. This would be beneficial, for instance, to control the plague of rabbits in Australia. Rabbits have been introduced in Australia by Europeans but lack predators and as a result the rabbit population increased enormously in number, which caused an ecological imbalance. It would be beneficial if the population of rabbits could be decreased in number by the prevention of their reproduction.

More knowledge about fertilization will also be useful in the breeding industry. Semen for artificial insemination (AI) purposes is often frozen to be stored for longer periods. Semen, however, is very sensitive to freezing and thawing and good protocols to freeze semen are not available. Unravelling the processes that happen during freezing and storage could contribute to improve freezing procedures. Besides the fact that better understanding of semen storage may be beneficial in an economical context, better understanding may also be of great importance to save endangered species from extinction.

Aim of the thesis

Ejaculated sperm cells are not yet able to bind and fertilize oocytes. Sperm cells have to be activated in the female genital tract in a process called capacitation. After capacitation, sperm cells can bind to the ZP. Which triggers a signal in the sperm cell that evokes the influx of calcium leading to the fusion of the plasma membrane with the underlying acrosome (large secretory vesicle in the sperm head). Fundamental knowledge about the molecules involved in capacitation, ZP binding and subsequent acrosome reaction are lacking (especially at the sperm

plasma membrane level). The sperm-ZP interaction can be subdivided in two phases. The sperm cell binds to the ZP first with its plasma membrane, the primary ZP binding. The primary binding evokes the acrosome reaction, leading to secretion of the acrosomal content (mainly hydrolytic enzymes) and the subsequent formation of vesicles (and removal) of the apical plasma membrane and the outer acrosomal membrane. As a consequence of the latter, the inner acrosomal membrane is exposed and this membrane contains proteins that are involved in secondary binding. Differentiation of primary and secondary ZP binding molecules is very difficult, since secondary binding molecules are ubiquitous compared to primary binding molecules. Therefore, the aim of this thesis was to develop a biochemical assay in which sperm capacitation and ZP binding could be studied at the level of the sperm plasma membrane in a context that resembles the physiological system of molecular regulation of these processes.

Results described in this thesis

A first goal was to isolate the sperm plasma membrane, because this is the exclusive site of the sperm cell that is involved in primary ZP binding. Chapter 2 describes the isolation of the plasma membrane of boar sperm cells using nitrogen cavitation and subsequent differential centrifugation. The plasma membrane is released from cells by compression with nitrogen and subsequent decompression, which leads to the formation of nitrogen (gas) bubbles and thereby tearing off of pieces of plasma membrane. This isolation method, also called nitrogen cavitation, and the subsequent differential centrifugation, has been used before. However, in those earlier studies the membrane characterization was only poorly performed. This was mainly due to the absence of markers for the outer acrosomal membrane. In the present study the binding specificity of lectins (carbohydrate binding proteins) for sperm membranes was tested. FITC conjugated lectins were localized using fluorescence microscopy, flow cytometry and transmission electron microscopy (the latter in combination with antibodies against FITC and protein A conjugated to gold). Two lectins, WGA and PNA, proved to be specific for the plasma membrane and the outer acrosomal membrane, respectively. An enzyme linked lectin binding assay was developed to quantify the binding capacity of sperm homogenates and isolated plasma membranes. Subsequently, the purification rate of both plasma membranes and outer acrosomal membranes could be calculated. Purification rates of these marker lectins in combination with marker enzymes showed that the isolated plasma membranes were very pure (>90%) and largely devoid of outer acrosomal membrane.

Since freshly ejaculated sperm cells do not bind to the zona pellucida, plasma membranes of in vitro capacitated sperm cells were isolated. Chapter 3 describes the isolation of plasma membrane from capacitated sperm cells. In vitro capacitation normally occurs in presence of large amounts of BSA. However, these quantities of external proteins disturb biochemical analysis of membrane components such as lipids and proteins. Therefore, capacitation was performed in a medium (Tyrode's) without BSA. Capacitation was monitored using merocyanine, a probe that detects the plasma membrane fluidity, which increases in capacitating sperm cells. Flow cytometry was used to simultaneously monitor the sperm cell's plasma membrane fluidity and viability. Bicarbonate was found to be essential to capacitate sperm cells, whereas, BSA and (extra added) calcium could be omitted. Only a subpopulation of sperm cells could be capacitated

with an average response of 40%, although, large differences existed between ejaculates. Marker enzyme and lectin analysis revealed that plasma membrane isolates from capacitated sperm cells contained only plasma membrane with minor amounts of outer acrosomal membrane. Earlier data in the literature had suggested that capacitation induces tyrosine phosphorylation of proteins in sperm cells. However, it was not known whether tyrosine phosphorylation takes place at the level of the sperm plasma membrane. In the present study we were able to show that capacitation induced tyrosine phosphorylation of three major plasma membrane proteins and several other plasma membrane proteins, which indicates that capacitation alters plasma membrane proteins. These alterations of plasma membrane proteins could be involved in the capacitation induced sperm-oocyte binding or the subsequent acrosome reaction.

Therefore, as described in chapter 4, a physiological relevant assay has been developed to study primary sperm-ZP interaction. Oocytes were isolated from ovaries obtained at the slaughterhouse and subsequently ZPs were isolated and purified. The isolated ZPs were pure as judged by analysis of ZP material by two-dimensional gel-electrophoresis, lectin affinity and phase contrast microscopy. The affinity of solubilized ZP proteins for sperm (plasma membrane) proteins could be blocked by elevation of the NaCl concentration. This finding was used to separate binding from non-binding sperm plasma membrane proteins on a ZP affinity column. The ZP affinity column was made of ZP fragments (not from solubilized ZP proteins) to ensure the correct 3-dimensional folding of ZP proteins and matrix texture. Both may be relevant for correct ZP recognition and binding by primary sperm ZP receptors. Elution of the ZP affinity column, loaded with solubilized plasma membrane proteins derived from capacitated sperm cells, revealed two ZP binding proteins (35 and 46 kDa) that were phosphorylated at tyrosine residues as judged by Western blotting. Both proteins did not bind to the ZP affinity column if solubilized plasma membrane proteins isolated from freshly ejaculated sperm cells were applied to the ZP affinity column. Most likely, capacitation induces tyrosine phosphorylation of both proteins that leads to increased ZP affinity. Future studies should focus on the possibility that these two plasma membrane proteins form dimers before or after capacitation-induced phosphorylation, which may lead to the increased ZP affinity. The exact role of these proteins in sperm-ZP interaction remains to be established.

The bicarbonate-induced increase of plasma membrane fluidity in a subpopulation of capacitating sperm cells (chapter 3) has been further investigated in chapter 5. The cause of this change in plasma membrane fluidity in only a subpopulation of sperm cells was not known. It has been described earlier that capacitation induces a cholesterol efflux in sperm cells in case acceptor molecules (e.g. BSA) are present in the capacitation medium. However, the increased plasma membrane fluidity can be evoked by bicarbonate in absence of BSA as lipid acceptor. In other words, the increase in plasma membrane fluidity (increase in merocyanine fluorescence) is not due to cholesterol efflux. In order to investigate the role of cholesterol in capacitation, subpopulations of viable sperm cells were sorted, one subpopulation showing a capacitation response with high plasma membrane fluidity (high merocyanine fluorescence) and the other one that lacked this response and remained low in plasma membrane fluidity (low merocyanine fluorescence). Lipid analysis revealed that the phospholipid composition (both classes and species) was identical in both sorted subpopulations. However, the cholesterol content in the

subpopulation showing high plasma membrane fluidity was 18% lower when compared to the subpopulation showing low plasma membrane fluidity. Furthermore, the distribution of cholesterol (visualized using filipin in combination with fluorescence microscopy) changed by incubation with bicarbonate from a more general localization in the sperm head to the apical site of sperm cells in the high merocyanine fluorescence subpopulation. Moreover, a relatively large number of sperm cells in the subpopulation with low merocyanine fluorescence contained cytoplasmic droplets, an indication that sperm cells are not fully matured in the epididymis. The cholesterol content of sperm cells incubated with bicarbonate and BSA decreased dramatically (approximately 50%) and showed virtually no filipin staining. In conclusion, bicarbonate induces a cholesterol redistribution only in fully matured sperm cells (i.e. without cytoplasmic droplets and with a relatively low cholesterol content). Furthermore, addition of a lipid acceptor (e.g. BSA) in combination with bicarbonate enables the extraction of cholesterol in these responding cells. These membrane changes may be relevant for the induction of high ZP affinity for instance by enabling tyrosine phosphorylation of primary ZP receptors (see chapter 4).

General discussion and perspectives

Capacitation and sperm-ZP interaction are important events in fertilization. However, the molecular mechanism and the exact sequence of events are still not fully understood. To study capacitation and ZP interaction we used boar semen as a fertilization model. One of the reasons to choose the porcine system is the availability of freshly ejaculated sperm cells. Large numbers of sperm cells are needed to collect enough material to isolate and study the plasma membrane and boars produce large volumes of semen containing up to 125 billions of sperm cells. An additional advantage of boar semen, compared to rodent semen (e.g. mouse), is the possibility to obtain freshly ejaculated sperm cells. In the rodent system, often used to study fertilization, this is not possible since ejaculates from rodents contain a spermicidal coagulation plug. Therefore, rodent semen is collected from the cauda epididymidis and often contains large proportions of immature sperm cells. A second reason to choose for the porcine system is the availability of enough biomaterial from the female side. For instance the ovaries used to isolate the large amounts of ZPs described in this thesis were collected at the slaughterhouse.

Sperm cells lack the appropriate organelles to synthesize proteins or lipids. Therefore, it is not possible to study fertilization with techniques that act via protein expression or mRNA/DNA. In general sperm-ZP interaction is investigated using sperm cells and ZPs. Primary sperm-ZP binding is still not completely understood yet, likely due to complexity of the binding and the interference of secondary binding proteins that are present inside the sperm cell. Much more information would have been revealed, when this binding would be just a simple protein-protein interaction. In that case, Western blotting or affinity chromatography would allow identification of the proteins that are involved in primary sperm-ZP binding. However, the sperm plasma membrane most likely contains ZP binding proteins that are parts of protein complexes (di- or multimers). These complexes are probably not resistant to biochemical analysis like SDS-PAGE. Moreover, the presence of secondary ZP binding proteins also disturbs simple identification of primary ZP receptors from entire sperm cell preparations. These secondary binding proteins are probably more abundant when compared to primary binding proteins as can be concluded from

chapter 4. In conclusion, pure plasma membranes from capacitated sperm cells and ZP fragments seemed to be the material of choice in order to pick up ZP binding sperm proteins. The production of monoclonal antibodies directed to the two ZP binding proteins and other sperm plasma membrane proteins is the logical next step in the study on porcine sperm-oocyte interaction. These antibodies can be used in inhibition studies to confirm the ZP affinity. Furthermore, the antibodies can be employed for localization of the two ZP binding proteins or to reveal whether capacitation induces oligomerization of these proteins in order to achieve high ZP affinity. Interaction of these proteins with the ZP and subsequent oligomerization may also be relevant for the induction of the acrosome reaction. In this case, oligomerization activates directly or indirectly calcium channels, resulting in increased intracellular calcium levels. This hypothesis can be tested by the combination of calcium imaging and a technique to examine oligomerization (e.g. experiments with co-localization or fluorescence resonance energy transfer).

The lack of protein and lipid synthesis in sperm cells can also be considered as an advantage when studying membrane fusion without the interference of newly synthesized proteins or lipids. The sperm cell is a model for intracellular membrane fusion (acrosome reaction: plasma membrane and outer acrosomal membrane) and intercellular membrane fusion (sperm-oolemma fusion), e.g. the acrosome as a large model vesicle to study exocytosis. Several techniques like calcium imaging and flow cytometry are used in sperm research to study the concentration of intracellular calcium (high calcium concentration is needed for membrane fusion). Calcium detection in much smaller vesicles, like vesicles in nerve cells, is not easily accomplished and flow cytometry with these cells is even impossible. Increasing the fundamental knowledge of fusion events in fertilization thus may also contribute to better understanding of fusion events in and between somatic cells (e.g. in cell secretion or in myofibril formation).

Sperm capacitation is one of the items investigated and described in this thesis. A step forward in capacitation research is the possibility to discriminate between capacitated and non-capacitated sperm cells using the plasma membrane fluidity probe, merocyanine first described by Harrison et al. [46]. The use of merocyanine made clear that not all sperm cells in an ejaculate respond in a similar way to capacitation stimuli. The bicarbonate-induced increase in plasma membrane fluidity is only detectable in fully matured sperm cells with a ‘low’ cholesterol content. Identification and isolation of this capacitated subpopulation, enables the separation of capacitation events from other events. Using flow cytometry, dead and acrosome reacted sperm cells can be separated from viable, capacitated sperm cells, which excludes artefacts by cell death or acrosome reaction. A disadvantage of merocyanine is that this probe detects an early event in capacitation (maximal response already after 15 minutes), namely the redistribution of lipids (lateral redistribution of cholesterol and seminolipid, and a bilayer scrambling of phospholipids). Prolonged exposure of sperm cells to capacitation stimuli, probably initiates other slower events like tyrosine phosphorylation, protein oligomerization, cholesterol efflux and more. Mapping and linking all these events would be a great step forward in the comprehension of mammalian sperm capacitation.

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Abbreviations

AC	adenylate cyclase
ADAM	A Disintegrin And Metalloprotease (domain or protein)
AI	artificial insemination
AIA	*Artocarpus integrifolia* agglutinin
AKAP	A kinase anchoring protein
ANOVA	analysis of variance
BPA	*Bauhinia purpurea* agglutinin
BSA	bovine serum albumin
BSP	bovine seminal plasma protein
BTS	Beltsville thawing solution
cAMP	cyclic 3'5'-adenosine mono phosphate
CL	cardiolipin
ConA	*Canavalia ensiformis* agglutinin
CTC	7-chlorotetracycline
DAG	diacylglycerol
DBA	*Dolichos biflorus* agglutinin
DiI16	1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanin
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EGF	epidermal growth factor
ELLBA	enzyme-linked lectin binding assay
ELISA	enzyme-linked immuno sorbent assay
ER	endoplasmic reticulum
FSC	forward light scatter
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
GPI	glycosylphosphatidylinositol
GS-I/II	*Griffonia simplicifolia* I/II agglutinin
HBS	hepes buffered saline
HBSS	hepes buffered sucrose solution
HBT	hepes buffered Tyrode's
HDL	high density lipoproteins
HPLC	high performance liquid chromatography
HRP	Horseradish peroxidase
ICSI	intra cytoplasmic sperm injection
IEF	iso-electric focussing
IP_3	inositol-1,4,5-triphosphate
IVF	in vitro fertilization
LEA	*Lycopersicon esculentum* agglutinin
LPA	*Limulus polyphemus* agglutinin
LP(C,I,S)	lysophosphatidyl (-choline, -inositol, -serine)
M540	merocyanine-540
MAP	mitogen-activated protein
MPA	*Maclura pomifera* agglutinin

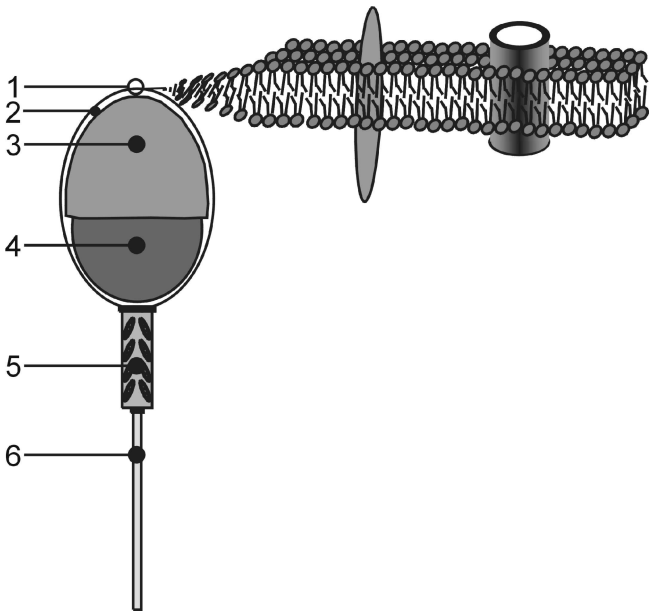
ODAF	5-(*N*-octadecanoyl) aminofluorescein
PAF	platelet activating factor (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phoshocholine)
PBS	phosphate buffered saline
PC	phosphatidylcholine (phosphocholineglyceride)
PE	phosphatidylethanolamine (phosphoethanolamineglyceride)
PI	phosphatidylinositol
PI3-kinase	phosphoinositide 3 kinase
PIC	phosphoinositidase C
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PL(A_1,A_2,C,D)	phospholipase (A_1,A_2,C,D)
PMSF	phenylmethylsulfonyl fluoride
PNA	*Arachis hypogaea* (peanut) agglutinin
PP(1,2A)	protein phosphatase (1,2A)
PS	phosphatidylserine
PTK	protein tyrosine kinase
ROS	reactive oxygen species
SBA	*Glycine max* (soybean) agglutinin
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gelelectrophorese
SGG	sulfogalactosyl glycerol
SLIP	seminolipid immobilizing protein
SM	sphyngomyelin
SSC	sideward light scatter
TBS	tris buffered saline
TBSS	tris buffered sucrose solution
UEA-I	*Ulex europeaeus* agglutinin
WGA	*Triticum vulgare* (wheat germ) agglutinin
ZP	zona pellucida
ZP1,2,A,...	zona pellucida protein 1,2,A,...

Samenvatting

Inleiding

De geboorte van een baby is vaak een blije gebeurtenis. Een lange tijd voor de bevalling is het begin van het nieuwe leven reeds begonnen: de versmelting van de spermacel met de eicel. Ondanks dat dit gegeven al erg lang bekend is, weten we niet precies wat er gebeurt tijdens deze interactie die moet leiden tot de bevruchting. Helaas geldt niet voor elke man en vrouw die kinderen willen, dat dit ook zal lukken. Hoe dat komt, is lang niet altijd duidelijk. Om vast te kunnen stellen wat er misgaat is het van belang om allereerst te weten wat er normaal gesproken gebeurt tijdens de sperma-eicel interactie op het niveau van de cel en het molecuul. Het hier beschreven onderzoek heeft plaats gevonden om fundamentele kennis te verkrijgen over de sperma-eicel interactie en de moleculen die daarbij een rol spelen.

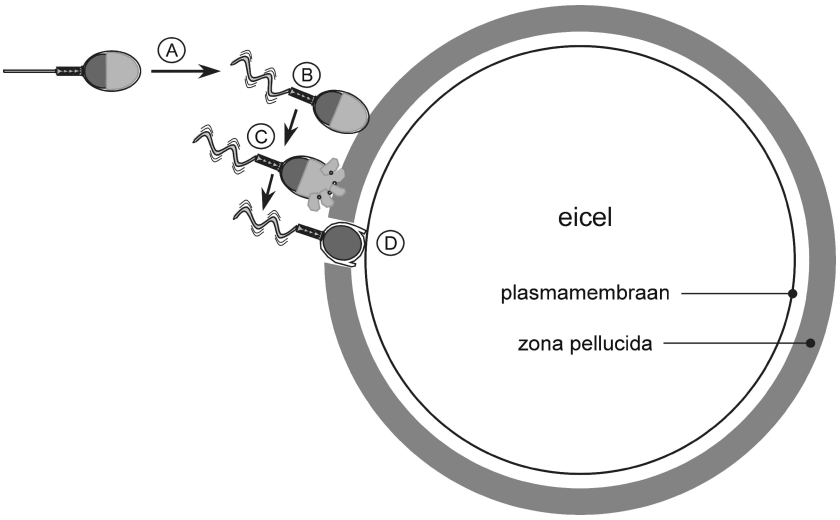
Hoe ziet een (sperma)cel er eigenlijk uit? Een cel kan men zich voorstellen als een kleine eenheid met een vaak specialistische functie. Zo is er de rode bloedcel voor zuurstoftransport, de witte bloedcel om het lichaam te verdedigen tegen indringers zoals bacteriën en de spermacel voor de voortplanting. Iedere cel bestaat globaal uit water met allerlei kleine structuren (cytoplasma, inclusief structuren zoals o.a. de celkern) met de plasmamembraan daaromheen als een jasje. In het geval van een spermacel kunnen we de kop, het middengedeelte en de staart onderscheiden (zie figuur 1). Met name de spermakop is van belang voor de binding aan en het binnendringen van de eicel. Het middengedeelte en de staart zijn er voor de voortbeweging.



Figuur 1. De spermacel en zijn plasmamembraan. De spermacel wordt in zijn geheel omgeven door de plasmamembraan (1). De plasmamembraan bestaat uit een dubbele laag lipiden, die met de waterafstotende vetzuurstaarten naar elkaar toe gericht zijn en de waterminnende kopgroepen zijn gericht naar de buitenkant van de cel en naar binnen (cytoplasma, 2). Deze flexibele dubbellaag bevat naast lipiden ook eiwitten die allerlei functies hebben. Naast de plasmamembraan bestaat de kop van de spermacel uit het acrosoom (3) en de celkern (4). Het acrosoom is een structuur die omgeven is door een membraan en eiwitten bevat, die nodig zijn om de eicel binnen te dringen. De celkern bevat het erfelijk materiaal (DNA). De beweeglijkheid van de spermacel wordt veroorzaakt door het middelgedeelte (5) dat de energie produceert en de staart (6) die de beweging veroorzaakt.

In hoofdstuk 1 is de spermaplasmamembraan geïntroduceerd welke voor iedere cel van levensbelang is. De plasmamembraan kan worden gezien als een vetlaagje op soep: het onderscheidt de soep (cytoplasma) van de omgeving (alles buiten de cel), de laag is heel elastisch en er kunnen soepballen (eiwitten) doorheen steken. Eiwitten, die overal in de cel zitten, zijn het gereedschap van een cel. Zo zijn er eiwitten die bepaalde moleculen kunnen knippen of aan elkaar plakken. De grote blaasvormige structuur in de spermakop, het acrosoom (zie figuur 1), is nodig

om de buitenste schil rond de eicel (zona pellucida) te kunnen doorboren. Na binding van de spermacel aan de zona pellucida zal door een signaal in de spermacel het acrosoom en de plasmamembraan op verschillende plaatsen fuseren (zie figuur 2). Daardoor kunnen de eiwitten die in het acrosoom zitten naar buiten en deze knippen plaatselijk de zona pellucida in kleine stukjes. Zo kan de spermacel naar binnen om te binden en te versmelten met de eicel. Het fuseren van de plasma- en acrosomale membraan en het daarop volgende naar buiten vloeien van eiwitten wordt de acrosoomreactie genoemd.



Figuur 2. Sperma- eicel interactie. De spermacel wordt geactiveerd in het vrouwelijk geslachtskanaal (A). Activering leidt tot verhoogde bewegelijkheid en affiniteit voor de zona pellucida (B). Na binding aan de zona pellucida fuseren de plasmamembraan en de acrosomale membraan, waardoor eiwitten uit het acrosoom vrijkomen (C). Deze acrosomale eiwitten knippen de zona pellucida lokaal kapot, waardoor de spermacel de plasma membraan van de eicel kan bereiken en vervolgens binden, waarna de sperma- en de eicel zullen versmelten (D).

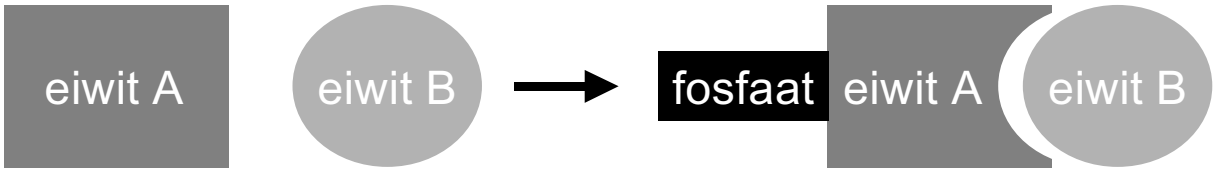
Het is van belang dat de acrosoomreactie op het juiste tijdstip en bij de juiste cel geïnitieerd wordt, namelijk na binding aan de zona pellucida. Herkenning op cellulair niveau gaat altijd via de plasmamembraan en veelal via specifieke eiwitten. De ene cel heeft een eiwit in de plasmamembraan dat een bepaald eiwit op een andere cel kan binden: beide eiwitten passen precies op elkaar zoals de juiste sleutel in een slot. De herkenning tijdens de sperma-eicel interactie gaat waarschijnlijk ook zo. Het is echter nog steeds niet bekend welke sperma-eiwitten de zona pellucida binden. We weten ook niet hoe het komt dat deze binding pas optreedt na sperma-activering. In het hier beschreven onderzoek is gebruik gemaakt van het varken als model voor de sperma-activering en de daarop volgende binding aan de zona pellucida. De voortplanting bij het varken lijkt in veel opzichten op die bij de mens. Bovendien brengt dit een aantal voordelen met zich mee: (i) biochemisch onderzoek noodzaakt vaak het gebruik van grote hoeveelheden biomateriaal (in dit geval sperma- en eicellen) en dit is van het varken gemakkelijk en in grote hoeveelheden te verkrijgen; (ii) onderzoek aan menselijk materiaal, met name de toepassing van in vitro fertilisatie technieken, gaat gepaard met veel ethische bezwaren.

Resultaten

Om heel specifiek de sperma-eicel interactie te kunnen bestuderen is het nodig om de spermaplasmamembraan te isoleren. In hoofdstuk 2 wordt beschreven hoe dit wordt gedaan en hoe deze gekarakteriseerd wordt. De isolatie is uitgevoerd met behulp van stikstofcavitatie (opblazen van de spermacel) en differentieel centrifugeren. Om te achterhalen welke structuren van de cel geïsoleerd zijn, moet de oorsprong van het materiaal worden onderzocht. Dit kan gedaan worden met behulp van markers, zoals eiwitten die alleen voorkomen in bepaalde

structuren. Voor de karakterisering van de plasmamembranen werd een geheel nieuwe methode ontwikkeld. Dit laatste was nodig omdat er geen marker bestond voor de acrosomale membraan. Verschillende lectines (eiwitten die aan bepaalde suikers binden) zijn getest op het vermogen om aan specifieke structuren van de cel te binden. Zo kwam aan het licht dat er een lectine is dat aan de plasmamembraan bindt en een ander dat aan de acrosomale membraan bindt. Met behulp van deze twee lectines en ander conventionele markers kon de zuiverheid van de geïsoleerde plasmamembraan worden bepaald. Het bleek dat de geïsoleerde membranen voor meer dan 90% uit plasmamembraan bestond.

In hoofdstuk 3 is gekeken of de plasmamembraan van geactiveerde spermacellen geïsoleerd kon worden en of op de plasmamembraaneiwitten van geactiveerde cellen veranderingen waren opgetreden ten opzichte van niet-geactiveerde cellen. Om te bekijken of spermacellen geactiveerd werden in de door ons gebruikte media (speciale vloeistof voor incubatie), hebben we met een (fluorescente) indicator de vloeibaarheid van de plasmamembraan bepaald. Bij spermacellen die geactiveerd worden, heeft de plasmamembraan namelijk een iets hogere vloeibaarheid. Uit deze experimenten kwam naar voren dat opgelost koolzuur (bicarbonaat) onontbeerlijk is voor activering van de spermacellen. Ook bleek dat ongeveer 50% van alle spermacellen werd geactiveerd. De plasmamembraan van geactiveerde spermacellen kon volgens de eerder beschreven methode geïsoleerd worden. Sommige plasmamembraaneiwitten in de geactiveerde spermacellen waren gefosforyleerd (een fosfaat-ion was gekoppeld aan deze eiwitten) terwijl dit bij niet-geactiveerde spermacellen niet kon worden waargenomen. De fosforylering van eiwitten kan van belang zijn voor de activering van de spermacel omdat bekend is dat eiwitten die gefosforyleerd worden van vorm kunnen veranderen. Deze vormverandering kan een eiwit activeren of deactiveren (een eiwit kan nu bijvoorbeeld knippen of juist niet). Ook kan een eiwit zodanig van vorm veranderen dat het nu een ander eiwit herkent (zie figuur 3). De sleutel past daardoor als het ware wel op het slot.



Figuur 3. Fosforylering van een eiwit kan leiden tot de verandering van de vorm van dat eiwit. Deze vormverandering kan zo zijn dat het gefosforyleerde eiwit in staat is om te binden aan een ander eiwit.

Vervolgens is in hoofdstuk 4 de binding van sperma-eiwitten aan de zona pellucida bestudeerd met behulp van een affiniteitsmatrix. Dit kan men zich voorstellen als een grote verzameling eiwitten (bijvoorbeeld het eiwit B in figuur 3: zona pellucida eiwitten in hoofdstuk 4) die met een netje bij elkaar gehouden worden. Als er andere eiwitten (deze uit de spermaplasmamembraan) bij de affiniteitsmatrix gebracht worden, zullen eiwitten met affiniteit voor de matrix binden (zoals het gefosforyleerde eiwit A in figuur 3). De niet-gebonden eiwitten kunnen weggewassen worden en met een ‘handigheidje’ (spoelmiddel met veel zouten) kunnen de bindende eiwitten vervolgens van de affiniteitsmatrix gehaald worden. Twee eiwitten uit geïsoleerde spermaplasmamembranen bonden aan de zona pellucida matrix en beide waren alleen in staat om te binden als de plasmamembranen afkomstig waren van geactiveerde spermacellen. Uit analyse bleek dat het gefosforyleerde eiwitten betrof. Een mogelijk model is dat deze twee

eiwitten tijdens activering gefosforyleerd worden en daardoor kunnen binden aan de zona pellucida. Mogelijk spelen deze eiwitten ook weer een rol bij de start van de acrosoomreactie.

In hoofdstuk 5 is getracht om het mechanisme op te helderen achter de verhoging van de plasmamembraanvloeibaarheid, welke tijdens de activering van spermacellen optreedt. Het belang hiervan werd duidelijk uit de resultaten van hoofdstuk 3, die beschrijven dat de verhoging van de plasmamembraanvloeibaarheid gelijktijding optreedt met de fosforylering van eiwitten in de spermaplasmamembraan. Het was reeds bekend dat cholesterol, een belangrijke component van de plasmamembraan, een regulerende rol heeft in de membraanvloeibaarheid. Spermacellen konden via een nieuwe procedure na activering gescheiden worden van niet-geactiveerde cellen. Daarna werd het cholesterolgehalte in deze twee gesorteerde populaties bekeken. Geactiveerde cellen hadden ongeveer 18% minder cholesterol (percentage van totaal gehalte aan lipiden) vergeleken met niet-geactiveerde cellen. Doordat in het gebruikte activeringsmedium geen acceptor voor cholesterol aanwezig was, moesten deze cellen reeds minder cholesterol bezitten tijdens ejaculatie. Microscopisch onderzoek liet zien dat in de groep geactiveerde cellen veel vaker goed gerijpte cellen voorkwamen, vergeleken met de groep die niet geactiveerd konden worden (veel cellen met cytoplasmatische druppels wat een teken van niet rijp zijn is). De plasmamembraan van niet goed gerijpte spermacellen bevat dus meer cholesterol. Met een (fluorescente) indicator kon cholesterol zichtbaar gemaakt worden. Cholesterol bleek zich te verplaatsen tijdens activering. Voor activering was cholesterol min of meer gelijkmatig verdeeld over de spermakop. Tijdens activering verplaatste cholesterol zich naar de voorkant van de spermakop. Als er in het activeringsmedium een cholesterolacceptor aanwezig was, verdween zelfs een groot gedeelte aan cholesterol. Dit laatste gebeurde alleen in cellen die geactiveerd konden worden. Het verplaatsen en verdwijnen van cholesterol uit de plasmamembraan zou een manier voor de spermacel kunnen zijn om zich voor te bereiden op de acrosoomreactie. Ook is het mogelijk dat door de veranderde membraanvloeibaarheid eiwitten iets van vorm veranderen en daardoor affiniteit voor de zona pellucida krijgen.

Conclusies

Zoals beschreven in hoofdstuk 6 bleek uit het in dit proefschrift beschreven onderzoek dat het isoleren en karakteriseren van de spermaplasmamembraan goed mogelijk is. Na isolatie was er voldoende materiaal beschikbaar om de plasmamembraan verder te bestuderen. Het bleek dat tijdens de activering van spermacellen bepaalde eiwitten van de plasmamembraan gefosforyleerd worden. Twee van deze eiwitten lijken na fosforylering te kunnen binden aan de zona pellucida. Het principe dat fosforylering leidt tot vormverandering van het betreffende eiwit wat daardoor affiniteit voor andere eiwitten kan krijgen is bekend. Het is nog niet duidelijk hoe de activering van spermacellen met behulp van bicarbonaat tot fosforylering van eiwitten kan leiden.

Een van de veranderingen die plaats vindt naast de fosforylering van eiwitten in de plasmamembraan tijdens de activering van spermacellen, is de herverdeling van cholesterol. Deze herverdeling kan zelfs uitmonden in de afname van cholesterol in de plasmamembraan als er een cholesterolacceptor aanwezig is. Waarschijnlijk treedt een vergelijkbaar proces ook op in het vrouwelijk geslachtskanaal, omdat daar grote hoeveelheden cholesterolacceptoren aanwezig zijn (lipoproteïnen). Het is bekend dat de hoeveelheid cholesterol in de plasmamembraan effect kan hebben op de vorm van bepaalde eiwitten. Het kan dus goed mogelijk zijn dat de

cholesterolveranderingen via eiwitten een effect hebben op de zona pellucida affiniteit van de spermacel. Ook kunnen de cholesterolveranderingen voorbereidingen voor de acrosoomreactie zijn.

Het in dit proefschrift beschreven onderzoek heeft met name aangetoond dat het mogelijk is om de herkenning en binding van de spermacel aan de zona pellucida te bestuderen op moleculair niveau. Daartoe zijn de structuren die daar een rol in spelen geïsoleerd: de zona pellucida en de plasmamembraan van de spermakop. Twee eiwitten gelegen op de spermaplasmamembraan blijken tijdens activering gefosforyleerd te worden waarna ze kunnen binden aan de zona pellucida. Het belang van deze eiwitten in zona pellucida binding en de inductie van de acrosoomreactie moet evenals de karakterisering van de structuur van de eiwitten ontrafeld worden in toekomstig onderzoek.

Dankwoord

En dan gaan nu eindelijk de laatste paar bladzijden gevuld worden met woorden, het einde is in zicht. Ik zal deze ruimte niet besteden aan het beschrijven van mijn zoektocht naar juiste wetenschappelijke woorden. Dat kostte mij soms aardig wat moeite. Tenslotte moest het niet alleen naar mijn zin zijn, maar ook naar de zin van mijn drie begeleiders. Mijn beide promotoren en co-promotor verdienen onder andere mijn dank voor het doorploegen van mijn manuscripten.

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Professor van Golde bekeek mijn manuscripten door een meer biochemische bril: 'Het maakt toch niet uit of dit onderzocht is bij een paard of een varken'. Beste Bert (ik hoop dat ik je deze keer wel op deze manier mag bedanken), bedankt voor de begeleiding en de gastvrijheid die ik vele jaren (bijna 7 jaar) als student en AiO heb genoten op het lab.

Dan hebben we nog mijn co-promotor, Doctor Gadella: 'Ik heb maar weinig opmerkingen', bij het geven van een 'volkomen rood' nagekeken manuscript. Je maakte veel werk van dit nakijken en met jouw commentaar kon ik vaak wat doen en daar heb ik dan ook veel van geleerd. Bartsky, bedankt voor je begeleiding en natuurlijk de gezelligheid op het lab en tijdens onze gezamenlijke congressen. Op onze eerste vertelde jij mij dat Marieke er aan kwam, je eerste 'in vivo' experiment. Ik wens je veel geluk met de volgende kleine (n=2, is dat voldoende?), samen met Marieke en Marion natuurlijk.

Naast de officiële begeleiding zijn natuurlijk de sfeermakers op de werkvloer voor mij erg belangrijk geweest. Chris ik heb van jou als alchemist veel geleerd en niet alleen de bereiding van vuurwerk. Bianca, bedankt voor de Chouffes die we elkaar gaven (en nuttigden) tijdens onze weddenschappen om wat eerder te beginnen. Karin, ik hoop dat ik nog eens een boompje bij je mag komen snoeien. Ate, nogmaals bedankt voor het schoonmaken van de het vergistingsvat, Jos natuurlijk voor de assistentie tijdens het gebruik ervan en Inez (met vele anderen) voor het proeven van het eindproduct. Edita hoort nu ook echt bij de kraaksperma-groep als een naar biochemie 'overgelopen' veterinair. Ik vond het gezellig om samen met jou over biochemie te babbelen en te BBQ-en.

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Biochemie is groter dan de kraaksperma-groep. Zo heb ik gelukkig wat vaker promoties mee kunnen maken van collega aio’s (Jaap, weer bijna terug, Bellinda, Jos, kleine Bianca). De promotie van Edwin was natuurlijk de spannendste omdat dat een race tussen ons was (die ik bij voorbaat kansloos had verloren op minimaal 6 dagen). Ik ben blij dat ik nog samen met je mocht voetballen bij (oud-)Kampong. Het ga je goed in Vancouver. Bianca (grote) mag ik natuurlijk wel extra dankbaar zijn, omdat ik haar promotiedatum gratis mocht overnemen. Daar drinken we er nog ééntje op. Ik hoop straks nog wat meer promoties bij te kunnen wonen (Bianca, Anja, Rob, Koen, Martin (wie heeft de Griek-pool gewonnen?), Michiel, Marieke, Susan, Renske). Ik heb nog heel wat feestjes voor de boeg. Ook de vele stagiaires die overal vandaan leken te komen wil ik als sfeermakers niet onvermeld laten. Natuurlijk was er ook ‘vastigheid’ (zomeravond fietsmaatje Jos, pooldirecteur Rob, René (mijn fiets doet het nog steeds), Paul, Co, Dorothé, Wil, Marion) die ik dankbaar ben voor de steun en de gezelligheid: Marion, moet ik nog Engelse suikerzakjes opsturen?

Mijn verblijf op ‘biochemie’ werd afgewisseld met de tocht naar de overkant: de voortplantingsgroep. I thank all the people from ‘reproduction’ for their faith in me as chairman of the AI-presentations and their help with several ‘small jobs’. Een paar mensen wil ik toch nog even noemen. Peter en Arend, die mij altijd telefonisch te woord stonden als ik te lui was om naar de overkant te lopen om iemand te zoeken of me in te schrijven voor de microscoop. Andries, Jan en Jan mag ik niet vergeten als redders in nood tijdens de ‘spermaloze’ varkenspest periode. Ook veel dank gaat uit naar de volleyballers die het mogelijk maakte om eindelijk eens een volleybalprijs te mogen vasthouden al is meedoen natuurlijk ook belangrijk!

Hulp tijdens een promotie komt niet alleen van de werkvloer. De ontspanning daarbuiten is minimaal net zo belangrijk. Ik heb me dan ook heerlijk kunnen opladen tijdens mijn sportieve bezigheden (Kampong 5 en Switch Heren 3) en de ontspanning daarna. Ik zal dat erg gaan missen als ik een jaartje weg ben.

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Curriculum vitae

Frits Flesch werd geboren op 17 januari 1971 te Oirschot, waar na een aantal luier-jaren gestart werd met het volgen van onderwijs op de Maria school. Het Atheneum werd doorlopen op het Jacob Roelandslyceum te Boxtel en werd afgesloten met het uitreiken van het diploma op 8 juni 1989. Aan de (toen nog Rijks) Universiteit Utrecht werd vervolgens aangevangen met de studie Medische Biologie. Tijdens deze studie werd door een tweetal lange stages de interesse in wetenschappelijk onderzoek gewekt. De eerste stage (10 maanden) werd begeleid door Prof. Dr. WPM Hoekstra (Universiteit Utrecht) en Dr. GH van Geel-Schutten (TNO-Voeding, Zeist) en betrof het onderzoek naar de productie van exopolysacchariden door melkzuurbacteriën. De tweede stage (22 maanden) werd begeleid door Prof. Dr. LMG van Golde en Dr. AGM Tielens en werd uitgevoerd op de afdeling Biochemie aan de Faculteit Diergeneeskunde te Utrecht. Tijdens deze stage werd het energiemetabolisme van de vrij levende worm *Caenorhabditis elegans* bestudeerd. Ook werd tijdens deze periode de afstudeerscriptie geschreven met de titel: 'glycocalyx in (parasitic) helminths'. Het doctoraal diploma werd op 29 augustus 1995 uitgereikt. Op 1 januari 1996 werd aangevangen met het promotie-onderzoek beschreven in dit proefschrift. Beide promotoren Prof. Dr. B Colenbrander (Gezondheidszorg Landbouwhuisdieren) en Prof. Dr. LMG van Golde (Biochemie, Celbiologie en Histologie), en co-promotor Dr. BM Gadella (Biochemie, Celbiologie en Histologie) begeleidden de jonge onderzoeker dit maal. Vanaf 1 november 2000 zal een start gemaakt worden met een post-doc baan aan de Universiteit van Birmingham onder begeleiding van Dr. IA Brewis and Dr. CL Barratt.

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