Capacitation and acrosome reaction in equine spermatozoa

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Chapter 2

Abstract

During sexual reproduction, the sperm and oocyte must fuse before the production of a diploid zygote can proceed. In mammals such as equids, this fusion depends critically on complex changes in the plasma membrane of the sperm and, not surprisingly, this membrane differs markedly from that of somatic cells. After leaving the testes, sperm cease to synthesise plasma membrane lipids or proteins, and vesicle-mediated transport stops. When the sperm reaches the female reproductive tract, it is activated by so-called capacitation factors that initiate a delicate reorientation and modification of molecules within the plasma membrane. These surface changes enable the sperm to bind to the extracellular matrix of the egg (zona pellucida) and the zona then primes the sperm to initiate the acrosome reaction, an exocytotic event required for the sperm to penetrate the zona. This paper will review the processes that occur at the sperm plasma membrane before and during successful penetration of the equine zona pellucida. It is noted that while several methods have been described for detecting changes that occur during capacitation and the acrosome reaction in bovine and porcine sperm, relatively little has been documented for equine sperm. Special attention will therefore be dedicated to recent attempts to develop and implement new assays for the detection of the capacitation status of live, acrosome-intact and motile equine sperm.

Introduction

During insemination, enormous numbers of sperm are deposited in the mare’s genital tract. However, in the normal situation only one sperm will eventually fertilise the egg and, to ensure that this is the case, sperm-egg interaction and fertilisation are highly regulated processes. Moreover, the sperm plasma membrane plays a critical role in regulating sperm-egg interaction and, for this reason, it is an extremely dynamic structure. During spermatogenesis [for review see 1], the sperm plasma membrane and many of its other structures are tailored for their roles during transport through the female genital tract and interaction with the oocyte. However, due to the concurrent loss of most of the cell organelles and the cessation of DNA-transcription, spermatozoa are unable to produce proteins or maintain vesicular transport and, therefore, components of the plasma membrane cannot be newly synthesised. Nevertheless, the plasma membrane of sperm entering the epididymis is not yet fully "mature" [for review see 2] and during the passage of sperm through the epididymis the plasma membrane changes significantly 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 and, certainly, sperm only reach full maturity after entering the cauda epididymidis.

The mature sperm has three highly specialised regions (Fig 1A): (i) the DNA containing sperm head, which is vital to sperm-oocyte interaction; (ii) the midpiece, which contains
mitochondria and is involved in energy production; (iii) the flagellum, which is involved in motility. Other than the nucleus, the sperm head contains little except a small amount of cytoplasm and, at its apical extreme, the acrosome (Fig 1B), a large vesicle containing the hydrolytic enzymes necessary for penetration of the zona pellucida (ZP) [3]. The boundary between the plasma membrane that overlies the acrosome and that caudal to the acrosome is known as the equatorial segment. Sperm-oocyte interaction can be subdivided into a sequence of events (Fig 2) [for review see 4]. First, ejaculated sperm must be activated in the female genital tract in a process called capacitation because only capacitated sperm can bind to the ZP (Fig 2; process 1). This binding of sperm to oocyte in turn induces changes in the sperm
collectively termed the acrosome reaction (Fig 1C, 2). In particular, zona-binding triggers a 
Ca$^{2+}$-influx that causes the plasma membrane to fuse, at multiple sites, with the outer acrosomal 
membrane to form “mixed” vesicles that disperse into the ZP and release hydrolytic enzymes 
to digest the ZP (Fig 2; process 2-3). This loss of the outer two membranes leaves the inner 
acrosomal membrane as the new outer cell membrane and it becomes continuous with the 
plasma membrane of the post equatorial segment via an inflexion that resembles a hairpin [5]. Consequently, the inner acrosomal membrane is exposed to, and binds to, the ZP (secondary 
ZP-binding) and the sperm that have reacted properly are able to penetrate the ZP and enter the 
perivitelline space (Fig 2; process 4). Here the sperm binds to the egg plasma membrane 
(oolemma), firstly via its tip and subsequently via its equatorial region and, in particular, via 
the hairpin membrane structure. After this lateral binding, the sperm plasma membrane and the 
oolemma fuse and the sperm is incorporated into the oocyte that, in turn, is activated and 
initiates an effective block to polyspermy (Fig 2; processes 5 and 6).

**Fig. 2.** Schematic representation of the sequence of interactions between the male and female 
gamete required for fertilisation. 1. Sperm binding to the zona pellucida with its apical subdomain; 
2. The acrosome reaction, a multiple fusion event of the outer-acrosomal membrane with the 
apical and pre-equatorial plasma membrane; 3. The penetration of the sperm cell through the 
zona pellucida; note that the equatorial membrane hairpin structure remains intact. 4. Sperm 
binding and fusion with the oolemma (fertilisation) are both exclusive events for the equatorial 
plasma membrane; 5. Activation of the oocyte by cytosolic factors and fast poly-spermy block; 
6. Secretion of cortical granules (cortical reaction) causing a definitive (slow) poly-spermy block. 
Note that, although the apical subdomain is exclusively involved in primary zona binding, the 
bound sperm appears to have a more flattened position towards the zona surface as indicated 
in this cartoon. Consequently the zona penetration curve is rather diagonal than radial towards 
the oolemma.
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It is only relatively recently that the heterogeneity of the sperm plasma membrane has become clear. However, the dramatic differences in different regions clearly relate to physiological specializations of the plasma membrane. Moreover, the composition and lateral organization of the plasma membrane regulate its affinity for adhesion factors, its permeability to hydrophilic solutes and its role in cell signalling and cell fusion events.

This review will compare events that occur during the capacitation of stallion sperm with those reported in other mammalian species. Furthermore, we will review the current understanding of the relationship between the dynamics of sperm plasma membrane organization and the physiology of capacitation and the acrosome reaction.

Capacitation induced alterations in sperm lipid organisation and their role in sperm-zona interaction

The mature sperm lacks the major organelles involved in lipid synthesis and breakdown. Furthermore, its surface membrane is not in contact with intracellular membranes because vesicle-mediated membrane transport has ceased and will only briefly resume when the apical plasma membrane fuses with the underlying outer acrosomal membrane during the acrosome reaction (Fig 1). The unusual composition and organization of lipids in the sperm plasma membrane are almost certainly reflections of these limitations.

Lipid composition

There is considerable variation in the lipid composition of the sperm plasma membrane in different mammalian species. However, there is a general similarity. For example, the plasma membrane of boar sperm contains approximately 67% phospholipids, 25% neutral lipids and 8% glycolipids [6] while that of a stallion sperm contains approximately 57% phospholipids, 37% cholesterol and 6% glycolipids, such that the stallion sperm differs primarily with regard to its relatively high cholesterol content.

Phospholipids can be divided into phosphoglycerolipids and sphingomyelin and the phospholipid class composition of sperm is generally similar to that of somatic cells. More specifically, stallion sperm contain approximately 48% phosphocholineglycerides (PC), 16% phosphoethanolamineglycerides (PE), 13% sphingomyelin (SM), 15% phosphatidylserinocardio/erin, 5% phosphatidyglycerol and approximately 3% phosphatidylinositol. Moreover, not only do the PC and PE fractions of mammalian sperm have fairly unique structural characteristics, but stallion sperm differ from those of the other mammalian species that we have investigated so far, by having a surprisingly very high content of 22:5 fatty acids in their PC and PE fractions and relatively few 22:6 fatty acids [Gadella and Brouwers; unpublished data]. In sperm from other mammals species the situation was just reversed in that the PE and PC fractions contained a profound amount of 22:6 and much less 22:5 PC fatty acids [7,8]. Stallion and other mammalian sperm share the property that PE and PC contain almost
exclusively 22:5 or 22:6 fatty acids which is fairly unique for sperm (with the exception of retina cells; [6]).

Major variations in the neutral lipid composition of sperm membranes are found not only between different species but also between different males within a species and even between different ejaculates from a single male. The major variable is the amount of cholesterol in the sperm plasma membrane. For instance, human sperm contain much higher amounts of cholesterol (>40 % of total lipids) than boar sperm (24 %) while equine sperm have relatively high cholesterol content (37 %). Furthermore, the cholesterol content seems to be related to the rate of capacitation (Yanagimachi, 1984) possibly because cholesterol must be depleted from the plasma membrane during this process.

The remaining class of lipids, the glycolipids, are found in sperm almost exclusively in the form of seminolipid, a structurally unique glycolipid found only in mammalian sperm and Schwann cells [for review see 6]. Stallion sperm contains similar amounts of seminolipid as sperm from other mammalian species (approximately 6.8 mol % of total lipid in equids versus approx. 8 mol % of total lipids in man, bull, mouse, rat, boar; [9]). Both seminolipid and its desulfated counterpart are believed to participate in certain fertilisation processes [for review see 4] and to this end accessory sex gland secretions contains low amounts of arylsulfatase. However, it has yet to be shown whether seminolipid can be desulfated in equine sperm after ejaculation, as it can in human and porcine sperm.

Lateral membrane topology

The functional division of the sperm head surface into lateral domains and subdomains, based on the topology of the surface molecules, was first recognized in the 1970’s and the domains are summarized in Fig 1. This lateral heterogeneity has been clearly demonstrated by lectin binding patterns and freeze-fracture studies and revealed that integral membrane proteins are unevenly distributed among different regions of the sperm head and other parts of the sperm [10, 2]. Furthermore, this delicate sperm surface organization alters during capacitation as a result, at least in part, of decoating (i.e. removal of glycocalyx components [11]), adsorption of new components from female genital fluids and enzymatic modification of glycocalyx components [12,13,]. In addition, a lateral reorganization of transmembrane proteins occurs across the sperm head [14, 15,]. These phenomena have been shown to occur in stallion sperm [16, 17], although it is difficult to understand how the lateral heterogeneity is maintained and altered specifically when there are few obvious structural means to divide areas within the sperm head. It has been variously proposed that the sperm head cytoskeleton [18] or differential electrostatic interactions help to maintain the regional differences [4]. More recently, it has been found that the lipids within the sperm head plasma membrane are also organized into distinct domains [19, 20, 21, 22, 23, 24,] This uneven distribution of lipids within the equine sperm plasma membrane was first detected using probes that complex with unesterified sterols (e.g. filipin, a fluorescent molecule that emits blue light when exited by UV light; [25]). Filipin/cholesterol complexes can be visualized on freeze-fracture analysis (by electron
microscopy) or by UV fluorescence confocal microscopy. In freshly ejaculated sperm, filipin complexes are distributed over the entire sperm head, but a lower density is apparent in the post-equatorial segment. Following capacitation, the post-equatorial region becomes devoid of filipin complexes while the amount in the apical region increases slightly. Although, it has been suggested that lipid segregation and reorientation in the sperm plasma membrane can be explained by lipid phase transitions (from the fluid to the gel phase), such transitions are only observed after cooling of sperm [24]. It has alternatively been suggested that, the organization of the glycocalyx is important for the lateral lipid heterogeneity of the equine sperm head plasma membrane and that capacitation dependent reorganization of lipids is most likely to reflect changes in the glycocalyx and a collapse of the phospholipid asymmetry (see below).

**Membrane bilayer topology**

As in somatic cells, the lipids of the sperm plasma membrane are distributed asymmetrically across the lipid bilayer. More specifically, choline phospholipids, SM and, to a lesser extent, PC are found predominantly in the outer lipid leaflet [26] while the aminophospholipids PE and, in particular, PS are located in the inner lipid leaflet. The inner leaflet concentration of PE and PS is probably due to active inward transport by an aminophospholipid translocase. Recently, we performed a set of experiments to determine whether lipid asymmetry in stallion sperm is affected by capacitation in vitro [27] and, indeed, a partial scrambling of lipid asymmetry was observed. After 5 h in capacitating conditions, sperm showed a marked increase of PC and SM levels in the inner leaflet and the normal inward movement of PE and PS was considerably slowed down (up to 10 times). These changes probably result from bicarbonate mediated activation of a non-specific bidirectional phospholipid scramblase during capacitation and, certainly, this would explain the increased inward translocation of PC and SM and also the decrease in net PS and PE translocation. Indeed, PS and PE are only found in the outer leaflet of the plasma membrane of equine, or other mammalian, sperm during capacitation (see also Figure 3). That scrambling of phospholipids is controlled by a bicarbonate mediated signalling pathway is demonstrated most clearly by the dose dependent increase in phospholipid scrambling that accompanies increased bicarbonate levels. In the absence of bicarbonate, scrambling can be induced by phosphodiesterase inhibitors (inhibit cAMP breakdown), protein kinase A (PKA) activators, protein phosphatase 1 and 2a inhibitors and by the addition of cAMP analogues [28]. From a practical point of view, this lipid scrambling can be monitored using the fluorescent probe merocyanine-540, a marker for disordered lipid packing of membranes [29]. Non-capacitated sperm with maximal lipid asymmetry show no fluorescence when stained with merocyanine, whereas scrambling allows intercalation of the dye into the membrane and, therefore, results in high fluorescence. In fact, the merocyanine response mirrors the scrambling response, because it is dependent on the same bicarbonate induced signalling cascade [28]. Indeed the merocyanine-540 response in stallion sperm correlates well with the number of cells that have either a capacitated or an acrosome-reacted CTC labelling pattern [27].
Fig. 3. Lipid reordering in capacitating stallion sperm cells. Panel A: The lipid ordering of the apical plasma membrane of freshly ejaculated sperm cells. Note the asymmetric bilayer distribution of phospholipids and the lack of phospholipid scramblase activity. Relatively high levels of cholesterol are present in the phospholipid bilayer and this cholesterol cannot be depleted by albumin. The sperm specific glycolipid seminolipid is concentrated in the apical plasma membrane (outer leaflet) and trans membrane proteins are not phosphorylated at tyrosine residues (Y). Panel B: Changes in lipid ordering during capacitation in Tyrode’s media containing albumin, bicarbonate and calcium. Scramblase is activated via a bicarbonate adenylyl cyclase protein kinase A driven signalling pathway, as a result the phospholipid asymmetry collapses. This phospholipid asymmetry collapse permits albumin-mediated depletion of cholesterol, the lateral membrane removal of seminolipid, phosphorylation of tyrosine residues (PY) in membrane proteins (inducing conformational changes and dimerisation of such proteins).

**Lipoprotein mediated cholesterol transport**

The group of Langlais found that inclusion of albumin in the in vitro fertilisation (IVF) media developed by them improved embryo production rates and they therefore proposed a model for capacitation and acrosome reaction based on the hypothesis that lipoproteins extract sterols from the sperm plasma membrane [30]. Certainly, albumin decreases markedly (up to 40%) the cholesterol content of sperm of various mammalian species [31] including stallion sperm.
capacitated in vitro (Gadella and Brouwers, unpublished results). However, this albumin-medi- ated decrease in cholesterol content occurs only when sperm are incubated in the presence of bicarbonate and the extraction of cholesterol is very specific since it does not affect the phospholipid content of the membranes. The decrease in cholesterol content only takes place in bicarbonate-stimulated sperm. In fact, only merocyanine positive subpopulations of sperm allow albumin mediated cholesterol depletion. Therefore, it appears that albumin mediated cholesterol depletion from sperm membranes is a later event in sperm capacitation than the bicarbonate mediated changes in membrane fluidity and the induction of a collapse of phospholipid asymmetry (see Fig 3).

Cholesterol can also be extracted from the sperm plasma membrane in the absence of bicarbonate (i.e. in non-capacitating conditions) using cyclodextrins [32, 33]. Both albumin and cyclodextrin-treated sperm incubated under capacitating conditions showed a very rapid and pronounced activation of PKA and protein tyrosine kinase (PTK) is observed [33] (see Figure 3), thereby demonstrating that, at low cholesterol concentrations, capacitation and the acrosome reaction proceed rapidly. It is, therefore, possible that the rate of sperm capacitation relates to the rate of cholesterol efflux from the plasma membrane and, in this respect, sperm with high cholesterol contents (e.g. from man and bull) are slow to capacitate (respectively 8 and 6 h) whereas those with lower cholesterol contents (boar and ram) are much faster (1 and 2 h, respectively) [3]. Probably, stallion sperm capacitate slowly due to their relatively high content in cholesterol (see section Lipid composition). It is also worth noting that seminal plasma of men, stallions and perhaps other mammals contains cholesterol rich vesicles secreted by the prostate (prostasomes) [34, 35], which block cholesterol efflux from sperm and probably help to delay capacitation until the appropriate time.

**Lipid peroxidation**

Because of their high polyunsaturated fatty acid content and relatively poor antioxidant defences [36, 37], mammalian sperm are somewhat prone to oxidative stress. Of course sperm will contact free radicals during their passage towards the oocyte and, for example, neutrophils and leucocytes in the seminal plasma secrete reactive oxygen species (ROS) [38, 39] and sperm themselves secrete intracellular ROS as a result of flagellar activity [40, 41]. The resultant oxidative attack is believed to regulate sperm function in two ways (i) Beneficial; mild peroxidation may promote capacitation by activating the sperm specific PKA [42, 43,] and switching on tyrosine kinases [42], while superoxide anions induce sperm hypermotility and increase their affinity for the ZP. (ii) Detrimental; on the other hand excessive peroxidation results in sperm deterioration [44]. Unfortunately, while the detrimental effects of lipid peroxidation for sperm have been extensively described [45] the importance of peroxidation for normal fertilisation remains to be investigated.
Fig. 4. The role of the glycocalyx in zona induced acrosome reaction. Panel A: In freshly ejaculated sperm cells a highly complex extracellular matrix is attached to the sperm plasma membrane (note the lipid ordering of the freshly ejaculated sperm cell; see also Fig. 3A). This glycocalyx prevents sperm zona binding and progesterone binding to the sperm surface receptor (CF = coating factor). Panel B: In capacitated sperm cells large parts of the glycocalyx are released (and lipids are reordered as detailed in Fig. 3B). As a result the progesterone receptor is unmasked now can bind and progesterone (P). The conformation changes in tyrosine phosphorylated (PY) transmembrane proteins enable binding of the zona pellucida (ZP3). Progesterone coupling to its receptor is coupled to the opening of Ca\(^{2+}\) channels and therefore involved in Ca\(^{2+}\) influx. This Ca\(^{2+}\) synergistically with the activated zona receptor induces the GTPase driven synaptotagmin I, synaptobrevin like induced exocytosis (acrosome reaction).

Capacitation induced alterations in sperm glycocalyx and its role in sperm-oocyte interactions

On the extracellular side of the sperm plasma membrane, there are a number of carbohydrate structures that are bound to plasma membrane proteins (glycoproteins) or specific lipids (see Fig 4) and are known collectively as the glycocalyx. The glycocalyx has been proposed to play an important role in sperm-oocyte interaction because it is the part of the sperm that first comes...
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into direct contact with the oocyte. Furthermore, the glycocalyx may help to organize the sperm plasma membrane with which it is in direct contact via the transmembrane proteins and glycolipids.

During capacitation the glycocalyx, or at least its lectin binding properties, change markedly [reviewed by 3]. However, whether these changes in lectin binding reflect modification of carbohydrate structures, (un)covering of carbohydrate structures, or repositioning of glycoproteins or glycolipids is not yet known. Certainly, glycolipid repositioning during capacitation (Fig 3) [20, 21] is thought to involve the active removal of decapacitation factors [46, 47] and other factors bound to the sperm plasma membrane [48] and this almost certainly alters the glycocalyx (Figure 4).

Glycocalyx and tyrosine phosphorylation

During capacitation, tyrosine phosphorylation of proteins occurs in the plasma membrane of the flagellum and of the sperm head and it is thought that conformational changes in these proteins may alter their properties and contribute to the increased ZP affinity [49] and hyperactivity [50] and the induction of the acrosome reaction [51] that are characteristic of capacitated sperm. Indeed, a large number of proteins are tyrosine phosphorylated during in vitro capacitation of sperm from several mammalian species including man, mouse, boar, and bull [see 4]. It is currently believed that removal of the glycocalyx allows oligomerisation of transmembrane proteins, which is due to their conformational changes, and they become active tyrosine kinases. While, these data are not yet available for stallion sperm, preliminary experiments in our lab show clearly that bicarbonate and albumin are also involved in the induction of tyrosine phosphorylation in capacitating stallion sperm.

Glycocalyx and progesterone binding

Progesterone, like other steroid hormones, classically exerts its biological effects on protein expression via intracellular receptors [52]. However, it now appears that in several mammalian cell types biologically active progesterone (and other steroid hormone) receptors can also be found on the plasma membrane [53]. In this respect, the presence of progesterone binding sites/receptors on the plasma membrane of mammalian sperm heads has been shown using both FITC labelled BSA conjugated to progesterone (PBF) and the monoclonal antibody C-262, raised against the human intracellular progesterone receptor [17, 54, 55]. Although the specificity of both these labelling techniques has been questioned, isolation and identification of the putative progesterone receptor(s) may prelude the discovery of a new family of plasma membrane hormone receptors with low affinity for steroids but an ability to elicit rapid physiological changes. Isolating the progesterone receptor must be a major goal of studies to further detail the signalling events that lead to the acrosome reaction. Certainly, progesterone will induce the acrosome reaction in the sperm of several mammalian species such as human, boar or stallion [17]. However, the progesterone-binding site seems to have a rather low affinity for progesterone compared to the cytosolic progesterone receptor of somatic cells (µM versus
pM respectively [17, 56, 57, 58, 59]. Nevertheless, progesterone stimulation of the acrosome reaction may be physiologically relevant because the follicular fluid surrounding an ovulated oocyte contains enormous quantities of progesterone (horse: ~0.5 \( \mu \text{M} \) in follicular fluid vs < 6 nM in simultaneously harvested serum [60]. Mechanistically, progesterone induces a rise in the intracellular Ca\(^{2+}\) of capacitated sperm [61]. However, it is not sure how this Ca\(^{2+}\)-influx is triggered. Recent evidence favours mediation via the \( \gamma \)-aminobutyric acid (GABA\(_\gamma\)) receptor /Cl\(-\)channel complex [62]. It also appears that progesterone primes the sperm plasma membrane to acrosome react more readily in response to ZP binding [60].

Finally, it appears that progesterone receptor exposure is regulated by accessory sex gland secretion because dog epididymal sperm, which expose the progesterone receptor are coated by prostate fluid during ejaculation and cease to bind progesterone [63]. Similarly, freshly ejaculated sperm from other mammalian species (including horses) will not bind progesterone but, during capacitation in vitro, they regain their affinity for progesterone and will acrosome react in response to progesterone exposure [17, 55, 60]. These results suggest that some factor picked up from seminal plasma is removed during capacitation, thereby leading to the uncovering of the progesterone receptors and enabling the cells to respond to the hormone (Fig 4).

Sperm zona binding and acrosome reaction

The acrosome reaction is an exocytic event initiated immediately after primary binding of a sperm to the oocyte. The sperm plasma membrane fuses to the underlying acrosomal membrane at multiple sites and the acrosome contents are released. (Fig 1 and 2). The hydrolytic enzymes released are required to dissolve the ZP-matrix around the penetrating sperm and allow that sperm to enter the perivitelline space. However, if the acrosome reaction is initiated too early (i.e. prior to ZP-binding) the enzymes will be lost and the sperm will no longer be capable of zona penetration or, therefore, fertilisation.

Induction of the acrosome reaction

Several chemical or physical agents have been reported to induce the acrosome reaction in vitro [4]. However, it should be remembered that capacitated sperm have a destabilised plasma membrane, which is sensitive to even small environmental stresses. Thus exposure to calcium ionophore in the presence of Ca\(^{2+}\) will induce fusion of the plasma membrane to the underlying acrosome membrane of capacitated, but not of uncapacitated sperm, because the former have such fragile plasma membranes. The fragility of the plasma membranes of capacitated sperm is clearly demonstrated by the fact that they will undergo spontaneous acrosome reaction as a result of simply cooling them from 38°C to 30°C. Therefore, if we are to study physiological induction of the acrosome reaction, sperm must be kept at oviductal temperature. Furthermore,
it should be noted that calcium ionophore induced membrane fusion bypasses much of the
signal-transduction pathway involved in the ZP-induced, physiological acrosome reaction.
Thus, dramatic differences exist between the ZP-induced (physiological), spontaneous
(detrimental) and calcium ionophore-induced acrosome reactions [64, 65] and if research
concentrates on how the acrosome reaction proceeds after the forced introduction of Ca$^{2+}$ into
the sperm we will probably miss numerous changes that may be critical to normal fertilisation.

**G-proteins**

Guanine nucleotide binding proteins (G-proteins) are involved in signal transduction in nearly
all mammalian cells and plasma membrane receptors frequently exert their effect on cells via
G-protein coupling. Not surprisingly then, G-proteins appear to play a role in the signal
transduction which leads to the acrosome reaction. Sperm from men, mice and bulls all possess
G-proteins in the plasma membranes of their acrosomal and equatorial segments [66, 67] and,
G-proteins have been implicated in the Ca$^{2+}$-influx that occurs during the acrosome reaction
[61].

**Ion channels**

The acrosome reaction is a membrane fusion event that requires rather high (mM-range)
cytosolic Ca$^{2+}$-levels. In capacitated sperm, the intracellular Ca$^{2+}$-concentration is very much
lower (µM-range) and, since sperm do not possess an intracellular pool of mobilisable Ca$^{2+}$,
extracellular Ca$^{2+}$ has to pass through the plasma membrane prior to the initiation of the
acrosome reaction [for review see 68]. In this respect, it appears that sperm voltage-dependent
Ca$^{2+}$-channels are activated by ZP-binding, although it is also thought that a secondary Ca$^{2+}$-
response of separate origin is necessary to induce membrane fusion [69].

**Fusion proteins**

Recently, studies of the proteins involved in the acrosomal fusion machinery have been
reported in the literature, and they have likened the acrosome reaction to synaptic vesicle
exocytosis. The reports suggest that an increase in intracellular Ca$^{2+}$ is a priming step that
enables a conformational shift in the proteins that make up the membrane fusion complex (i.e.
that span between the plasma membrane and the acrosomal outer membrane). These proteins
include the soluble $N$-ethylmalmeide-sensitive factor attachment protein receptors (SNARE)
proteins [70] and are controlled by the activation of small GTPases [71] (see also Fig 4). The
control of the fusion machinery in the secretory-pathway has been reviewed in detail by Harter
and Reinhard [72].

**Detection of the acrosome reaction**

Over the years, a variety of assays have been used to detect occurrence the acrosome reaction
[see 73]. However, it is now most common to monitor acrosome integrity in living sperm using
lectins with acrosome specific binding characteristics. For example, peanut agglutinin-fluorescein conjugates (PNA-FITC) bind specifically to the outer acrosome membrane of stallion sperm [74]. Thus, incubated sperm samples can be stained with FITC-PNA, and counterstained with a supravital stain such as propidium iodide, and the still living cells can then be monitored using an inverted confocal microscope equipped with a life cell chamber (equilibrated at 38°C and 5% CO₂) or monitored by flow cytometry [27]. This life cell staining method differs from the commonly used method in which sperm are first subjected to membrane solubilisation before FITC-PNA staining [28]. Solubilisation procedures as well as alternative staining methods such as Triple stain [75] or CTC [76] are less reliable because the staining can only be detected on fixed cells after washing off the unbound dye. Such fixation and preparation is likely to induce cell deterioration, especially in the fragile capacitated sperm.

Conclusions

This paper gives an overview of the dynamic changes that occur in the plasma membrane of an equine sperm during capacitation and the acrosome reaction. These changes in this highly organized membrane are vital to subsequent membrane adhesion and fusion processes. Unfortunately, the fragility of the sperm plasma membrane after capacitation makes it extremely difficult to study, and this may explain some of the conflicting data on the roles, and indeed existence of, surface molecules. Artefacts are easily introduced when primed cells are studied under non-physiological conditions. However, at least three of the intracellular signalling pathways that have been described to occur in capacitating or acrosome reacting sperm deserve more detailed study: (i) Bicarbonate activation of adenylate cyclase. The subsequent induction of PKA seems to cross-talk with tyrosine kinase and is involved in the collapse of the normal phospholipid asymmetry in the apical plasma membrane. These effects seem to be important for both sperm binding and the acrosome reaction. (ii) Cholesterol efflux from the sperm plasma membrane. This process appears to sensitise sperm to bicarbonate induced capacitation. However, it is not yet known how cholesterol efflux is regulated or what proteins are involved in this process. (iii) The cell surface progesterone receptor. Sperm contain a specific cell-surface progesterone receptor that enables Ca²⁺ influx by opening voltage dependent Ca²⁺-channels. This receptor may belong to an entirely new family of steroid receptors.

Sperm-ZP binding is divided into more than one phase, each apparently involving different proteins at different locations of the sperm plasma membrane. Furthermore, the various regional plasma membrane specializations, and reorganizations thereof, may be important not only for sperm adhesion and acrosome fusion but also for intracellular signalling. However, because zona binding is initiated by sperm plasma membrane proteins, whereas secondary binding is mediated by acrosomal matrix proteins, it may be sensible to isolate the sperm plasma membrane from the acrosome before attempting to study its role in primary zona binding. A similar membrane isolation approach may also help to distinguish primary sperm
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tip binding to the oolemma, which is probably initiated by an inner acrosomal membrane protein, from secondary oolemma binding which is mediated by a plasma membrane protein located in the equatorial region of the sperm head. Similarly, it may be better to study sperm-zona binding using intact ZP rather than solubilized zona proteins.

If there is still much to learn about sperm-zona binding, even less is known about how the sperm plasma membrane fuses with the outer acrosomal membrane and what proteins are involved in this fusion; this may be a useful area for future research.

In conclusion, a better understanding of how the sperm plasma membrane is organized and of the relevance of rearrangements in this organization to the signalling events that lead to sperm capacitation, zona binding and the physiological acrosome reaction will help greatly in understanding how the sperm and oocyte normally interact prior to fertilisation and how this can be optimised in the in vitro situation or how we can protect against functionally disastrous damage during semen preservation and storage.

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