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Regulation of the sperm acrosome reaction

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Department of Cellular and Molecular Medicine
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ABBREVIATIONS

6XHis tag=tag of 6 Histidine
Aa=amino acid
AII= angiotensin II
AKAP= A-kinase anchoring protein
AR= acrosome reaction
BSA= bovine serum albumin
Ca^{2+}= calcium
CAMKII= Ca^{2+}- and Calmodulin-dependent protein kinase II
cAMP= cyclic 3',5'-monophosphate
Cap=capacitated
CKII= casein kinase II
Cplx= complexin
(F)-actin= filamentous actin
FPP= fertilizing promoting peptide
GPI= glycosylphosphatidylinositol
GTP= guanine triphosphate
H^+= hydrogen
$HCO3^-$= bicarbonate
HDL= high density lipoprotein
IAM= inner acrosomal membrane
IP3= inositol 3,4,5-triphosphate
K^+= potassium
kDa= kilodalton
KO= knock-out
KRB= Krebs Ringer Bicarbonate
Na^+= sodium
NAD^+= nicotinamide adenine dinucleotide
NC=non-capacitated
NSF= N-ethylmaleimide sensitive fusion protein
OAM= outer acrosomal membrane
PC= phosphatidylcholine
PE= phosphatidylethanolamine
PKA= protein kinase A
PLD= phospholipase D
PM= plasma membrane
PS= phosphosphingomyelin
sAC= soluble adenylyl cyclase
SDS= sodium dodecyl sulphate-polyacrylamide
SEM= standard error mean
SLO= Streptolysin O
SM= sphingomyelin
SNAP= soluble N-ethylmaleimide sensitive fusion attachment protein
SNAP-25= synaptosome associated protein of 25kDa
SNARE= soluble N-ethylmaleimide sensitive fusion attachment receptor protein
Syt= synaptotagmin

TMD= transmembrane domain
TSSK= testis-specific serine/threonine kinase
VAMP= vesicle associated membrane protein
VGCC= voltage-gated Ca^{2+} channels
VCP= Valosin-containing protein
ZP= zona pellucida

ABSTRACT

The acrosome is a large secretory granule that undergoes exocytosis when receptors on the sperm surface bind ligands found on the oocyte's extracellular matrix. Acrosomal exocytosis resembles stimulated neurotransmitter release in neurons in that it is triggered by a rise in intracellular calcium. In neurons, a core complex composed of the SNARE proteins – syntaxin, SNAP-25 and VAMP is involved in synaptic vesicle fusion. Regulation of this protein complex is accomplished through the action of accessory proteins, including complexin, which is thought to stabilize the core SNARE complex prior to fusion. Recent evidence has revealed that isoforms of the SNARE proteins and their accessory proteins are present in mammalian sperm, where they might mediate the exocytosis of the acrosome. It has been hypothesized that the SNARE complex may be regulated as part of the process of capacitation, a set of physiological changes that occur within the female tract, during which the spermatozoon acquires its fertilizing ability. In this thesis, the hypothesis that complexin is involved in the regulation of the acrosome reaction is examined. To do so, streptolysin-O permeabilized sperm will be used to study the effect of recombinant complexin and of an antibody directed against complexin on the rate of the acrosome reaction, as well as on the sensitivity of this fusion machinery to calcium. Regulation may also occur through posttranslational modification or conformational change in one of the SNARE proteins. Syntaxin 2, which is the isoform present in sperm, showed a shift in apparent molecular weight on Western blots with capacitation. Alkaline carbonate extraction and dephosphorylation were used in an attempt to determine the kind of modification syntaxin 2 is undergoing during capacitation. Together, these data will shed light onto the

role of complexin in the regulation of the acrosome reaction as well as on the modifications syntaxin 2 undergoes during capacitation.

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INTRODUCTION

Fertilization is the first step toward the creation of many living organisms. In animals, it involves the fusion of the male and female gametes, the spermatozoon and the oocyte respectively, each containing one copy of the necessary genetic material, and subsequently results in the formation of an early embryo [1]. The spermatozoon is composed of three main parts: the flagellum, the midpiece and the head. The flagellum serves as a motility engine [1]. The midpiece, which has a high mitochondria content, provides the energy necessary for the diverse processes of the sperm mainly via the glycolytic pathway [1]. In addition to the nucleus, the head contains the acrosome, a Golgi-derived, lysosome-like organelle overlying the nucleus containing proteolytic enzymes, such as hydrolases and hyaluronidases, which will help the spermatozoon create a hole through the extracellular matrix of the oocyte, the zona pellucida [1, 2].

Fertilization is a multi-step process, where every stage is important for the precise timing of the process, from the activation of the sperm in the female reproductive tract to the binding of the acrosome-intact sperm onto the zona pellucida (ZP) and to the insertion of the male pronucleus (Figure 1) [3]. Once the sperm is ejaculated in the female reproductive tract, the sperm undergoes two essential processes: capacitation and the exocytosis of its acrosome, the acrosome reaction. These two steps are absolute prerequisites for the fertilization of the egg and involve biochemical, physiological and physical changes to the sperm.

Capacitation of the spermatozoon, a necessary step toward acrosome reaction

Following ejaculation and deposition into the female reproductive tract, spermatozoa are not capable of fertilization. First, they need to undergo a series of biochemical,

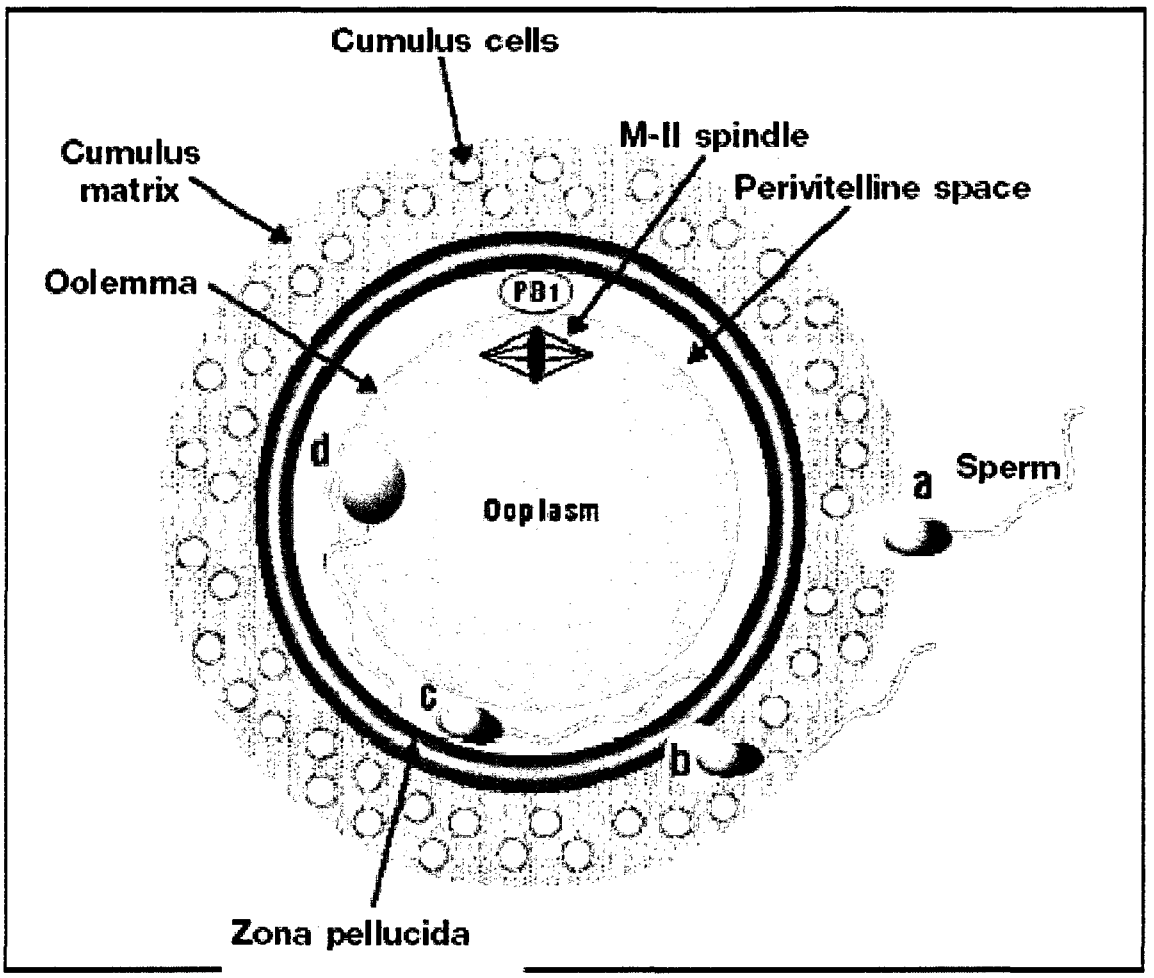


Figure 1: The different steps leading to fertilization. (a) The spermatozoon gets activated in the female reproductive tract via the capacitation process and penetrates the cumulus oophorus. (b) It contacts the zona pellucida and undergoes the release of hydrolytic enzymes contained in the acrosome. (c) Once the spermatozoon acrosome-reacts, it enters the perivitelline space and adhere to the oolemma. (d) Finally, the adhesion of the sperm head to the oolemma is followed by gamete membrane fusion and the engulfment of the sperm into the ooplasm. Adapted from EVANS J.P., FLORMAN H.M., *The state of the union: the cell biology of fertilization,* Nature cell Biology 2002, 4(1): S57-S63).

physiological and physical changes, collectively known as capacitation, during which spermatozoa acquire their fertilizing ability. *In vivo*, capacitation occurs in the isthmus of the oviduct, and once the spermatozoa reach the oviductal epithelium, they form the sperm reservoir, which provides an environment suitable for the changes associated with capacitation [4]. It has been stated that the spermatozoa can detach themselves from the sperm reservoir when they display the hyperactivated motility pattern, one of the main characteristics of capacitation [3-5]. However, capacitation can also occur *in vitro*, by incubating spermatozoa at temperature ranging from 34°C to 37-38°C in media that is supplemented with the species-specific compounds, such as the Krebs Ringer Bicarbonate media, a balanced salt medium supplemented with bicarbonate, albumin, calcium and energy substrates [4, 6]. Actually, the study of capacitation *in vitro* helped determine the factors that control capacitation and the effects of the different components of the tested media on the capacitative response of spermatozoa [1].

Even though capacitation prepares the spermatozoa for the encounter with the egg, there seems to be a differential response of individual spermatozoa to capacitate, so that a capacitating population is functionally heterogeneous [6]. As well, capacitation time varies among species (for example, mouse capacitation lasts one hour compared to rabbit capacitation that lasts 5-6 hours) [1]. It is important to point out that each species does not have the identical requirements for capacitation [4]. Since their discovery in the early 1950s [7], those changes have been studied thoroughly, but the precise molecular processes remain uncharacterized. Most investigators use the acrosome reaction as an indicator of the completion of capacitation. In order to capacitate, the spermatozoon needs a source of energy and capacitation-promoting factors to trigger the changes associated with the capacitation process at the biochemical level, intracellular ion concentrations, intracellular pathways and

the plasma membrane. Several studies have identified several compounds deemed essential for the capacitation process. Amongst those are Ca^{2+}, bicarbonate and albumin. These three compounds have to be present in the capacitative media for there to be a successful capacitation, even though the relative importance of each individual compound depends greatly on the specific needs of different species [4, 6].

Role of calcium and other ions

During the capacitation process, sperm exhibit a change in the intracellular concentrations of various ions, via a number of transporters, channels and antiporters [8]. Changes in potassium (K^+) and sodium (Na^+) concentrations occurs and the movement of these ions is thought to have an impact on sperm motility and the initiation of capacitation [1, 8].

Changes in the intracellular Ca^{2+} concentration, however, has been the most studied [9, 10]. Ca^{2+} concentration is low in both head and tail, mostly because of the presence of ATPase-mediated Ca^{2+} pump, Na^+/Ca^{2+} antiporter and Ca^{2+}/H^+ exchange system in the plasma membrane which keeps the Ca^{2+} concentration low [1, 8, 10]. As capacitation is triggered, the change in the Ca^{2+} concentration plays an important role in the capacitative process by functioning as an activator of key pathways, such as the soluble adenylyl cyclase (sAC)/cyclic adenosine 3',5'-monophosphate (cAMP)/protein kinase A (PKA) pathway (Figure 2) [1, 4, 10-12]. To allow the Ca^{2+} concentration variation, specific Ca^{2+} channels must be present to help regulate this influx. It has been postulated that this Ca^{2+} influx results from voltage-gated Ca^{2+} channels (VGCC), Ca^{2+}-ATPase inactivation and Na^+/H^+ pump [4, 8, 9, 13]. In fact, VGCCs, named Catsper 1 and Catsper 2, were found in the sperm flagella, reinforcing the implication of Ca^{2+} in the hypermotility of sperm upon capacitation [13, 14].

Aside from being implicated in the activation of the hypermotility pattern in capacitated sperm, Ca^{2+} seems to be implicated in the regulation of pH and membrane potential as well. Uncapacitated sperm maintain low acidic intracellular pH, which in turn acts as a negative regulator of the capacitative Ca^{2+} influx [4, 15], whereas during capacitation, pH has been shown to become more alkaline in murine and human spermatozoa [15]. Alkalinisation combined with membrane depolarization (a consequence of ion movement across the membrane), seems to cause an increase in the intracellular Ca^{2+} [4, 15]. Common theories shared by many investigators suggest that the primary downstream effect of increased intracellular Ca^{2+} concentration, bicarbonate and increased intracellular pH during capacitation is the activation of sAC and increase in cAMP [4, 16]. In fact, there seems to be a cross-talk between cAMP, PKA and tyrosine kinase that leads to protein tyrosine phosphorylation (Figure 2) [17].

Two of the most recognized characteristics of sperm capacitation are the changes in sperm motility and the phosphorylation pattern (tyrosine and serine/threonine). Activation of cAMP-dependent protein kinases, like PKA, and phosphorylation of sperm proteins play a vital role in the initiation and maintenance of sperm motility, as well as various capacitative changes [1]. The predominant source of cAMP in mammalian sperm is a bicarbonate- and Ca^{2+}-responsive soluble adenylyl cyclase, sAC [4, 12, 18]. Most certainly, bicarbonate and Ca^{2+} work together to modulate the cAMP levels produced during capacitation (Figure 2).

Bicarbonate and capacitation

Bicarbonate levels in the epididymis are maintained at low concentrations (3-4 mM) whereas in the uterus and oviduct, bicarbonate levels are high (more than 20 mM) [1, 4, 19]. Only spermatozoa that have reached complete epididymal maturation are sensitive to

bicarbonate and the effect of bicarbonate is different within the sperm structure (for example, bicarbonate will have a different effect on the tail than on the sperm head) [1]. Bicarbonate has been shown to participate in the process of capacitation by activating the sAC/cAMP/PKA pathway. sAC then produces an increase in intracellular cAMP concentration, which in turn stimulates PKA, a serine/threonine kinase, to phosphorylate unknown protein substrate on tyrosine or serine/threonine residues [6, 17-21]. It is not known how bicarbonate enters the cell but, in murine sperm, a Na^+/HCO_3^- cotransporter which could act in concert with a Na^+/H^+ antiporter may be involved [4, 19]. Typically, phosphorylation of tyrosine residues occurs about one hour after exposure to bicarbonate [1, 4, 16, 17].

Bicarbonate has also been shown to be implicated in membrane stability and fluidity associated with capacitation. It was suggested that bicarbonate increased the membrane fluidity by scrambling of phospholipids via activation of the sAC/cAMP/PKA pathway [19]. This scrambling seems to favor the cholesterol efflux by relocating cholesterol at the apical region of the sperm head [19].

Albumin and capacitation

Albumin and high density lipoproteins (HDL) are believed to act as cholesterol acceptors, mediating cholesterol efflux from plasma membrane after redistribution of cholesterol via the bicarbonate-induced sAC activation [1], thus promoting sperm capacitation [1, 4]. Cholesterol may be associated with the alteration of lipid raft distribution [1]. This alteration could in turn, facilitate the redistribution of cholesterol in the plasma membrane. The resulting decrease of cholesterol in the plasma membrane seems to

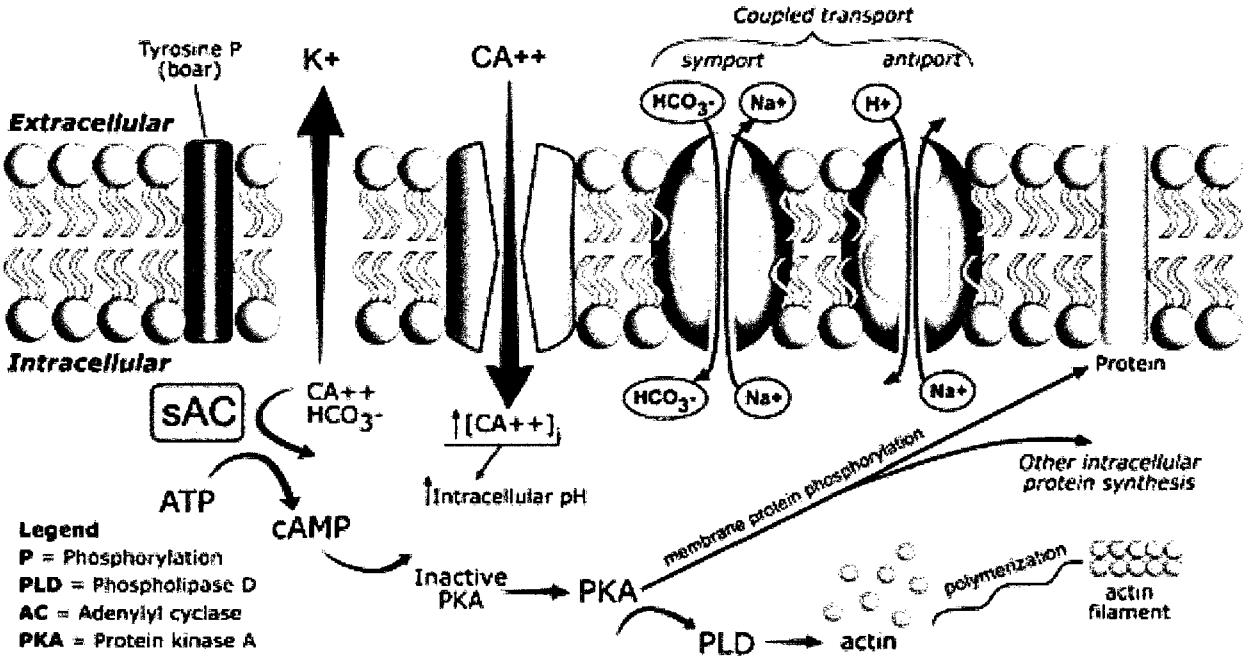


Figure 2: Signalling pathways taking place during capacitation. Hyperpolarization activates voltage-gated calcium (Ca^{2+}) channels and allow the influx of Ca^{2+}. Entry of bicarbonate ($HCO3^-$) is ensured by the coupled action of cotransporters and antiporters (in murine spermatozoa, $Na+/HCO3^-$ and Na^+/H^+). The soluble adenylyl cyclase (sAC) gets activated by Ca^{2+} and/or $HCO3^-$, and produces cAMP, which in turn activates the protein kinase A (PKA). PKA is thought to be responsible for the extensive protein phosphorylation observed during capacitation. Taken from Vadnais, M. L., Galantino-Homer, H. L. and Althouse, G. C. (2007) Current concepts of molecular events during bovine and porcine spermatozoa capacitation. Archives of andrology. **53**, 109-123.

contribute to an increase in the membrane fluidity and perhaps an increase in ion permeability and a subsequent shift in the membrane potential [4, 20, 22-24]. In murine sperm, membrane potential becomes increasingly negative as K^+ leaves the cell and the hyperpolarization resulting from this K^+ current was shown to be important in the development of ZP-activated signalling during capacitation and AR [4, 15].

Changes associated with capacitation

The activation of sAC/cAMP/PKA pathway by Ca^{2+} and bicarbonate stimulates protein phosphorylation and it appears that protein tyrosine phosphorylation has been linked to capacitation and sperm hyperactivated motility [4, 16, 17, 25]. A phosphoproteome analysis on human sperm has identified several substrates of tyrosine phosphorylation, including valosin-containing protein (VCP), a member of the ATPase associated with a number of cellular activities (AAA), NSF, a homolog of the SNARE-interacting protein, and A-kinase anchoring protein (AKAP) [4, 26]. VCP and NSF, which belong to families of proteins that mediate fusion events could be involved in the AR. In addition VCP localizes in the principal piece of non-capacitated sperm but is detected in the anterior head region of capacitated sperm [26]. AKAPs anchor PKA to its site of action, in particular in the fibrous sheath of the sperm flagellum [4, 17, 26] and have been shown to be phosphorylated on their tyrosine residues during capacitation, possibly playing a role in the hyperactivated motility pattern [4, 26]. This reinforces the probability of cross-talk between cAMP, PKA and protein tyrosine phosphorylation (Figure 2). Serine/threonine kinases and phosphatases as well as serine/threonine phosphorylation have been demonstrated to be present in sperm. In fact, PKA and PKC, are present in sperm and their cross-talk with protein tyrosine phosphorylation may contribute to the capacitation process [4, 16, 21, 25]. In support of this

theory, a family of kinases has been found to be expressed in the testis; the testis-specific serine/threonine kinase (TSSK) [4, 16, 27]. TSSK2 appears to be localized in the equatorial segment of ejaculated sperm [4]. As suggested by Jha *et al.* [28], at least in the phosphorylation of serine/threonine residues preceding a proline residue, serine/threonine phosphorylation seems to be dependent on cholesterol removal by albumin and on the membrane reorganization observed during capacitation [4, 16]. It is clearly stated in the literature that there is an increase in protein phosphorylation activity during capacitation, thus the regulation of this process would be modulated not only by protein kinases but also by protein phosphatases. Cytosolic and membrane-associated protein phosphatases have also been detected in sperm as well [16, 25, 27, 29]. For example, on the cytosolic side, PP1, a serine/threonine phosphatase seems to have a role in the motility pattern via dephosphorylation of cytoskeleton proteins [27] whereas, a membrane-associated tyrosine phosphatase has been detected in hamster sperm and is thought to contribute in the overall velocity and motility of sperm [29]. Together with protein kinases, the phosphatases might help in the regulation and modulation of the protein tyrosine phosphorylation events observed during capacitation as well as the activity of key proteins during the preparation of the AR by acting as switch of signalling pathways. Furthermore, it has been suggested that serine/threonine kinases are positive regulators of AR whereas serine/threonine phosphatases, like PP-2A or PP1, seem to be negative regulators [27].

Changes can be observed as well, on the plasma membrane structure. In the fluid mosaic model of biological membranes, proteins are non covalently associated with the lipid bilayers that form the matrix of the membrane [1]. Intrinsic or integral proteins are firmly embedded in the bilayer whereas peripheral proteins are associated with the membrane, primarily through electrostatic interactions, that can be disrupted by an increase in pH [1]. In

capacitative media, integral membrane proteins change their distribution in both head and tail, and areas of the plasma membrane that are devoid of integral membrane proteins are also free of sterols and anionic lipids [1]. It has been shown that bicarbonate alters the plasma membrane lipid architecture during capacitation, through an increased membrane lipid disorder caused by a change in the phospholipid composition of the plasma membrane during capacitation, and does so via the sAC/cAMP/PKA pathway [1, 4, 19, 24, 30]. These membrane changes include scrambling of the four major phospholipids: phosphatidylethonalamine (PE) (mainly inner leaflet), phosphatidylserine (PS) (solely inner leaflet), sphingomyelin (SM) (solely outer leaflet), and phosphatidylcholine (PC) (mainly outer leaflet) [4], which is mainly an inversion of PS to the outer leaflet. However, it appears that lipid scrambling allows cholesterol to relocate to the apical region and this relocation may be required for cholesterol removal [24]. This is confirmed by the fact that sterols are found to be abundant in the plasma membrane over the acrosome and the density of sterols in this area is greatly reduced after capacitation [1]. Lipid rafts, mainly composed of glycosphingolipids, sphingomyelin, glycosylphosphatidylinositol (GPI)-anchored proteins, caveolins, Src family kinases and cholesterol, have been shown to be present in sperm and to be altered during capacitation, along with the cholesterol redistribution and depletion by albumin [4]. Several groups have studied the presence and movement of lipid rafts during sperm capacitation [4, 30, 31]. They found that presence of bicarbonate and albumin causes GPI-anchored proteins to be redistributed in the plasma membrane, as well as a redistribution of syntaxin and VAMP [30, 31]. It has been shown that SNARE proteins are associated to some extent to lipid rafts but that this association increases after capacitation, as the cholesterol depletion seems to trigger the clustering of SNARE proteins in lipid rafts [32]. The AR is an exocytosis event, therefore proteins involved in membrane fusion must be

implicated in the process. The fact that the SNARE proteins, a set of three proteins known to govern membrane fusion, were shown to be associated and clustered in those lipid rafts after capacitation is seductive in the sense that this clustering may achieve a high local concentration of SNARE proteins, that might result in a more efficient fusion [4, 32, 33]. This would be of particular interest and convenient in the context of the AR.

Overall, capacitation comprises many steps and processes that are essential to prepare the sperm for the AR, and ultimately, the fertilization of the egg.

The acrosome reaction, a prerequisite for the fertilization of the egg

The acrosome is a membrane-bound, cap-like structure that overlies the anterior portion of the sperm nucleus and originates from the Golgi complex during early spermiogenesis [1, 5]. It is delineated by the outer acrosomal membrane (OAM) and the inner acrosomal membrane (IAM). It is thought to be analogous to a lysosome or a zymogen granule of pancreatic cells but to have the characteristics of a secretory vesicle. Its basic structure is the same for all mammals, and only differs in the size and shape of the acrosome [1]. The acrosome covers the anterior portion of the nucleus and extends to the posterior equatorial segment. It is loaded with a large array of powerful hydrolyzing enzymes, such as hyaluronidase, acrosin and arylsulfatase, synthesized before the acrosome vesicle is formed at the round spermatid stage during spermatogenesis [1, 5, 34].

The eggs of many mammals are surrounded by a coat of glycoproteins, named the zona pellucida (ZP), and a cumulus oophorus, composed of cumulus cells, cells that surround pre-ovulatory oocyte, interconnected by an extracellular matrix primarily composed of hyaluronic acid covalently attached to a protein backbone, which the sperm must pass

through before reaching the egg plasma membrane [5]. To be able to fuse with the egg, the spermatozoon must make its way through the cumulus oophorus and the ZP, by dissolving it locally. It does so using the hydrolytic enzymes contained in the acrosome. The acrosome has been defined as a secretory vesicle, which means that in order to release its content, sperm has to undergo the exocytosis of its single vesicle, the acrosome, termed the AR [5]. *In vivo*, the AR of capacitated spermatozoa is stimulated upon contact with the zona pellucida glycoprotein ZP3, which is the natural agonist that initiates the AR [5]. The egg binding protein on the sperm head to which ZP3 binds to has not yet been identified although multiple candidates were put forward [2]. The AR can be triggered by progesterone as well, via an unidentified pathway. Upon stimulation by ZP3 or progesterone, there is a rise in the intracellular Ca^{2+} concentration via Ca^{2+} channels and internal Ca^{2+} stores, which will trigger the fusion event [18, 35]. The OAM and the PM will fuse at multiple points, which results in the extensive formation of hybrid membrane vesicles, the exposure of the IAM. This exposure is thought to maintain the penetration of the sperm through the ZP, via binding to secondary binding sites present on the IAM [3], and the release of the acrosomal content (Figure 3) [1, 2, 34].

Actually, the AR shares common features with regulated secretion of somatic cells: they both result in the release of their vesicle content, they are both triggered by a rise in the intracellular Ca^{2+} and many molecules involved in secretion in somatic cells participate in the initiation of the AR. These include several signal-transducing proteins, such as G proteins, IP3 and IP3 receptors, phospholipase C, Ca^{2+} and VGCC [1-3, 14, 18, 35]. In addition, recent studies showed that proteins known to govern membrane fusion and their regulators are present in the spermatozoon, in the acrosomal region, and seem to

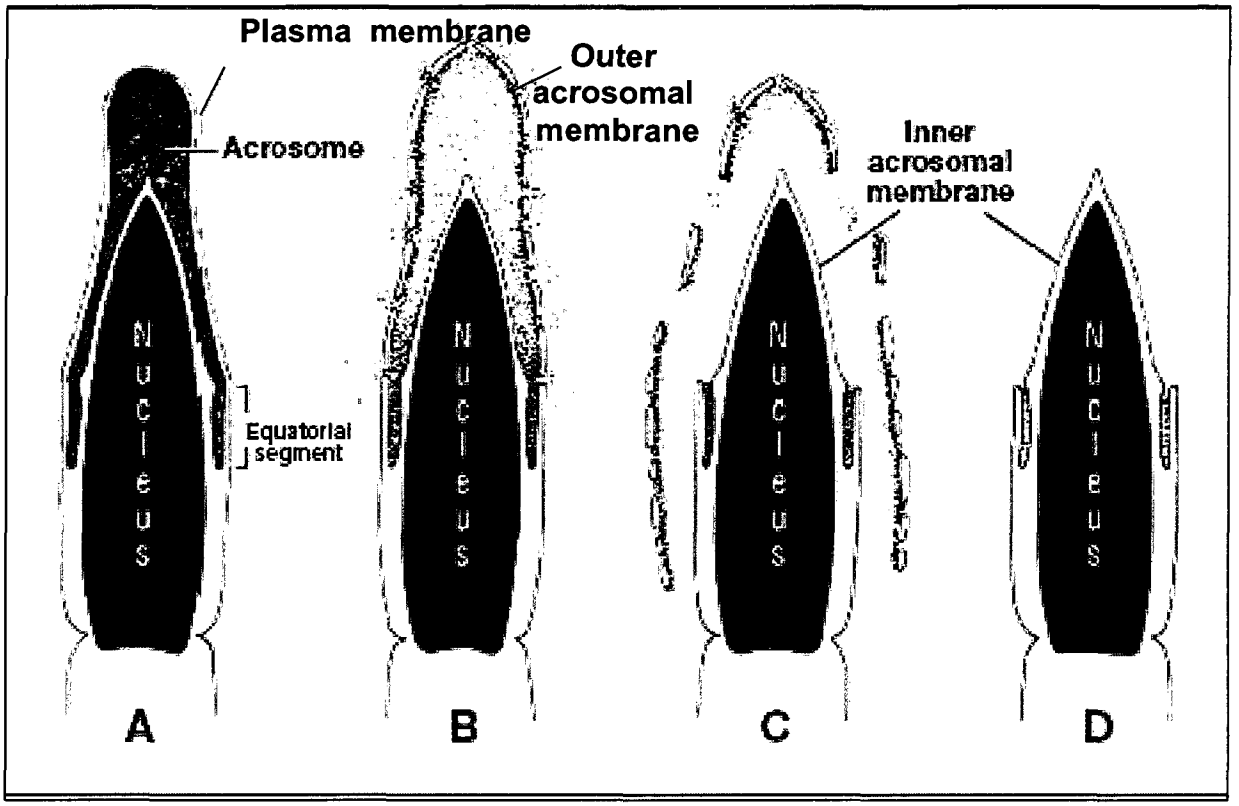


Figure 3: The acrosome reaction, a prerequisite for fertilization. (**A**) spermatozoon with intact acrosome. (**B**) Upon contact with the zona pellucida, the outer acrosomal membrane fuses at multiple points with the plasma membrane. (**C**) It results in the formation of hybrid vesicles and the loss of membrane and acrosomal content. (**D**) The inner acrosomal membrane is revealed along with its receptors, the latter thought to be necessary for the good progression of the sperm through the zona pellucida. Modified from Yanagimachi. (1994) Mammalian fertilization. In: Knobil E., Neil JD, editors. Physiology of reproduction, 2nd Edition. New York: Raven Press., A189-1317.

participate actively in the fusion of OAM and PM, during the AR. Those proteins are the GTPase Rab 3A, the soluble N-ethylmaleimide sensitive fusion attachment receptor proteins (SNARE) and their regulatory components, such as complexin I and synaptotagmin VIII [2, 36-39]. In fact, the AR can be qualified as a regulated exocytosis, specific to sperm.

The SNARE proteins, the minimal exocytosis machinery

Membrane fusion is part of most processes involved in the regulation or the maintenance of a living organism or cell (i.e. mitosis, cell growth or division) [40]. Vesicle fusion involves many coordinated steps that require the concerted action of numerous protein families. Another level of regulation is found in the need for Ca^{2+} stimulation, since it is known that regulated exocytosis is triggered by second messengers, such as Ca^{2+}, in response to activation of Ca^{2+} channels or membrane depolarization, as in the case of synaptic neurotransmitter release [41]. Since late 1980s, the SNARE proteins were identified as key elements in membrane fusion, and are considered to form a conserved core protein machinery that mediates fusion in all the trafficking steps of the secretory pathway [33, 42]. They are also linked directly to Ca^{2+}-induced exocytosis, most likely in conjunction with a Ca^{2+} sensor. The SNARE proteins are a superfamily of small proteins with 25 members in *Saccharomyces cervisae*, 36 members in humans and 54 members in *Arabidopsis Thaliana* [33], that are abundant in the brain forming about 1% of the total brain protein content [33]. The core SNARE complex is composed of syntaxin and 25kDa synaptosome associated protein (SNAP-25), both located on the plasma membrane and termed target SNARE proteins (t-SNARE); and of vesicle associated membrane protein (VAMP) (also called synaptobrevin), located on the vesicle membrane and termed vesicle SNARE (v-SNARE) [40, 41, 43, 44]. The hallmark of all SNARE proteins is a conserved stretch of 60-70 amino

acids that are arranged in heptad repeats in their membrane-proximal regions that form coiled-coil structures, named the SNARE motif or domain [33]. More recently, the SNARE proteins have been reclassified based on the identity of highly conserved residues in this SNARE motif. In that sense, t-SNARE proteins have been renamed Q-SNARE proteins, due to conserved glutamine residue and the v-SNARE proteins have been renamed R-SNARE, due to a conserved arginine residue [40, 45]. The formation of the SNARE complex is crucial for membrane fusion and is thought to bring the opposing membranes in close apposition so that the repulsive ionic force and hydration between the two lipid bilayers are overcome [40, 46]. It has been noted that Q-SNARE proteins are not uniformly distributed throughout the membrane, but are clustered in microdomains, the stability of which depends on cholesterol [33, 47]. Furthermore, it has been suggested that secretory vesicles seem to selectively dock and fuse at such clusters. But whether the cluster formation is a hallmark of the SNARE proteins or specific to the PM and other membranes rich in steroid lipids remains to be elucidated. However, this clustering would generate a highly local SNARE concentration that might result in a more efficient fusion, which could be particularly convenient in the case of one exocytotic event, such as the AR [30-32, 47]. The importance of the SNARE proteins came forth with experiments involving the clostridial neurotoxins (botulinum and tetanus). The SNARE proteins are sensitive to clostridial neurotoxins and are the targets of clostridial neurotoxins light-chain proteases, like the botulinum neurotoxins. Proteolysis of SNARE proteins by these toxins causes disruption in the ability of the SNARE proteins to form the core complex, thus disrupting neurotransmission or a subsequent exocytosis event [48, 49].

The v-SNARE or R-SNARE protein, VAMP

VAMPs are small type II integral membrane proteins of approximately 18 kDa [41, 50, 51] and are anchored in the vesicle membrane by a transmembrane domain (Figure 4) [52]. The cytosolic domain is composed of a single extended or two short alpha helical domains, better known as the SNARE interacting domain, the SNARE motif [45, 51, 53]. The N-terminal domain of neuronal VAMP has been found to contain a proline- rich region of approximately 24 amino acids, whose function is not clear [41, 54]. In mammals, the v-SNARE family is composed of 8 isoforms of VAMP. Although the primary sequence diverge between VAMP1 and VAMP2, the proline-rich feature remains [54, 55]. A VAMP coil without the proline-rich domain inhibits exocytosis in cracked PC12 cells, but an antibody directed against the proline-rich region of VAMP2 does not affect exocytosis [54]. This suggests that the proline-rich region of VAMP is not involved in the membrane fusion process, but could be important for the interactions with VAMP regulators. The importance of VAMP in vesicle fusion has been shown after experiments using botulinum and tetanus toxins to specifically cleave VAMP, impaired vesicle fusion [48-50, 56] and that VAMP KO mice showed an impaired fusion of secretory vesicles [33, 50].

The t- or Q-SNARE proteins, SNAP-25 and syntaxin

In mammals, the SNAP-25 family comprises three isoforms, SNAP-23, SNAP-25 and SNAP-29 [33, 57-59] whereas in yeast, one ortholog, Sec9 exists [60, 61]. Unlike VAMP or syntaxin, SNAP-25 is not anchored in the membrane but rather peripherally attached to it by palmitoylation of four cysteine residues [33, 62]. In fact, SNAP-25 consists of two SNARE domains (also termed sn1 and sn2) attached together by a palmitoylated linker region (Figure 5) [40]. This loop region is dispensable for SNAP-25

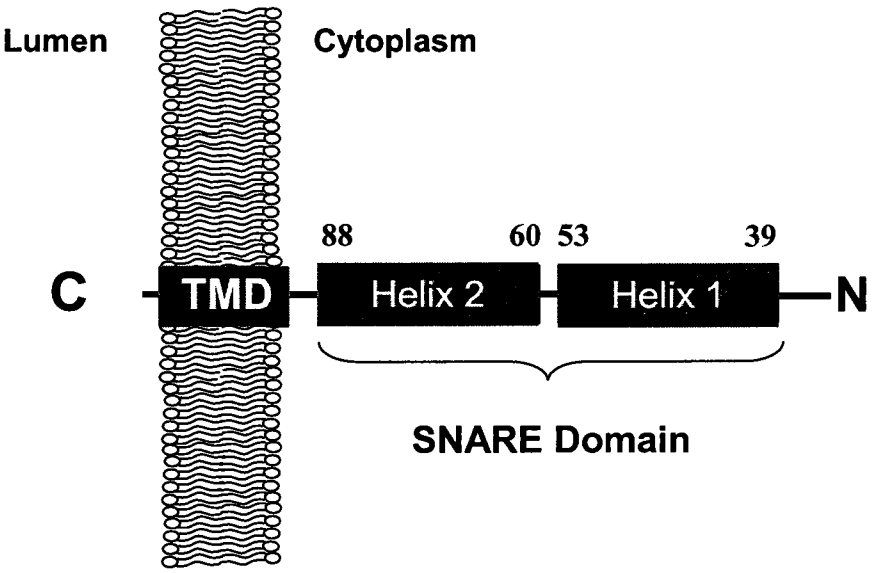


Figure 4: Schematic representation of the v- or R-SNARE protein, synaptobrevin or VAMP. VAMP is anchored in the vesicle membrane via a transmembrane domain (TMD, red). The cytoplasmic segment of VAMP is composed of the SNARE domain, a 60 amino acid characteristic of the SNARE proteins (residues 39 to 88), which can be composed of two short or a single extended helical segments in VAMP (green). The N-terminal region of the neuronal VAMP is rich in proline.

fusogenic activity, but it is crucial for the availability of SNAP-25 to form the SNARE complex, as it generates a high local concentration of the two required SNAP-25 coils by linking them to the target membrane. Like VAMP, SNAP-25's role in vesicle fusion is disrupted upon application of botulinum toxins [48, 49, 56, 58].

The mammalian syntaxin family is composed of eighteen isoforms whereas 7 exist in yeast [40, 57, 58, 63]. Syntaxins are type II integral membrane proteins which are anchored to the membrane by a transmembrane domain, with the exception of syntaxin 11, which lacks a transmembrane domain [64]. The membrane proximal region is the coiled coil region involved in the formation of the SNARE complex, the SNARE motif [40, 45]. Several syntaxins, including syntaxin 2, also possess a regulatory amino-terminal domain composed of an anti-parallel bundle of three conserved coiled-coil domains, termed Ha, Hb and Hc (from amino to carboxyl end) [65, 66]. These domains are independently folded and are connected to the SNARE motif, also termed H3 in syntaxin, by a flexible linker [66]. It can fold back onto the SNARE motif, which gives the "closed" conformation of syntaxin (Figure 6). This conformation is required for the interaction of nSec1/Munc-18, a chaperone protein that binds to the closed conformation of syntaxin 2 and keeps it in the "closed" conformation [67-69]. However, upon a structural change in Munc-18, the chaperone protein is released and syntaxin can unfold to its "open"conformation, revealing its SNARE motif to its binding partner, SNAP-25 and VAMP [65, 68]. This mechanism, as well as Munc-18 syntaxin-independent interactions, may be involved in the regulation and in the proper timing of the SNARE core complex assembly [65, 68, 70]. Syntaxin has been shown to bind to synaptotagmin in a Ca^{2+}-dependent manner and to voltage-gated potassium and Ca^{2+} channels, via its C-terminal SNARE domain [41, 71-76]. The direct interaction of syntaxin with Ca^{2+} channel provides a suitable molecular mechanism between the fusion machinery

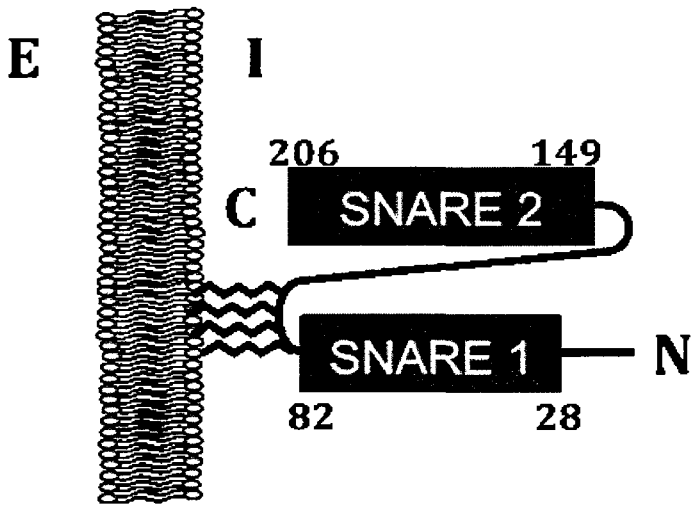


Figure 5: Schematic representation of the t- or Q-SNARE protein, SNAP-25. SNAP-25 is associated to the plasma membrane by palmitoylation of four cysteine residues found in the middle linker piece. It has two SNARE domains of approximately 60 amino acid, termed sn1 (residues 28 to 82) and sn2 (residues 149 to 206), going from the N- to the C-terminal. E= extracellular and I= intracellular milieu

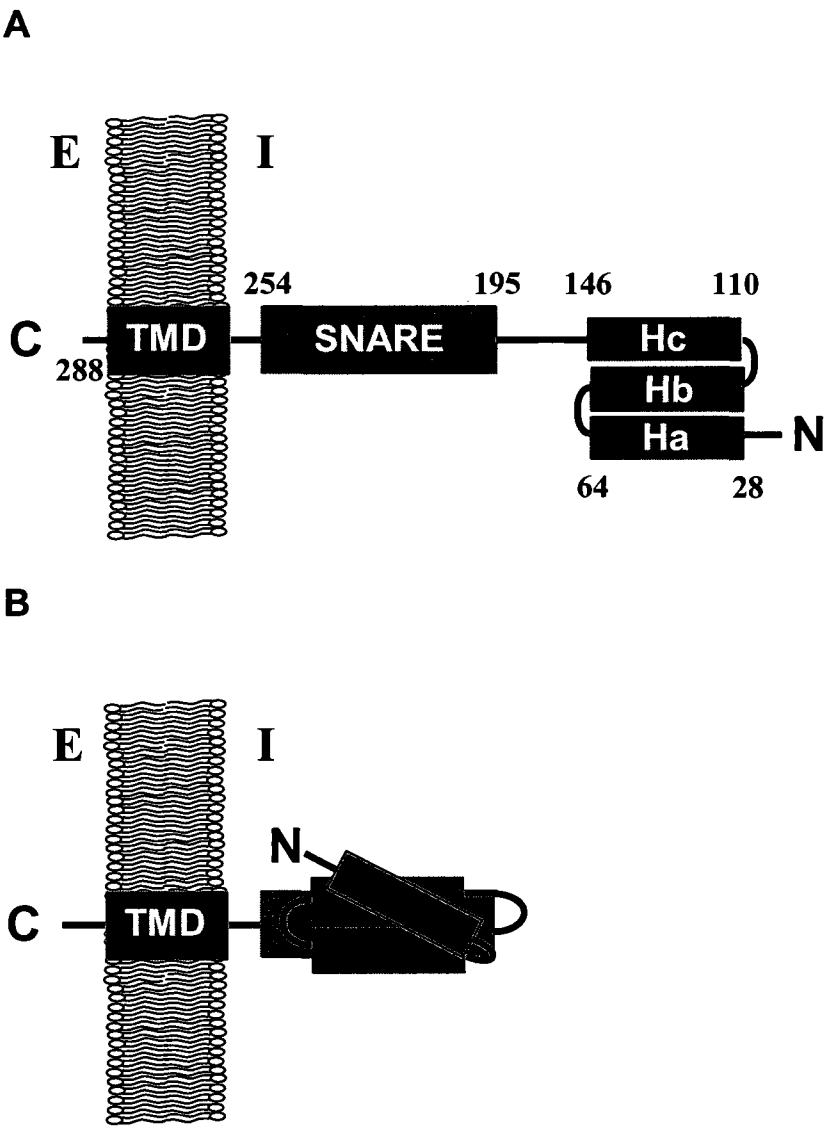


Figure 6: Schematic representation of the t- or Q-SNARE protein, syntaxin.
(**A**) Syntaxin is anchored in the plasma membrane via a transmembrane domain (TMD, red). The cytoplasmic part contains the membrane proximal SNARE domain (residues 195 to 254), a 60 amino acid motif via which syntaxin interacts with VAMP and SNAP-25 to form the four-helix bundle SNARE complex (green). The N-terminal of some syntaxins, like syntaxin 2, forms a bundle of 3 helix termed Ha (residues 28 to 64), Hb (residues 68 to 105) and Hc (residues 110 to 146) (purple). (**B**) The N-terminal 3-helix bundle can fold back and bind to the SNARE motif via a groove formed by Hb and Hc, preventing its SNAREs binding partner to interact with syntaxin, via the SNARE motif. This is the closed conformation of syntaxin and this allows the chaperone proteins Munc-18 to bind to syntaxin and to modulate the availability of the syntaxin SNARE motif for SNARE complex formation.

and the gates of Ca^{2+} entry during depolarization of the target membrane, as the physical proximity would ease the interaction of Ca^{2+} and Ca^{2+} channels. Similarly to VAMP and SNAP-25, the crucial role of syntaxin in vesicle fusion has been revealed through its sensitivity to cleavage by botulinum toxins [48, 49, 77].

The SNARE core complex

The SNARE core complex is formed by the three SNARE proteins VAMP, SNAP-25 and syntaxin. Isolated monomeric SNARE proteins have SNARE motifs that are largely unstructured, but upon complex formation, they undergo major structural changes and spontaneously associate with one another to form helical core complexes with high stability [41, 43, 53]. This results in the core complex, a parallel four-helix bundle, composed of the coiled-coil domains of each SNARE proteins; syntaxin and VAMP each contributing one domain and SNAP-25 contributing two domains (Figure 7), which are resistant to SDS denaturation, protease digestion and clostridial neurotoxin cleavage and are stable at heat up to 90°C [53]. The core of the four-helix bundle is composed of sixteen primarily hydrophobic layers formed by interacting side-chains from each of the four helices. At the center of the core complex, a conserved ionic layer, the "zero" layer, is present consisting of one arginine and 3 glutamine residues contributed from the SNARE domain of each of the four helices originating from the PM and the vesicle membrane [53, 78-80]. This ionic layer is sealed off against solvent by adjacent hydrophobic layers, but it contains a buried water molecule. It is further stabilized by hydrogen bonds or salt bridges between the SNARE proteins (Figure 8) [78, 80]. The SNARE complex is assembled in a multi-step manner: the vesicle docks to the target membrane via the interaction of the small GTPase Rab3A with Rab3 Interacting Molecule (RIM) [81]; this results in the hydrolysis of the GTP-bound Rab3A, which disrupts

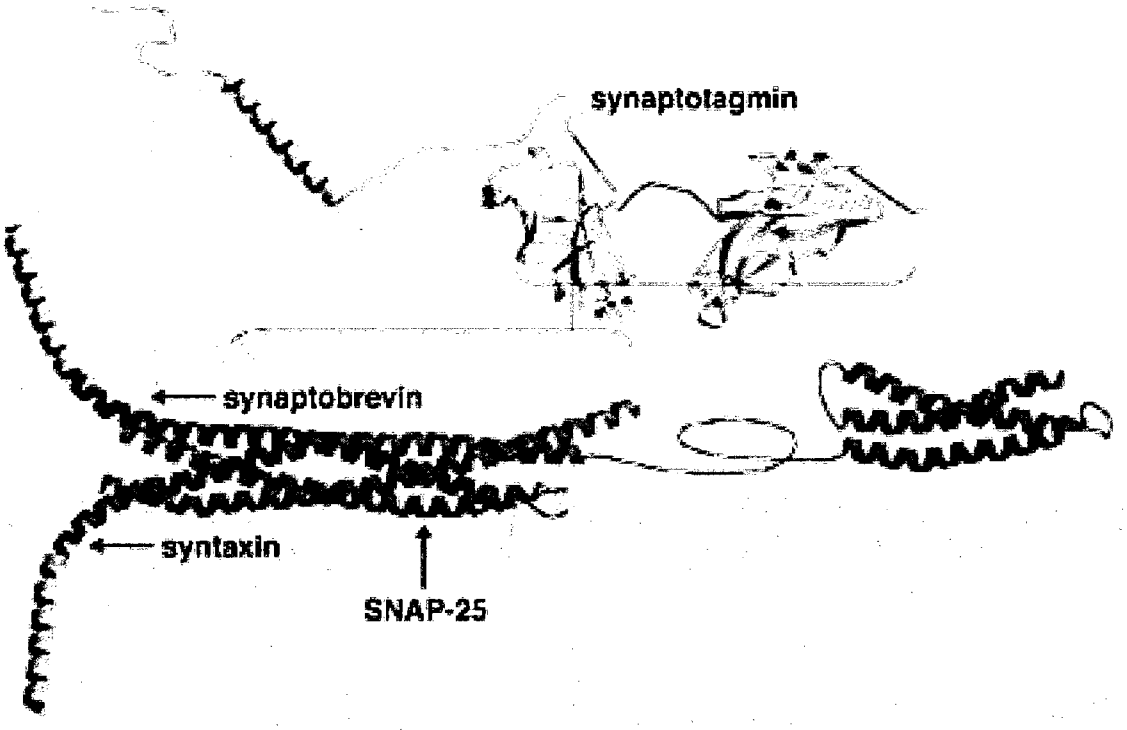


Figure 7: The four-helix SNARE complex. Syntaxin (red), SNAP-25 (green) and VAMP (blue) interact through their SNARE domains and associate in a parallel orientation. This results in the formation of highly stable SDS-resistant complex, which is thought to bring the two membranes in close apposition and to drive the fusion of the vesicle to its target membrane. The TMD of syntaxin and VAMP are shown in yellow. Synaptotagmin is the Ca^{2+} sensor of this fusion machinery. Adapted from Sutton, R. B., Fasshauer, D., Jahn, R. and Brunger, A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature. **395**, 347-353.

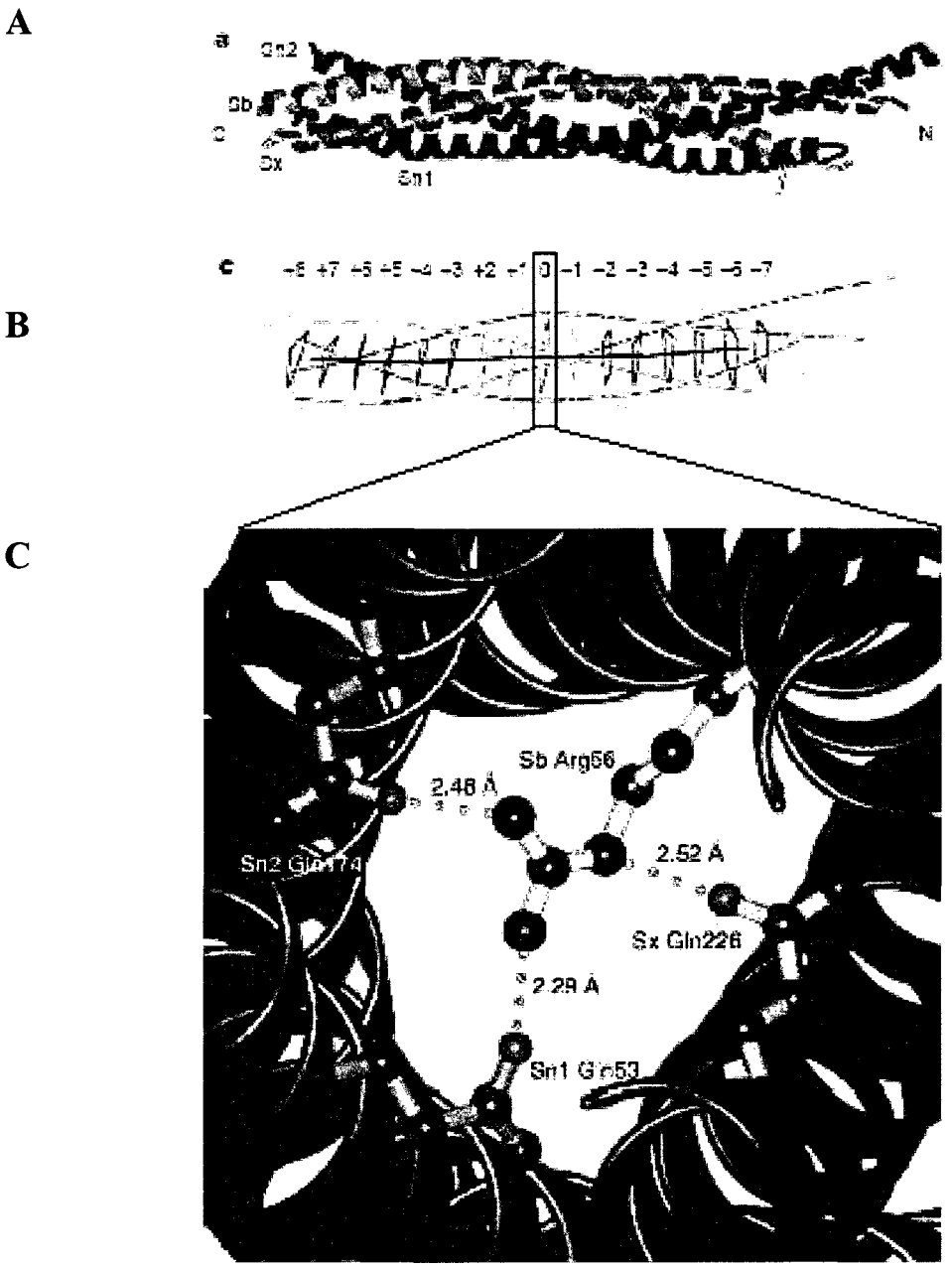


Figure 8: The ionic layer of the four-helix SNARE complex. (**A**) the SNARE complex formed by syntaxin (red), SNAP-25 (green) and VAMP(blue) is shown as a ribbon diagram. (**B**) Ionic layers found in the core complex superhelical structure. The ionic "zero"layer is shown as a red diamond. (**C**) Magnification of the ionic "zero" layer. The Arginine 56 of VAMP (Arg56), the Glutamine 226 of syntaxin (Glu226) and the Glutamine 53 (Glu53) and 174 (Glu174) of the two SNARE domains of SNAP-25 are shown in ball and stick diagram whereas the remainder of the helices are represented by ribbons. Taken from Sutton, R. B., Fasshauer, D., Jahn, R. and Brunger, A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature. **395**, 347-353.

the interaction of Munc-18 with syntaxin by recruiting Munc-13. Munc-13 helps syntaxin 2 to adopt its "open conformation", revealing its SNARE domain to SNAP-25 and forming a Q-SNARE hetero-dimer complex [67, 68]. This transient binary complex can now interact with the R-SNARE protein VAMP to form a *trans* SNARE complex (Q-SNARES and R-SNAREs residing on opposite membranes). SNARE proteins assemble in a "zipper" manner from the N- to the C-terminus to form the stable coiled-coil complex. The energy released during the assembly of the SNARE proteins is thought to provide the energy necessary to overcome the repulsive forces preventing the membrane fusion and to bring the two membranes in close apposition to allow lipid mixing and fusion [82]. Due to its stability, the core SNARE complex requires the help of a chaperone protein to ensure its disassembly. The ATPase NSF, which is a cytosolic hexamer, binds to the *cis* SNARE complex (Q-SNARE and R-SNARE residing on the same membrane) and along with the chaperone protein SNAP, which is not related to the t-SNARE protein SNAP-25, it unravels the coiled-coil structure [41, 83]. The SNAP protein binds to the SNARE complex and recruits NSF. Then, it activates its ATPase activity which separates the SNARE proteins, recycling them and making them available for future rounds of exocytosis (Figure 9).

The SNARE proteins and sperm

The AR has been hypothesized to be similar to a Ca^{2+}-mediated exocytosis, in that they are both triggered by a rise of intracellular Ca^{2+} and by the activation of the SNARE proteins [84, 85]. There has been several indications that the SNARE proteins are involved in the fusion of the OAM with the PM in mammalian sperm [39, 85, 86]. The three SNARE proteins, VAMP, syntaxin and SNAP-25, have been found in the acrosomal region and treatment of permeabilized sperm with clostridial toxins or antibodies directed against one of

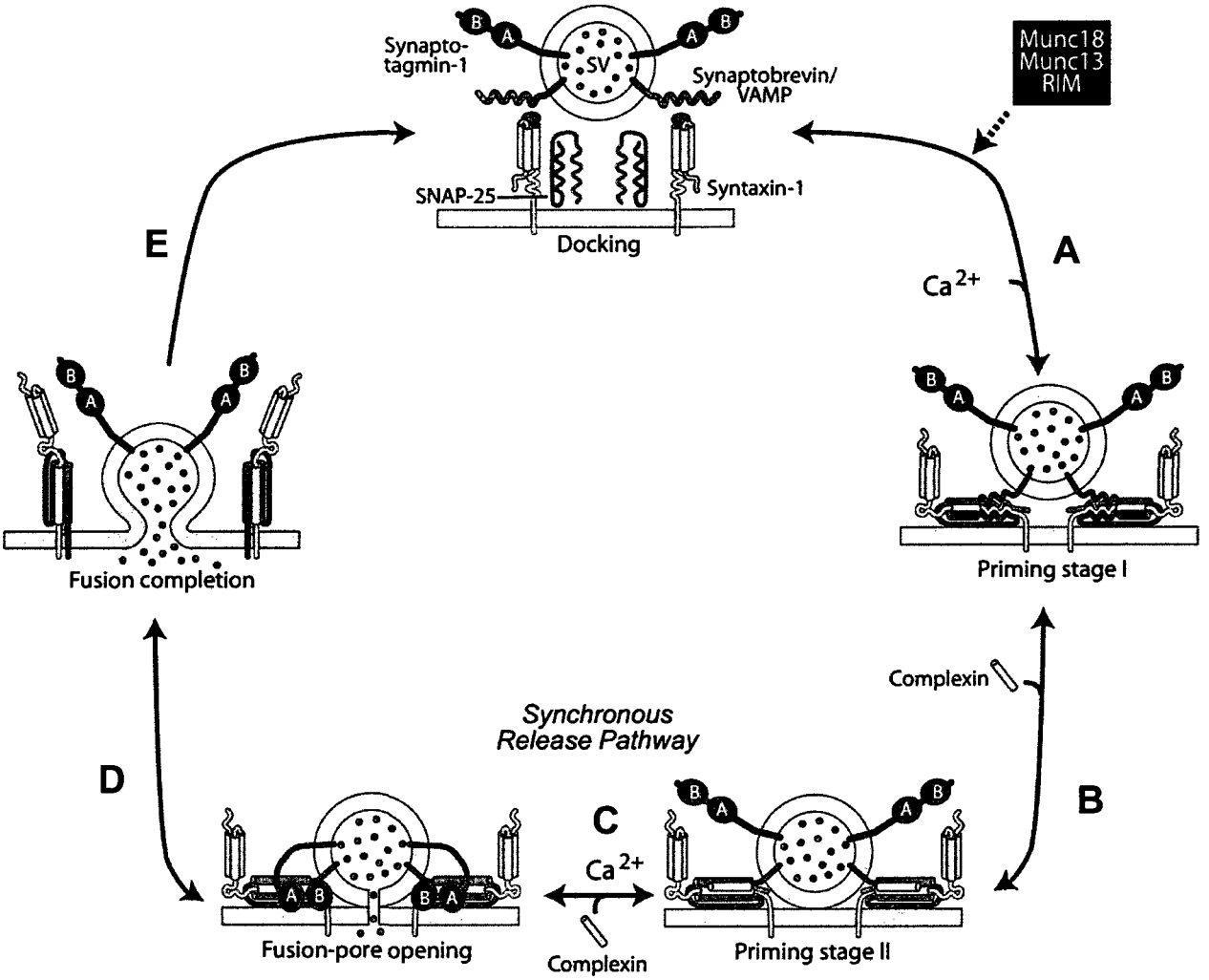


Figure 9: SNARE-mediated fusion. (**A**) First, the vesicle docks to its target membrane via the interaction of GTP-Rab3A with Rab3 Interacting Molecule (RIM). The hydrolysis of GTP-Rab3A will recruit Munc-13, which disrupts the interaction of Munc-18 with the "closed" syntaxin (yellow). This will "open" syntaxin and reveal its binding domain to its cognate t-SNARE partner SNAP-25, which results in the formation of a Q-SNARE complex. VAMP will bind to this Q-SNARE dimer to form a loose *trans* complex. (**B**) Complexin is going to come into play to stabilize the complex and keep it in a fusion competent state. (**C**) When the fusogenic signal Ca^{2+} is triggered, it will be sensed by synaptotagmin, thought to be the Ca^{2+} sensor of the SNARE fusion machinery. (**D**) Synaptotagmin will bind to the complex and to phospholipids of the target membrane, relieving the complex from the complexin clamp and driving membrane fusion. (**E**) Once fusion is accomplished, the *cis* SNARE complex will be disassembled by an ATPase NSF and its chaperone protein alpha-SNAP, recycling the monomeric SNARE proteins or preparing another run of fusion. Adapted from Tang, J., Maximov, A., Shin, O. H., Dai, H., Rizo, J. and Sudhof, T. C. (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell. **126**, 1175-1187

the SNARE proteins inhibited AR, proving that the SNARE proteins are implicated in the acrosomal exocytosis [39, 86-90]. The SNARE isoforms thought to be implicated in AR are VAMP 2 [91], SNAP-25 and syntaxin 2 [38, 92], even though mRNA for other syntaxins were detected in the testis [38]. This has been solved by the fact that the syntaxin 2 protein was detected in sperm, but not syntaxin 4 [38]. SNARE-interacting proteins, such as Rab3A [87, 90], synaptotagmin (isoform 6 and 8), a Ca^{2+} sensor [93], alpha-SNAP, NSF [88, 89] and complexin [39], have also been detected in sperm, suggesting a need for assembly and disassembly of the SNARE complex.

The difference between the neuronal exocytosis and the AR is that the AR is a single exocytotic event and there is no need for recycling the SNARE proteins. The assembly of the SNARE complex has to be tightly regulated, in order to prevent the immature acrosome reaction of the spermatozoon. How the SNARE proteins assemble to tether the acrosome to the PM is still unclear. Some suggest that the SNARE proteins are assembled in a *cis* SNARE complex, which becomes disassembled by NSF and alpha SNAP, in order for the SNARE proteins to reassemble in a *trans* SNARE complex in response to Ca^{2+} stimulation [88, 90]. But how the SNARE-mediated fusion is regulated is unknown. In the case of the sperm AR, one could think of two possible regulation patterns of the AR: either a regulatory protein comes into play to stabilize the SNARE complex and to keep it as a metastable fusion complex until the fusion signal is triggered, or key proteins could be the targets of posttranslational modifications, in order for the regulation of the SNARE complex to occur at the level of one of the SNARE proteins for example.

Regulation via a regulatory protein, Complexin

Complexins, also known as synaphin, are found throughout the body and characterized as small, highly charged cytosolic proteins of 134 amino acid in length and weigh approximately 20 kDa [94-96]. Free complexin lacks a tertiary structure but contains a conserved α-helical domain at the center of its sequence, around residues 48-70 [97], and it was shown that this α-helical domain mediates the binding of complexin to the SNARE complex [94, 95, 98-100]. Complexins bind tightly to the assembled *trans* SNARE complex in a 1:1 fashion, but not to the monomeric v-and t-SNARE subunits [94, 95, 97, 98], even though complexin I has been reported to bind to partially assembled SNARE complex, but the affinity depends on how complete the assembly of the SNARE complex is [101]. The helical segment of complexin binds to the groove formed by VAMP and syntaxin of the SNARE complex in an anti-parallel orientation. More specifically, complexin interacts at its C-terminus with syntaxin and VAMP chains, around the ionic zero layer, more specifically around the layers -3 and +1, of the SNARE complex (Figure 10) [94, 97, 98]. Recent studies have proposed that once complexin binds to the C-terminal part of SNARE complex, it activates it into a superprimed metastable complex and only complexin-activated SNARE complex can be "clamped" by complexin, and this would be a substrate for synaptotagmin [95, 99, 102-105]. There seems to be an interplay by which complexin and synaptotagmin work together to accomplish regulated exocytosis. Synaptotagmin is a family of proteins with two Ca^{2+}-binding C2 domains, thought to be the Ca^{2+} sensors of synchronous synaptic transmission and exocytosis in general [102, 103, 106-108]. KO mutations for synaptotagmin abolish or greatly reduce vesicle exocytosis [103]. Phenotypes of complexin and synaptotagmin deletions are virtually identical, suggesting they cooperate in some fashion in Ca^{2+}-triggered exocytosis [103-105]. Therefore, complexin has been suggested to be a

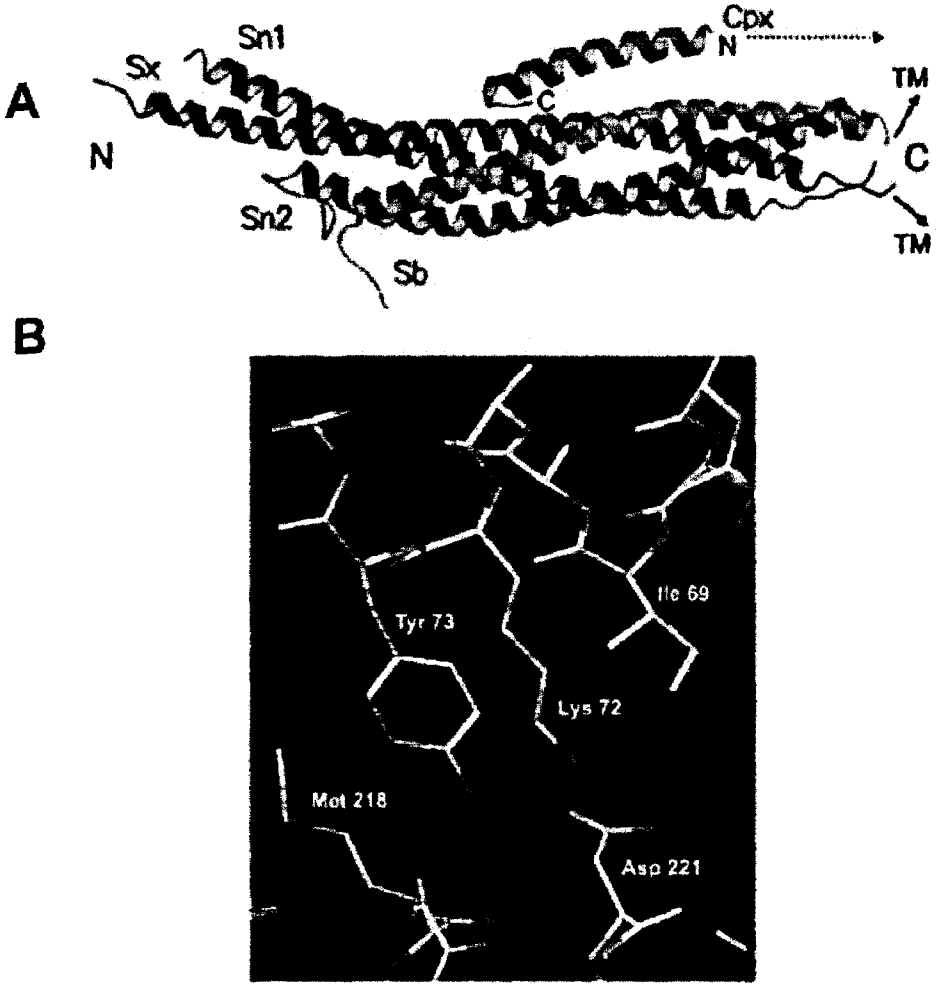


Figure 10: Complexin interaction with the SNARE complex. (**A**) Ribbon diagram of complexin (yellow) bound to the SNARE complex, composed of syntaxin (red), SNAP-25 two helices sn1 and sn2 (green) and VAMP (blue). Complexin binds to the groove formed by syntaxin and VAMP, in an anti-parallel orientation and with its C-terminal forming the closest contact point with the SNARE complex. The N- and C-terminals are indicated for the SNARE proteins and for complexin. (**B**) Electron density diagram of complexin bound to the SNARE complex, with the complexin residues Isoleucine 69 (Ile69), Lysine (Lys72) and Tyrosine 73 (Tyr73) interacting with syntaxin residues Methionine 218 (Met218) and Asparagine 221 (Asp221). Modified from Bracher, A., Kadlec, J., Betz, H. and Weissenhorn, W. (2002) X-ray structure of a neuronal complexin-SNARE complex from squid. The Journal of biological chemistry. **277**, 26517-26523

positive regulator of the SNARE complex, as shown by KO experiments, where neurons lacking complexin showed a severely reduced neurotransmitter release [103, 109], but also a negative regulator of the SNARE complex, as suggested by the "clamping" hypothesis [95] where an excess of complexin inhibits synchronous release. Once Ca^{2+} ions bind to synaptotagmin, synaptotagmin simultaneously binds to the SNARE complex and to phospholipids, thereby dislodging complexin and pulling the fusion pore open. These pieces of evidence reconcile the contradicting results of KO experiments and the "clamping" hypothesis by showing that the inhibition of synchronous release with an excess of complexin is likely due to complexin outcompeting synaptotagmin and that only complexin-activated SNARE complex can be stabilized [95, 103-105]. Therefore, complexin seems to have two roles in regulating exocytosis: the activation of the SNARE complex by complexin and the "clamping" of previously complexin-activated SNARE complex (Figure 9).

There are four complexin isoforms, which all have their own pattern of expression: complexin I is expressed in sperm [39, 104] and neurons [98]; complexin II is expressed more widely throughout the body [100, 102, 105]; and complexin III and IV are the only complexin isoforms found in the retinal ribbon synapses [105]. Complexin III and IV are membrane anchored by a C-terminal farnesylation. They can functionally replace complexin I and II and their C-terminal farnesylation is thought to regulate their synaptic targeting and their modulatory function in transmitter release [110]. Complexin I and II are soluble proteins, highly homologous and conserved among mouse, rat and human with close to 83% identity [104, 111]. In the brain, complexin I and II appear to have similar function, but are expressed in distinct subsets of neurons: complexin I is predominantly a marker of axosomatic, thus inhibitory synapses , and complexin II is mainly a marker of axodendritic synapses, hence excitatory synapses [111, 112]; this was hypothesized after studies showed a

transient increase in complexin I and II following traumatic injury to the brain and these alterations in complexin levels could play an important role in neuronal cell loss following traumatic brain injury and thus contributing to the pathophysiology of cerebral damage following brain injury [112]. In fact, complexin I and II have been found to be involved in the pathogenesis of several brain psychiatric disorders, such as Huntington's disease (HD) [113, 114], Parkinson's disease [114], Alzheimer's disease [115], Wernicke's encephalopathy [114], schizophrenia [114], major depression and bipolar and unipolar disorders [114], as well as in behavioural problems that could be associated with different stages of these disorders (this seems to be more restricted to mice exhibiting low levels of complexin I) [113, 114, 116-118]. In fact, decreased level of complexin I was found in brains of patients with schizophrenia and major depression and decreased level of complexin II was found in the anterior cingulate cortex and hippocampus of patients with major depression and more consistently in bipolar disorder, indicating a particular vulnerability of excitatory connections [114]. In fact, treatment with the anti-depressant fluoxentine, increased the levels of complexin I in the brain of rat model for depression [114]. It seems that complexin I and II are not only implicated in neuronal disorders but also in diabetes, pancreatic cells and mast cells secretion as well [108, 117, 119, 120]. KO mice lacking complexin I or complexin II exhibited different phenotypes in terms of behavioural habits. Complexin I KO mice displayed a strong ataxia and behavioural abnormalities in terms of motor and "emotional" deficits while complexin II KO mice displayed abnormalities in exploration and social behaviours and light progressive deficit in motor learning [81]. Interestingly, KO mice lacking complexin II have normal reproductive ability, whereas mice lacking complexin I are unable to reproduce due to a defect in the AR [104].

Regulation via modification of one of the SNARE proteins

Another possible regulation mechanism of the SNARE complex involved in the AR could be at the level of one of the proteins involved in the fusion machinery. Given that sperm is a cell lacking transcriptional and translational machinery, the only way for it to modify its proteins is by posttranslational modifications. Syntaxin is one of the t-SNARE and is known to be the target of some posttranslational modifications, such as proteolytic cleavage and phosphorylation.

Epimorphin, also known as syntaxin 2, has been shown to act as an epithelial morphogen when secreted by stromal cells in mammary glands, lungs, liver, pancreas, colon and other tissues [121]. But at the same time, the same protein mediates fusion within the cell. Intracellular epimorphin is complexed with synaptotagmin and annexin II, both of which can bind to phosphatidylserine at the inner leaflet of the PM [122]. In response to cell stress or Ca^{2+} influx, this complex translocates across the PM and epimorphin is then cleaved at the SNARE domain [122]. In the context of AR, translocation of syntaxin 2 is highly improbable but cleavage could render its commitment to the SNARE complex more permanent.

On the other hand, syntaxin 2 has been shown to be phosphorylated, mainly at the N-terminus, by serine/threonine kinases such as casein kinase II (CKII) and Ca2+- and calmodulin-dependent protein kinase II (CAMKII) [123, 124]. Although studies have indicated that the phosphorylation of syntaxins does not seem to have an effect on the SNARE complex assembly, it seems to modulate SNARE complex function through other proteins such as synaptotagmin or Ca^{2+} channels [71, 73, 74, 123, 125]. In this case, phosphorylation/dephosphorylation could help modulate the function of the SNARE

complex by adding another level of regulation to the SNARE complex assembly and stabilization.

The characteristics of AR as being a single and unique exocytotic event emphasize on the need for a tight regulation of the SNARE complex assembly and function. In order for the sperm to undergo AR, the SNARE proteins need to assemble into the core SNARE complex at the right time, to be stabilized until the fusogenic signal is triggered and to complete the fusion event at the doorstep of the oocyte, the ZP. To do so, it needs the help of regulatory proteins, that are going to modulate the process and ensure proper SNARE function, and the regulation of the fusogenic abilities of the SNARE proteins.

HYPOTHESES AND OBJECTIVES

Given the regulatory role of complexin on SNARE complex, my first hypothesis is that complexin stabilizes the SNARE complex and contributes to the acquisition of the Ca^{2+} sensitivity of sperm during capacitation, and in the regulation of the acrosome reaction.

In order to prove this, the effect of complexin on the sperm Ca^{2+} sensitivity and the acrosome reaction will be examined, before and after capacitation.

Syntaxin 2, as a member of the SNARE complex, is crucial for acrosome reaction. The second hypothesis consists in the fact that, to ensure another degree of regulation, syntaxin 2 is modified during capacitation and this modification is capacitation-dependent.

In order to prove this second hypothesis, the mechanism by which syntaxin 2 is modified between non-capacitated and capacitated state will be examined and determined.

MATERIALS AND METHODS

Reagents.

Recombinant Streptolysin O (SLO) was purchased from Dr S. Bhakdi from the institute of Medical Microbiology (Mainz University, Germany)[126]. Polyclonal affinity purified rabbit anti-complexin I (purchased from ProteintechTech Group Inc), polyclonal rabbit anti-syntaxin 2 was purchased from Sigma mouse monoclonal affinity purified anti-phosphotyrosine (pY100) was generously given by Dr Sorisky (purchased from Sigma). Rabbit IgG was purchased from Sigma. Secondary Alexa 488 goat anti-rabbit and anti-mouse were purchased from Sigma.

Subcloning and Protein Purification:

Cloning complexin I into pQE9

The full length complexin I construct of 134 amino acids (aa) was generated by PCR amplification using PCMVSPORT6/cplxI as a template. For bacterial expression, after PCR amplification, the DNA sequence was subcloned into the pQE9 vector using the following oligonucleotides: cplxI (forw) 5'-GCG AGA TCT ATG GAG TTC GTG ATG AAA CAA G-3' and cplxI (rev) 5'-GCA AGC TTT TAC TTC TTG AAC ATG TCC TGC-3'. The full length fragment was isolated using the BamHI/Hind III restriction sites, and cloned into pQE9 vector (Qiagen), containing a tag of 6 histidines (6XHis tag). The vector was previously cut with BglII/HindIII, BamHI having compatible sticky ends with BglII. This vector is useful in that following the multiple cloning site, there is a tag of 6 histidine, the 6XHis tag, at the C-terminal region of this promoter, therefore allowing the resulting protein to be bound to Ni-NTA beads.

Purification of pQE9 Complexin I protein

After subcloning complexin I into the pQE9 vector, the protein was successfully purified from the XL1 Blue *Escherichia coli* (*E.Coli*) strain through binding the His tag to Ni-NTA beads (purchased from Sigma).

A 1.5% YT + ampicillin (100 μg/ml) plate was streaked with the bacterial stock containing pQE9/CplxI and was incubated overnight at 37°C. The next day, single colonies were selected from the plate and inoculated into 200 ml of sterile 2YT (yeast tryptone) + ampicillin (100 μg/ml), which were allowed to grow overnight at 37°C in a shaking incubator. The next day, to 900 ml sterile 2YT, ampicillin was added at a final concentration of 100 μg/ml. 100 ml of the overnight culture was added to each flask, and grown at 37°C until the absorbance at 600 nm gave an optical density (OD) reading between 0.6 and 0.8 upon which, cells were induced.

After inducing the bacterial solution using 1 mM isopropyl β-D-thiogalactoside (IPTG) for four hours at 37°C, the bacterial solution was pelleted at 5,000 × g for 10 minutes at 4°C. The cells were then lysed using a lyzing buffer with lyzozyme at 2 mg/ml (in 25 mM HEPES-KOH pH 7.2, 7.22 mM phenylmethanesulphonylfluoride (PMSF) and 2 mM β-mercaptoethanol), pelleted at 10,000 × g for 10 minutes 4°C, and the supernatants were bound to equilibrated Ni-NTA beads for 2 hours at 4°C.

After being bound to the resin, the beads were washed first with a buffer containing 25 mM HEPES-KOH pH 7.2, 300 mM KCl, 0.1 % NP-40, 2 mM β-mercaptoethanol), followed by Wash Buffer 2 (25 mM HEPES-KOH pH 7.2, 100 mM KCl, 0.1 % NP-40, 2 mM β-mercaptoethanol). The fusion protein was finally eluted off the resin using 300 mM imidazole in WB2 and buffer exchanged with Krebs Ringer Bicarbonate (KRB) media (5.6 mM glucose, 0.55 mM sodium pyruvate, 25 mM sodium bicarbonate, 53 μM sodium lactate,

99.6 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM potassium dihydrogen phosphate and 1.2 mM magnesium sulphate) by dialysis.

To quantify the purified protein preparation, increasing amounts of His-tagged complexin I were loaded along with standard increasing amounts of purified sample of Bovine Serum Albumin (BSA) on a 15 % SDS gel and the gel was stained with Coomassie Brilliant Blue, as shown in Figure 14, and the concentration of the protein purification was evaluated according to the intensity of the BSA bands.

Preparation of mouse sperm.

Sperm were collected from cauda epididymis of 12-15 week old mice and allowed to "swim-up" for 15 minutes in 1 mL Krebs Ringer Bicarbonate (KRB) media (5.6 mM glucose, 0.55 mM sodium pyruvate, 25 mM sodium bicarbonate, 53 μM sodium lactate, 99.6 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM potassium dihydrogen phosphate and 1.2 mM magnesium sulphate). This technique is used to separate sperm based on their ability to swim through a culture medium, separating viable from non-viable sperm.

Sperm were then collected by centrifugation at 300 x g for 3 minutes at room temperature and counted. Sperm pellets were further incubated for 1h at 37°C/5%CO_2 in non-capacitating KRB media or capacitating KRB media which was supplemented with 3 mg/mL BSA and 1.7 mM $CaCl_2$ since these two reagents are required for capacitation. Sperm were then pelleted by centrifugation at 500 x g for 3 minutes and resuspended in KRB media without BSA and Ca^{2+}.

Detection of Complexin I in Non-capacitated and Capacitated Sperm samples.

After mouse sperm preparation as described above, detection of complexin I in non-capacitated sperm was first completed by sonication of samples at 3x15s on ice with Fisher Sonic Dismembranator model 300 and the samples were centrifuged at 500 x g for 5 minutes, to pellet insoluble materials. The equivalent of $10x10^6$ and $20x10^6$ sperm were mixed with sodium dodecyl sulphate (SDS) buffer and loaded on a 15% SDS-PAGE gel as described later in ***Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and western blot analysis.***

To determine the location of complexin I in sperm before and after capacitation, differential centrifugation was completed as follows. This procedure was revised from Baker *et al.* (2002) [37]. Following *Preparation of Mouse Sperm,* both non-capacitated and capacitated sperm samples were sonicated 3x15s and spun at 500 x g for 5 minutes to pellet cell debris. The samples were previously supplemented with a protease inhibitor cocktail of leupeptin (20 μg/ml), aprotinin (20 μg/ml), PMSF (1 mM), benzamidine (200 μg/ml). The supernatant was subjected to differential centrifugation: an initial spin at 30,000 x g for 30 minutes, resulted in the first pellet, P1. The supernatant of P1 was then spun at 100,000 x g for 1h, which yielded the pellet, P2 and the supernatant, S2. P1 and P2 correspond to the membrane fractions, large membrane fragments (plasma membrane) collected in P1 and smaller membrane fragments (vesicular membrane) collected in P2. Both P1 and P2 were dissolved in SDS buffer, while S2 was precipitated with 10% TCA (Trichloroacetic Acid), then dissolved in SDS buffer. The equivalent of $10x10^6$ sperm were loaded on a 12.5% SDS gel. The transferred nitrocellulose blot was cut around the 25 kDa and the upper part was probed with rabbit anti-syntaxin 2 at 1:500 as an indicator of the membrane fraction, while the lower part was probed with rabbit anti-complexin I at 1:500.

Ca^{2+} titration of the acrosome reaction in SLO-permeabilized mouse sperm.

Calcium buffers.

10X stock Ca^{2+} buffers were prepared by mixing the appropriate amount of 1 M $CaCl_2$ and 0.5 M EGTA to obtain the desired 10X final free Ca^{2+} concentration. The free Ca^{2+} concentration of the 1X buffer used was determined by a Ca^{2+} selective electrode (Corning) calibrated with Ca^{2+} standards. A standard curve was generated by plotting the predicted concentrations of the Ca^{2+} standards with the voltage measured by the electrode (mV) and the concentration of the prepared Ca^{2+} buffers was estimated with the help of the standard curve.

Acrosome reaction of SLO-permeabilized murine sperm.

Sperm were collected from cauda epididymis of 12-15 week old mice and allowed to "swim-up" for 15 minutes at 37°C/5%CO_2 in KRB medium supplemented (capacitating medium) or not (non-capacitating medium) with 1.7 mM $CaCl_2$ and 3 mg/mL BSA. Sperm destined to be capacitated were incubated in the capacitative medium for 1 h at 37°C/5%CO_2. Sperm were then pelleted at 300 x g for 3 minutes and resuspended in 50 µL Ca^{2+}-free KRB medium pH 7.4. $0.25x10^6$ sperm were sampled and permeabilized with 2 µg/ml recombinant SLO for 20 min at 37°C/5%CO_2 and stimulated with 1 mM $CaCl_2$ with an extra incubation time of 10 minutes. Sperm were then fixed with 4% formaldehyde and the assessment of acrosomal status was determined by Coomassie Brilliant Blue staining.

Ca^{2+} titration of the acrosome reaction in SLO-permeabilized mouse sperm.

Sperm were collected from cauda epididymis of 12-15 week old CD1 mice. Sperm for capacitative experiment were allowed to swim-up for 15 minutes at 37°C/5%CO_2 in KRB

medium supplemented with 1.7 mM $CaCl_2$ and 3 mg/ml BSA and capacitation was allowed to continue for 1 hour at 37°C/5%CO_2. Sperm for non-capacitative experiment were allowed to swim-up for 15 minutes at 37°C/5%CO_2 in KRB medium. Sperm were pelleted at 300 x g for 3 minutes and resuspended in 50 µl Ca^{2+}-free KRB pH 7.4 and diluted to a concentration of $0.25x10^6$ sperm. Sperm were permeabilized with 2 µg/ml recombinant SLO for 20 min at 37°C/5%CO_2 and subsequently supplemented with 10X $CaCl_2$, ranging from 19 µM to 10 mM. Sperm were incubated for an extra 10 minutes at 37°C/5%CO_2 and fixed with 4% paraformaldehyde. The assessment of acrosomal status was determined by Coomassie brilliant blue staining.

Introduction of recombinant proteins and antibodies in SLO-permeabilized mouse sperm.

After collection, capacitated and non-capacitated sperm samples were pelleted at 300 x g for 3 minutes and resuspended in Ca^{2+}-free KRB pH 7.4 and diluted to a concentration of $0.25x10^6$ sperm/mL in Ca^{2+}-free KRB pH 7.4. Sperm were permeabilized with recombinant SLO at a concentration of 2 µg/mL for 20 minutes at 37°C/5%CO_2 in the presence of the antibody or recombinant complexin I protein, from a concentrated stock in KRB media. Briefly, complexin I recombinant protein was added at increasing concentrations (from 0 µM to 3.13 µM), while the antibody was added at different dilutions (1:200, 1:100, 1:50; anti-synaptotagmin VIII, used as a positive control) to the permeabilized sperm. Dilutions were used instead of quantities because the rabbit anti-complexin I antibody is affinity purified and the rabbit anti-synaptotagmin VIII is a serum antibody, from Hutt *et al.* (2005) [92].

The samples were subsequently supplemented with $CaCl_2$ to a final concentration of 1 mM and incubated an additional 10 minutes at 37°C/5%CO_2. To determine the percentage of AR at 0 µM $CaCl_2$ in the case of the antibody addition, both untreated and treated samples

were incubated with non-immune rabbit serum and non-immune rabbit IgG as a control. The samples were finally fixed with 4% formaldehyde.

Assessment of acrosomal status.

Fixed sperm were harvested by centrifugation at 300 x g for 5 minutes and washed twice with 0.5 mL of 0.1 M ammonium acetate pH 9.0. The sperm were subsequently resuspended in 0.1 M ammonium acetate pH 9.0 and air-dried on glass slides. The sperm were stained with 0.625% Coomassie Brilliant Blue G-250 in 50% methanol and 10% acetic acid for 5 minutes at room temperature. The Coomassie Brilliant Blue dye will stain all the proteins in the sperm but due to the density of proteins in the small acrosome compartment, the latter appears dark blue following the staining procedure. The slides were then washed three times with distilled water and mounted with 30% glycerol in PBS. This experimental procedure was revised from Hutt *et al.* (2005) [92]. Sperm were imaged by bright field microscopy using a Zeiss Axiophot microscope and scored for acrosomal staining. Counting at least 2000 sperm per slide assessed the percentage of AR by dividing the number of acrosome-reacted or non acrosome-reacted spermatozoa by the total number of counted spermatozoa. The data generated from treated spermatozoa were corrected for the number of spermatozoa that underwent spontaneous acrosome reaction (acrosome-reacted in non-inducing conditions) and analyzed as described in ***Statistical Analysis***. Multiple repeats were done to take account for the variation due to technical manipulation from day to day.

Detection of syntaxin 2 in non-capacitated and capacitated sperm.

Lysates of non-capacitated and capacitated sperm samples were obtained by sonication at 3x15s on ice and insoluble materials were pelleted at 500 x g for 5 minutes. The

supernatant was centrifuged at 30,000 x g for 1h and the pellets dissolved in 1X sample buffer. The equivalent of $20x10^6$ sperm were loaded on a 10% SDS gel. The transferred nitrocellulose blots were probed with a rabbit anti-syntaxin 2 antibody.

Treatments of Sperm following *Preparation of mouse sperm.*

For further treatments of sperm to determine the circumstances of the appearance of the fast migrating band in the capacitated sperm (ie. alkaline extraction), samples were sonicated 3x15s at 30 % power with Fisher Sonic Dismembrator model 300 on ice. The samples were previously supplemented with a protease inhibitor cocktail of leupeptin (20 μg/ml), aprotinin (20 μg/ml), PMSF (1 mM), benzamidine (200 μg/ml). For sonicated samples, cell debris were discarded by centrifugation at 500 x g for 5 minutes at 4°C. Crude membranes were pelleted by ultracentrifugation of the supernatant at 30,000 x g for 30 minutes at 4°C. Pellets were either resuspended in the appropriate buffer for the study or dissolved in 2X SDS sample loading buffer.

Alkaline extraction.

Crude membranes collected by differential centrifugation were resuspended in KRB medium and the equivalent of $20x10^6$ sperm were treated with 0.1 M Ca^{2+} carbonate pH 11 on ice for 20 minutes. The supernatant and pellet were then collected by ultracentrifugation at 100,000 x g for 30 minutes at 4°C [127]. The supernatant was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) and by incubating it on ice for 20 minutes. The sample was then subjected to centrifugation and the subsequent pellet was dissolved in 1X SDS sample loading buffer. The pellet obtained after the 100,000 x g centrifugation was dissolved in 1X SDS sample loading buffer for further Western blot analysis using anti-

syntaxin 2 antibody. The fact that syntaxin 2 is a membrane protein can serve as a quality control verification of the success of the extraction.

Alkaline phosphatase treatment of non-capacitated and capacitated sperm.

For this experiment, sperm were collected in modified KRB lacking potassium dihydrogen phosphate. After sonication at power 30%, a spin 500 x g for 5 minutes, and a final spin of the supernatant at 30,000 x g for 30 minutes as described above, the pellets were resuspended in dephosphorylation buffer. After quantification with the BioRad protein assay kit (BioRad), crude membrane extracts were aliquoted to 100 µg and the volume completed to 20 µl with dephosphorylation buffer (0.1 M Tris-HCl, pH 8.5). The protein samples were incubated with 50 unit of alkaline phosphatase (broad range serine/threonine phosphatase from calf intestine, at Roche Diagnostics, Germany) or equal volume of dephosphorylation buffer (mock treatment) for 2 h at 37°C, one unit being the amount of enzyme needed for the reaction of 1 nmol of substrate per minute. The reaction was stopped by adding 20 µl 2X SDS sample loading buffer. Samples were analysed by Western blotting.

Sodium dodecyl sulphate(SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis.

Samples intended for Western blotting were dissolved in SDS sample loading buffer and boiled for 10 minutes prior to Western blotting (Laemmli 1970). Proteins were separated on a 10 % and 15 % SDS-PAGE gels for detection of syntaxin 2 and complexin I, respectively, with a 4 % stacking gel. The proteins were then transferred onto a nitrocellulose membrane. After blocking for 30 minutes at room temperature in 5 % blocking buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5, 5% skim milk), the blots were probed with the specific

primary antibodies diluted in blocking buffer overnight at 4°C. For syntaxin 2 detection, rabbit anti-syntaxin 2 was used at 1:500, while for complexin detection, rabbit anti-complexin I was used at 1:500. The blots were then washed in Western Wash Buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5) containing 0.05 % Tween-20 three times; once for 10 minutes and twice for 5 minutes. Blots were subsequently incubated with Alexa 488 goat anti-rabbit secondary antibody (Sigma) and diluted at 1:2000 in blocking buffer for 1h at room temperature. Blots were washed again as previously described and protein staining was visualized by enhanced fluorescence.

Statistical analysis.

Data for AR in SLO permeabilized sperm was corrected by subtracting the percentage of AR in Ca^{2+}-induced SLO permeabilized sperm from the percentage of AR in non-induced SLO permeabilized sperm. Two models were used for fit of the data obtained from the Ca^{2+} titration of the AR of capacitated sperm. One model assumed a single Ca^{2+} binding site in the form of:

$$y = \max/(1+10^{(\log EC_{50}-x)*\text{Hill slope}}),$$

with y = percentage of AR, x = $\log[Ca^{2+}]$, max = the maximum AR at saturating Ca^{2+}, EC_{50} = the Ca^{2+} at half-maximal binding, and Hill slope being the Hill coefficient that reflects cooperativity. The data were fit by non-linear least square regression (SigmaPlot) with max and EC_{50} as parameters of the fit. The fit to the data was also compared assuming a unitary Hill slope (Hill slope =1 is equivalent to no cooperativity) or a variable Hill slope. The other model tested assumed two binding sites with different Ca^{2+} affinities (EC_{501} and EC_{502}), in the form of:

$$y = \max*(F1/(1+10^{(\log EC_{50_1}-x)\text{Hill Slope}_1})+(1-F1)/(1+10^{(\log EC_{50_2}-x)*\text{Hill slope}_2})),$$

where y = percentage of AR, x = log[Ca^{2+}], F1 is the fraction of the sperm with EC_{501}. To determine if a model with a fixed or a variable Hill slope was appropriate, the Hill coefficient of the regression analysis was compared to the extrapolated Hill slope of the plot of the log(AR/nAR) as a function of log[Ca^{2+}] where AR is the fraction of acrosome reacted sperm and nAR is the fraction of acrosome intact sperm. The fit comparison between the 1-site and 2-site model was done by an F-test of the residuals. The 1-site model with a fixed Hill slope of 1 was used for the fit of the Ca^{2+} titration of the AR of capacitated sperm, as well as the Ca^{2+} titration experiments supplemented with recombinant complexin I and anti-complexin I antibodies.

Data for AR in SLO permeabilized sperm and the EC_{50} estimated from non-linear regression of Ca^{2+} titration was compared using one-way ANOVA followed by the Tukey-Kramer multiple comparisons post-hoc test to compare the means of all paired data. Differences were considered significant for $P<0.05$ levels.

RESULTS

PART 1: complexin I

Complexin I has been shown to bind to a core complex formed by the three SNARE proteins, and seems to play a regulatory role in the subsequent membrane fusion by stabilizing the SNARE complex as a fusion competent intermediate, until triggered by a fusogenic signal [99, 100, 103]. Since the AR is a single and irreversible exocytotic event, it must be tightly regulated to ensure exocytosis at the appropriate moment. Complexin has been proposed to stabilize SNARE complex [99, 100, 103], making it an ideal molecule to promote SNARE complex formation in the non-capacitated sperm and to maintain the SNARE complex in a fusion competent intermediate in capacitated sperm. To test this hypothesis, first, the presence of complexin I in sperm was assessed, and the effect of complexin I on Ca^{2+} sensitivity of the acrosome reaction in non-capacitated and capacitated sperm was then examined.

Detection of complexin I in sperm

To confirm the presence of complexin I in sperm, non-capacitated sperm were sonicated; insoluble materials were spun down at low speed of 500 x g and the supernatant was run on a SDS polyacrylamide gel. The experiment was performed four times. Complexin I is a protein of approximately 18-20 kDa and the results of my immunoblotting reveal a band around the 19 kDa level, in both $10x10^6$ and $20x10^6$ sperm sample (Figure 11). This confirms the presence of complexin in sperm, as previously demonstrated by other researchers such as Redecker *et al.* [39] and Sousa *et al.* [89], via Western blotting and immunohistochemistry.

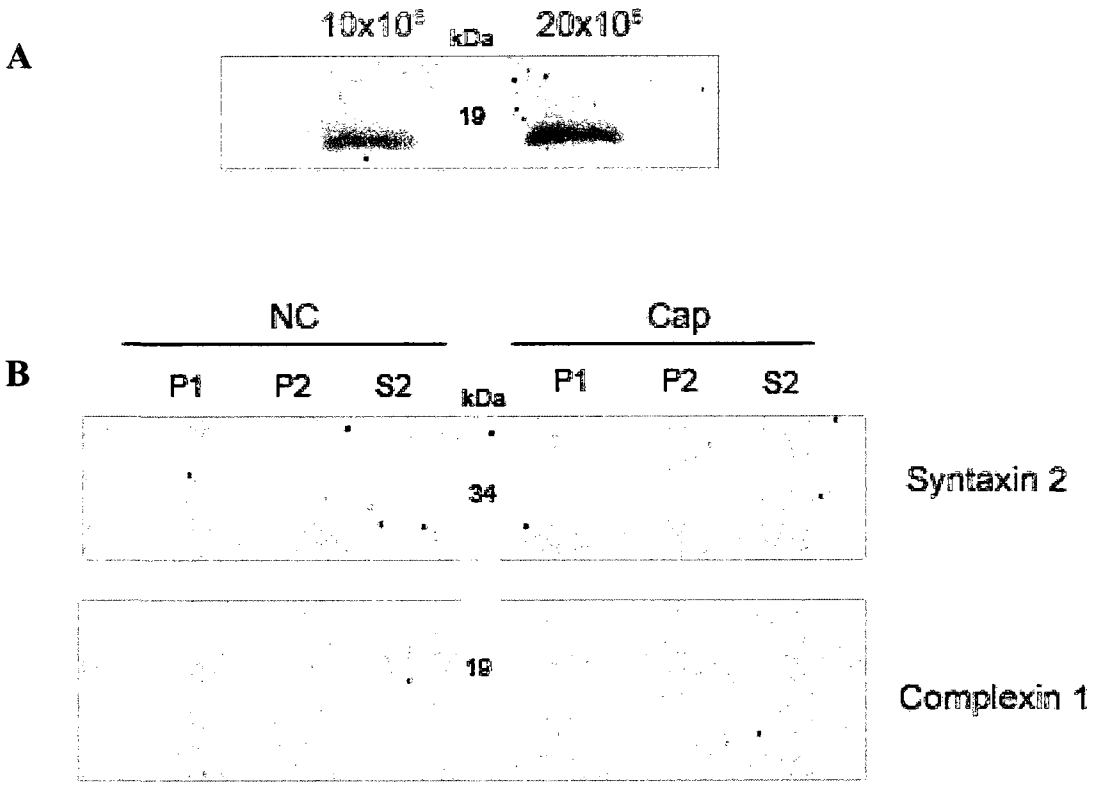


Figure 11: Detection of complexin I in sperm. **(A)** non-capacitated sperm were sonicated 3x15s and the insoluble materials were spun down by a brief spin at 500 x g for 5 minutes. The equivalent of $10x10^6$ and $20x10^6$ sperm were mixed with sample loading buffer and loaded on 15% SDS gel. The transferred nitrocellulose blot was probed with rabbit anti-complexin I at 1:500. **(B)** Non-capacitated and capacitated sperm were sonicated 3 x 15s and insoluble materials were spun down at 500 x g for 5 minutes. The supernatant of that low spin was then subjected to a differential centrifugation: a first spin at 30,000 x g for 30 minutes, which resulted in the first pellet P1 (microsome fraction). The supernatant of P1 was then spun at 100,000 x g for 1h, which gave the pellet P2 (small vesicular membranes) and the supernatant S2 (cytosolic fraction). Both P1 and P2 were dissolved in sample loading buffer and S2 was TCA-precipitated, then dissolved in sample loading buffer. P1 pellet corresponds to large membrane fragments, P2 pellet to vesicular membrane and S2 the cytoplasmic content. The equivalent of $10x10^6$ sperm were loaded on a 12.5% SDS gel. The transferred nitrocellulose blot was cut around the 25 kDa and the upper part was probed with rabbit anti-syntaxin 2 at 1:500 and the lower part was probed with rabbit anti-complexin I at 1:500 (n=4).

Complexin I is a cytosolic protein that only binds to assembled or partially assembled SNARE complexes. SNARE proteins and the SNARE complex have been shown to play a pivotal role in AR [90]. Knowing these two facts, I sought to determine whether the distribution of complexin I would change after capacitation. To do so, both capacitated and non-capacitated mouse sperm were sonicated and my samples subjected to a differential centrifugation at 30,000 x g for 30 minutes, which generated the supernatant S1 and P1 microsomal pellet. The S1 fraction was then subjected to centrifugation at 100,000 x g for 1h, which generated the P2 pellet and the supernatant S2. P1 represents most of the sperm membrane (plasma membrane), P2 smaller membranes (vesicle membrane) and S2 the cytosolic fraction. The blot was probed with a rabbit anti-complexin I antibody and a rabbit anti-syntaxin 2 antibody. The blot was also probed with an anti-syntaxin 2 antibody, as a control of the differential centrifugation, knowing that syntaxin 2 is an integral membrane protein. The rabbit anti-syntaxin 2 detects a protein of approximately 35 kDa, which corresponds to the apparent molecular weight of syntaxin in both pellets fractions P1 and P2. In addition, a second band was detected above the 25 kDa level as well but was shown to be an artefactual band that seems to appear in all blots probed with the secondary Alexa 488 Goat anti-rabbit antibody. When the blot was probed with a rabbit anti-complexin I, a complexin I band was detected in both P1 and S2 in non-capacitated and capacitated sperm (Figure 11). This suggests that complexin I is present in the cytosol, as expected, but also in the membrane. Since the SNARE proteins are the only known binding partners of complexin I when assembled into a core SNARE complex, it suggests that complexin I is bound to assembled or partially assembled SNARE complex in non-capacitated and capacitated sperm. Altogether, these results suggest that the SNARE proteins are assembled into a core complex, fully or partially, in the non-capacitated and capacitated state. This was surprising

because capacitation being the process by which sperm acquires its ability to undergo acrosome reaction, one could have thought that the SNARE proteins would have assembled into the core complex during capacitation in order to be functional at the right moment. The non-capacitated sperm used in this experiment could have reached late stages of the non-capacitated state, more specifically an intermediate stage between non-capacitated and capacitated conditions, especially since BSA and Ca^{2+} are needed for the sperm to capacitate [6]. Therefore, it seems that the SNARE proteins could be preassembled into a complex in "late" non-capacitated sperm, which is why complexin I is detected in the membrane fraction. It is worth mentioning that the intensity of P1 and S2 in the capacitated sample seems weaker than the intensity of the bands of P1 and S2 of non-capacitated sperm. Even though a protease cocktail was added to the sample prior to sonication, some degradation could have occurred in the capacitated sample. It seems as well that, according to the intensity of the complexin I bands, there could be less complexin I in P1 of capacitated sperm compared to the non-capacitated sample. This difference in intensity was not significant as the experiment was repeated four times and gave more or less the same level of intensity for both conditions.

Calcium titration of non-capacitated and capacitated sperm in AR

Sperm is a great system to study fusion events, because AR is a single event and it is the consequence of the fusion between the OAM and the PM, which results in vesiculation and shedding of the OAM and PM. To study the Ca^{2+} requirements and the effect of complexin I on the calcium sensitivity and AR of non-capacitated and capacitated sperm, the samples were subjected to a permeabilization step, done by the toxin Streptolysin O (SLO). Sperm is a transcriptionally and translationally silent system. Therefore, to study the specific

effects of a compound, such as a protein, it has to be able to penetrate the system [92]. SLO has been extensively used as permeabilization agent in many systems, where traditional procedures, such as transfection, were not possible, as for sperm [126, 128]. SLO is a member of a large family of cholesterol-binding cytolysins that forms pores in the cell's plasma membrane. SLO monomers bind to cholesterol molecules in the membrane and diffuse in its plane to form dimers with one another [126, 129, 130]. These dimers play the role of crystallization points where SLO complexes grow into arcs and rings to form transmembrane pores. These pores can have a diameter exceeding 30 nm, therefore introduction of macromolecules is possible. SLO will bind to the membrane at 4°C but will not form pores until the temperature is raised to 37°C [126, 129, 130]. Furthermore, since SLO is adsorbed to cholesterol and trapped within the plasma membrane, washing the sperm into SLO-free medium at 4°C and then raising the temperature to 37°C will specifically permeabilize the plasma membrane and not intracellular membranes. Thus, the sperm plasma membrane is selectively permeabilized by SLO, but the acrosome and other intracellular compartments are not perturbed.

Non-capacitated and capacitated sperm were permeabilized with SLO and adding increasing amount of free Ca^{2+} ranging from 0 mM to 1 mM was added to generate Ca^{2+} titration curves. Measuring the Ca^{2+} concentrations with a Corning Ca^{2+} electrode assessed the precise Ca^{2+} concentration of each buffer. The experiment was performed four times. Analysis of the data was done using the 1-site model described in the Methods section and the EC_{50} of non-capacitated and capacitated sperm was estimated to be 3.1 μM Ca^{2+} (Figure 12) and 9.8 μM Ca^{2+} (Figure 13) respectively. The distribution of the data points of the Ca^{2+} titration of the AR of capacitated sperm suggests that there could be two inflection points,

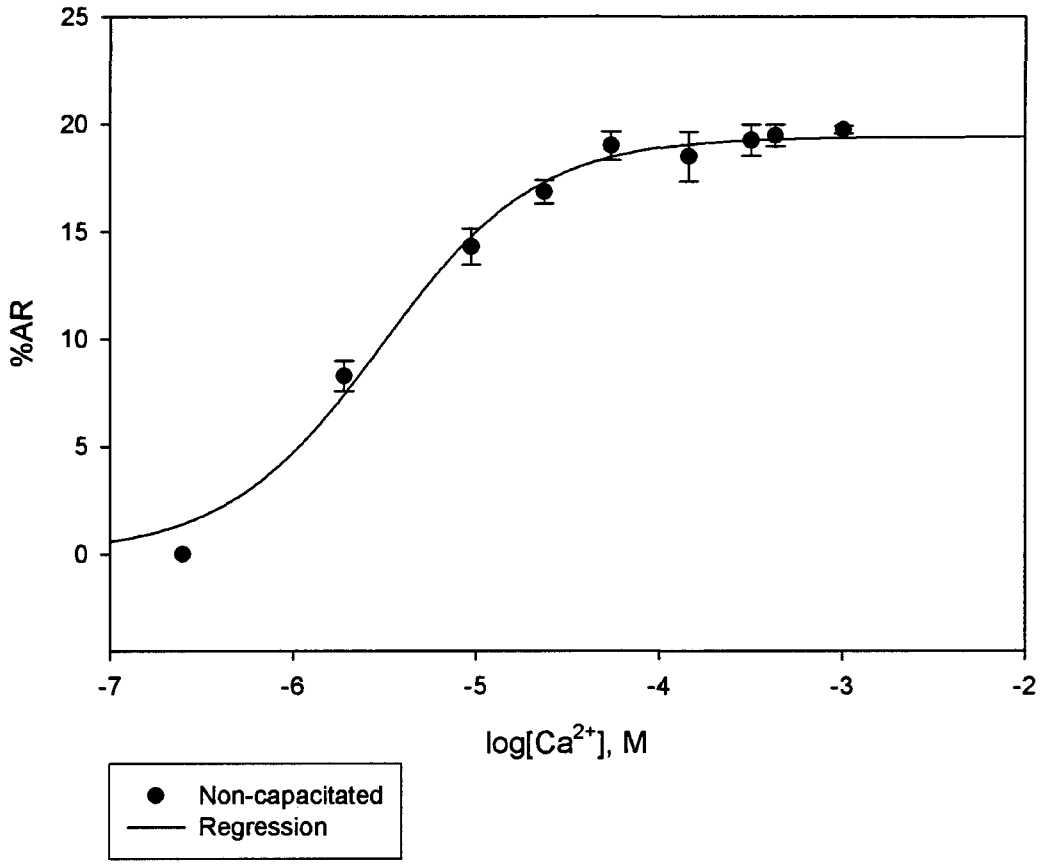
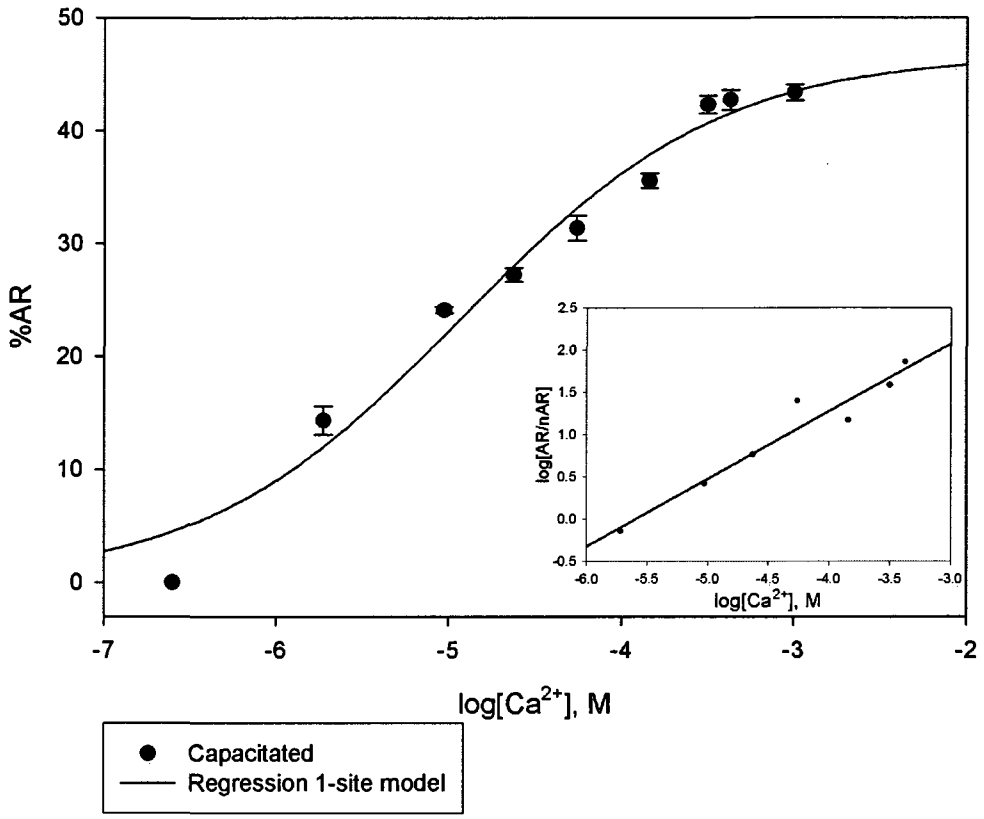


Figure 12: Calcium titration of non-capacitated sperm AR. Non-capacitated SLO-permeabilized sperm were incubated with KRB supplemented with increasing amounts of Ca^{2+} concentration ranging from 1.9 μM to 1 mM. Sperm were then fixed and stained with Coomassie. Counting at least 2000 sperm per slide assessed the percentage of AR. The value for EC_{50} was 3.1 μM Ca^{2+} for non-capacitated sperm. Data was corrected by subtracting the percentage of AR sperm to the percentage of spontaneous AR sperm at each Ca^{2+} concentration. The EC_{50} was determined by the non-linear least square regression, using the program Sigmaplot. The values have been corrected for spontaneous acrosome reaction and are expressed as means ± SEM (n=4)

A


%AR
log[Ca2+], M
log[AR/nAR]
log[Ca2+], M
Capacitated
Regression 1-site model

B

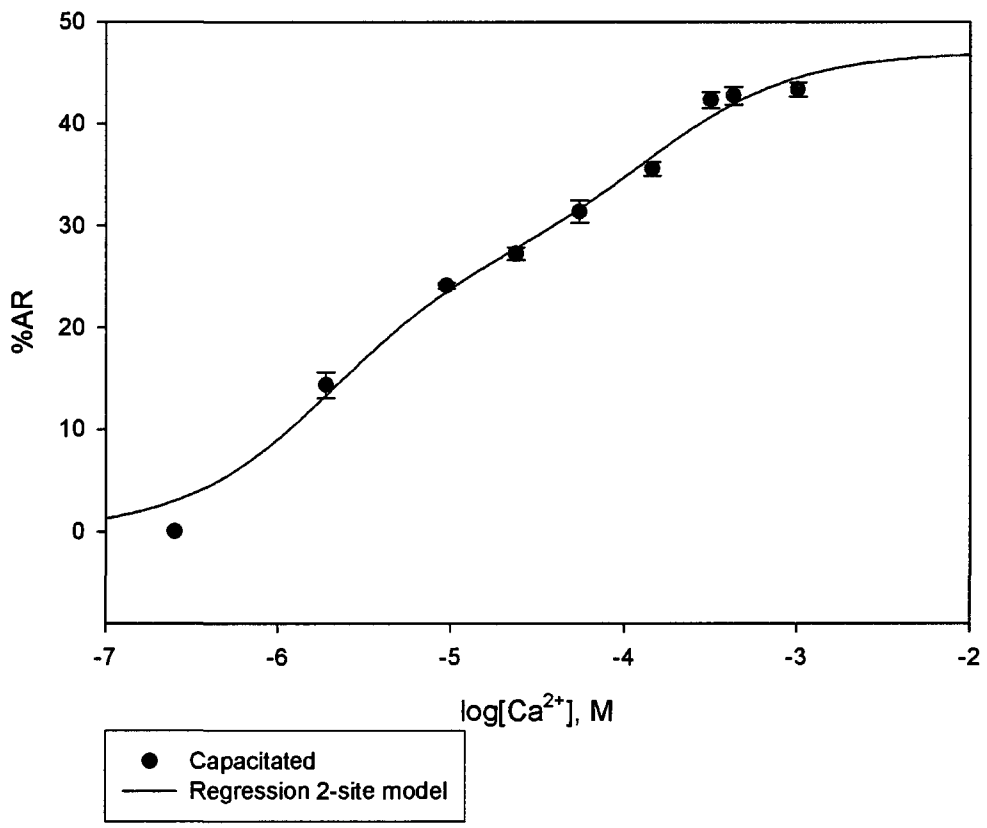

%AR
log[Ca2+], M
Capacitated
Regression 2-site model

Figure 13: Calcium titration of capacitated sperm AR. Capacitated SLO-permeabilized sperm were incubated with KRB supplemented with increasing amounts of Ca^{2+} concentration ranging from 1.9 μM to 1 mM. Sperm were then fixed and stained with Coomassie. Counting at least 2000 sperm per slide assessed the percentage of AR. The value for EC_{50} was 9.8 μM Ca^{2+} for capacitated sperm. Data was corrected by subtracting the percentage of AR sperm to the percentage of spontaneous AR sperm at each Ca^{2+} concentration. The EC_{50} was determined by the non-linear least square regression, using the program Sigmaplot. The values have been corrected for spontaneous acrosome reaction and are expressed as means ± SEM (n=4). **(A)** AR data was plotted using a 1-site model with variable slope. The EC_{50} value for the Ca^{2+} titration of the AR is 9.8 μM Ca^{2+}. Inset. A Hill plot for the Ca^{2+} titration of the AR is shown. The linear regression reveals a Hill coefficient of 0.79. **(B)** AR data plotted using a 2-site model with variable slope. The EC_{50} values for the Ca^{2+} titration of the AR are 1.6 μM Ca^{2+} and 96.8 μM Ca^{2+}.

therefore, two binding sites (Figure 13). First, the possibility of cooperativity was examined in the case of capacitated sperm. Regression analysis of the capacitated curve revealed a Hill coefficient of 0.58, which suggests that the AR does not exhibit cooperativity. This was supported by analysis of the Hill plot, which gave a linear regression with a similar slope of 0.79 (Figure 13A, inset). Analysis of the data with the equation using a unitary Hill slope provided a similar fit and gave an EC_{50} value of 11.7 μM Ca^{2+}, which is near the value found with the variable slope model. Using the 2-site model (described as well in the Methods section) (Figure 13B), the values of the EC_{50}s for the first and second binding site were estimated to be 96.8 μM Ca^{2+} and 1.6 μM Ca^{2+} respectively. A comparison of the fit with 1-site model (with fixed or variable Hill slope for both models) using F-test of the residuals suggests that the 2-site model does not provide a significantly better fit than the 1-site model. Therefore, the 1-site model was chosen to analyze the next Ca^{2+} titration experiments.

Effects of recombinant complexin I and anti-complexin I antibody on SLO-permeabilized non-capacitated and capacitated sperm

To study the effect of complexin I on the acrosome reaction, sperm membrane was permeabilized with SLO at a concentration of 2 μg/mL and the system was given time to equilibrate, in order to add recombinant complexin I or complexin I antibody to the permeabilized sperm cells [92, 108].

To express and purify recombinant complexin I, the cloning of complexin I (500 bp) cDNA into a pQE9 vector was performed. This vector contains a tag of six histidine (6XHis tag) attached to the N-terminus of the protein being expressed. Proteins containing the 6XHis tag are easy to purify because the 6XHis tag binds to NTA-nickel beads via strong interactions. To quantify and verify the purity of the protein purification, different amounts

of my purified recombinant proteins were loaded on a 15 % SDS gel, which was later stained with Coomassie Brilliant Blue. Equal amounts of 6XHis tagged complexin I from non-induced and induced cell culture were also loaded and the blot was probed with an anti-complexin I antibody, to assure that the anti-complexin I can recognize the recombinant protein. The first blot in Figure 14A shows that the 6XHis tagged complexin I is expressed in an inducible system and was not quantified. *E.Coli* XL-1 blue bacteria were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified via a complexin I small-scale protein purification using NTA-nickel beads. As expected, the induced sample generated a more intense band than the non-induced sample. Nevertheless, the signal in the non-induced lane was significant possibly due to the T5 promoter *lac* of our modified pQE9 vector, known to be "leaky". This background expression was suggested to be due to the insufficient repression of the *lac* operator by the *lac* repressor [131]. These issues have been known to cause a leak of gene expression in high number gene copy vectors [131, 132] (Figure 14A). Additional bands, with a high and low molecular weights, reactive to the rabbit anti-complexin I antibody were also observed. These bands were consistently present in repeat experiments. The high molecular weight band could be aggregates of complexin I, although complexin I is not known to form dimers, this option is not to be excluded. The lower molecular weight band could be the result of degraded fragments of complexin I protein, due to the overexpression of the vector carrying the complexin I cDNA. The second blot shown in Figure 14B is a quantification of the purified recombinant protein sample. The protein purification generated a concentration of 52.7 pmol/µl. Furthermore, the Coomassie-staining of the quantified gel clearly showed that the protein preparation is relatively clean (Figure 14B).

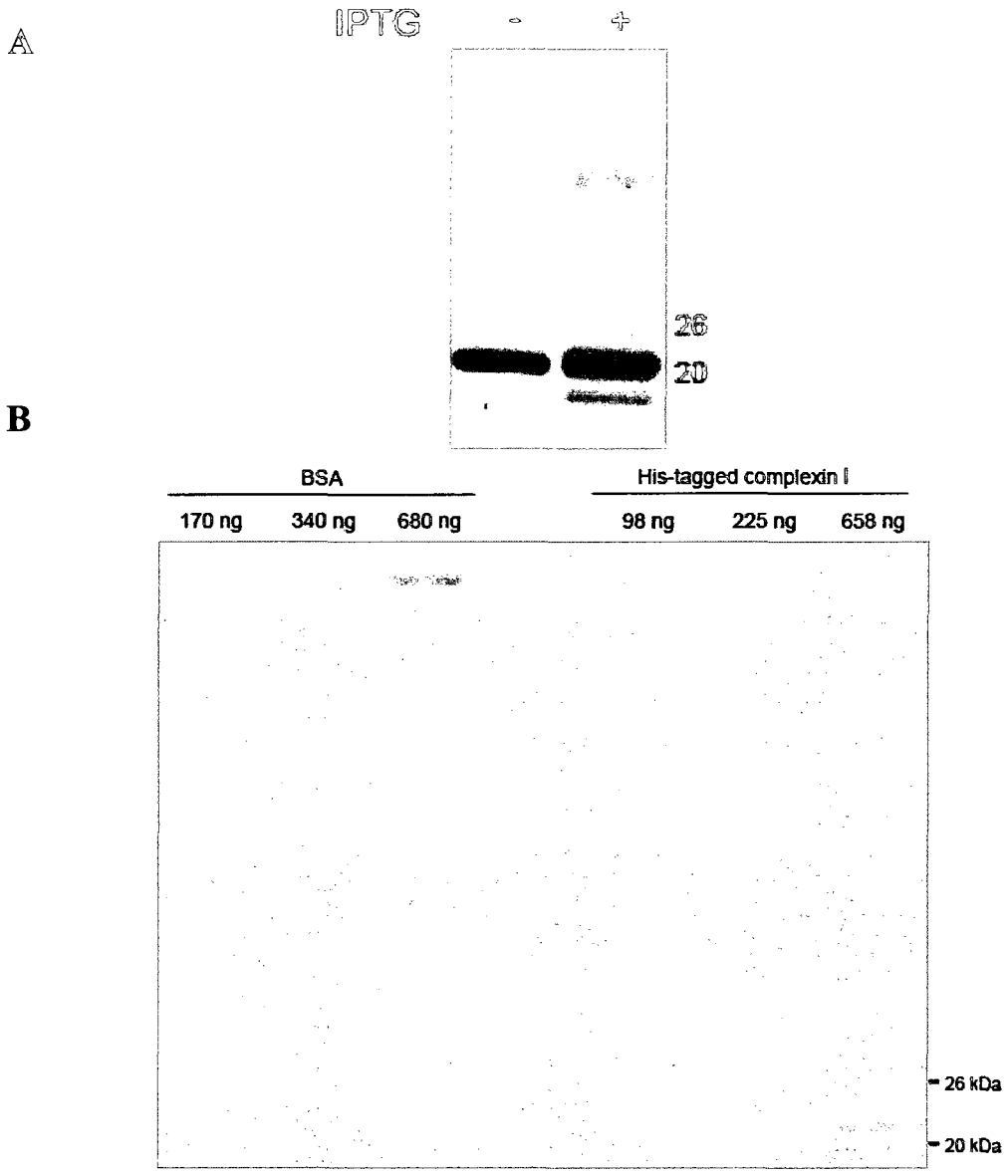
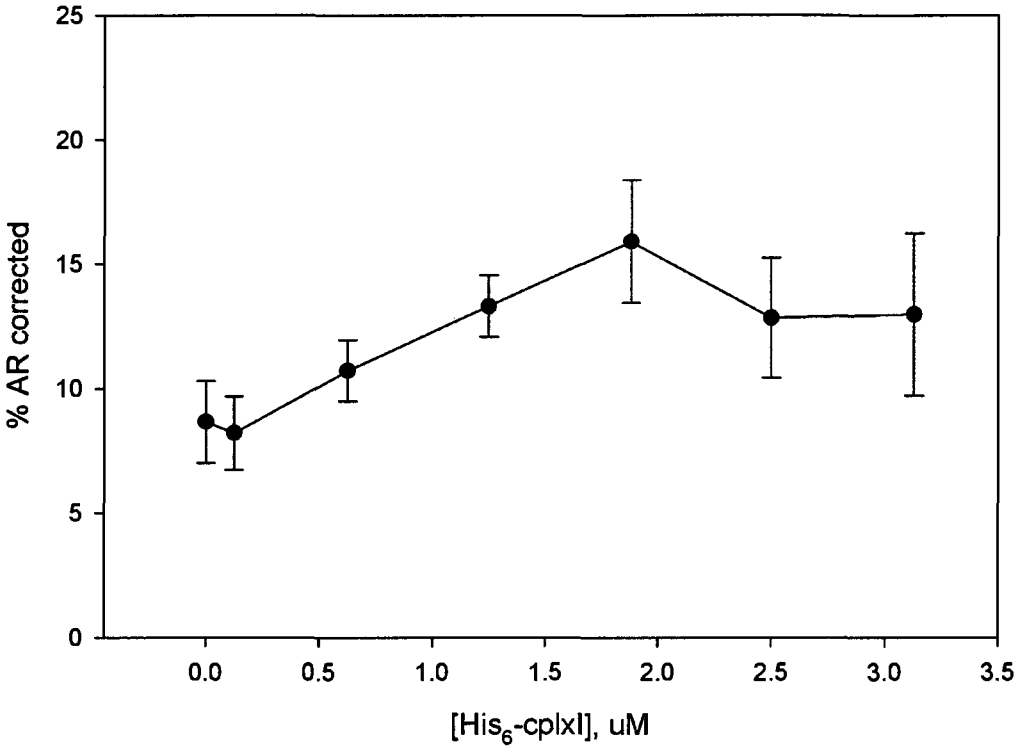


Figure 14: Expression and purification of His-tagged recombinant complexin I. (**A**) After subcloning complexin I gene product of 500 bp into the pQE9 vector, the vector was transformed into *E.Coli* bacteria by electroporation, that were induced with 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) and the resulted product is a recombinant complexin I protein with a 6 histidine tag at its N-terminus purified using Ni-NTA beads. To show the expression level under induction treatment, the bacteria were induced (+) or not (-), and after purification of the protein, the protein samples of both condition were loaded on a 15 % SDS gel. The blot was probed with rabbit anti-complexin I. (**B**) To quantify and verify the purity of the protein preparation, increasing amounts of His-tagged complexin I were loaded along with increasing amounts of purified sample of Bovine Serum Albumin (BSA) on a 15 % SDS gel and the gel was stained with Coomassie Brilliant Blue. The estimated size of His-tagged complexin I is approximately 20 kDa.

After permeabilizing sperm plasma membranes with SLO, increasing amounts of recombinant 6XHis tagged complexin I, from 0 μM to 3.13 μM, were introduced. For each amount of recombinant complexin I added, a control was done without the protein, where the equivalent volume of buffer was added. The purpose of this control is to assure that the effect observed after the introduction of the recombinant proteins to the permeabilized cells is not due to ionic or salt effect and to estimate the amount of spontaneous acrosome reaction. The experiment was performed 5 times, at different days with different mice. The percentage of acrosome-reacted spermatozoa at saturating Ca^{2+} concentration (1 mM) differs between non-capacitated and capacitated spermatozoa. When permeabilized with 2 μg/ml SLO and stimulated with 1 mM $CaCl_2$, non-capacitated sperm give an acrosome reaction percentage of about 25 % compared to 68 % in capacitated sperm, as shown in Figure 23 in Annex I. This difference in reactivity can be explained by the fact that capacitation is a needed process for sperm to acquire their fertilizing ability and comprises many changes, such as removal of adherent proteins on the sperm head, change in intracellular ionic concentrations or increase in the membrane fluidity. The fact that acrosome exocytosis can be stimulated in non-capacitated sperm suggests that it is a "premature" population of sperm that were stimulated and that this small fraction of non-capacitated sperm seems to possess a fusion-competent SNARE complex identical or similar to that found in capacitated sperm. This would imply that the main effect of capacitation is to increase the number of fusion-competent, pre-assembled SNARE complex. This population can be useful to assess the SNARE status as well as the role of complexin I in non-capacitated sperm and give greater insight into regulation of SNARE proteins before and after capacitation. It is important to remember that data displayed were corrected for spontaneous acrosome reaction (the amount of sperm that acrosome-reacted without being stimulated with Ca^{2+}), which is of 15 % and 24 % for

A


25
20
15
10
5
0
% AR corrected
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
[His6-cplxI], uM

B

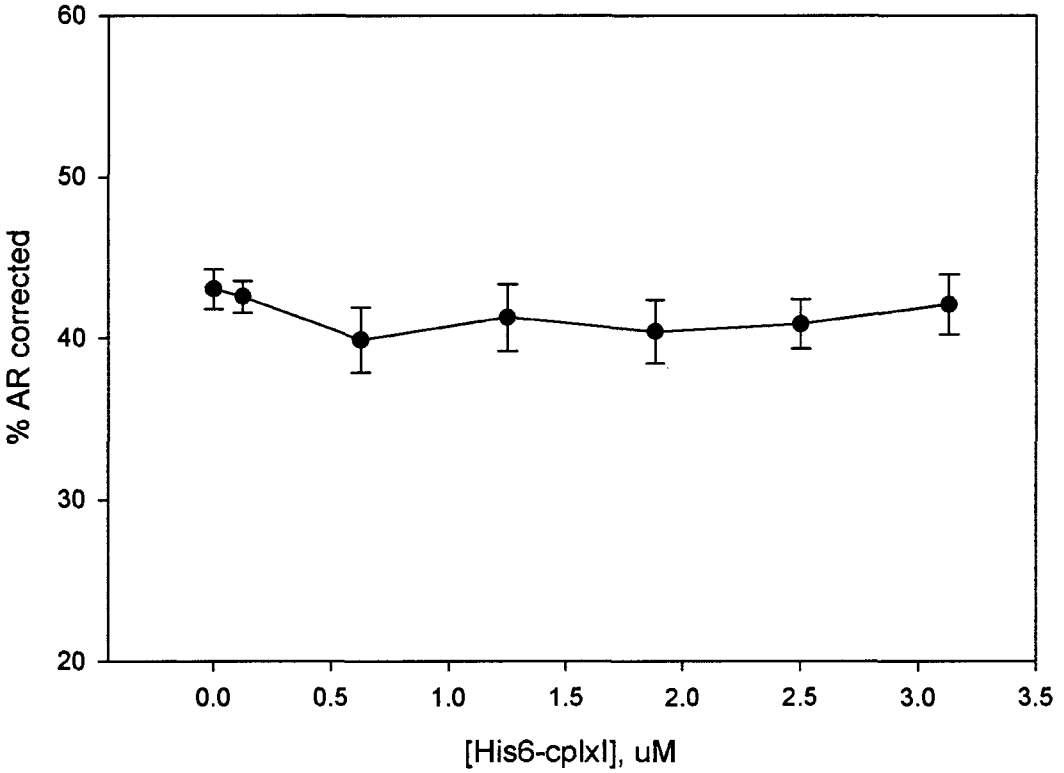
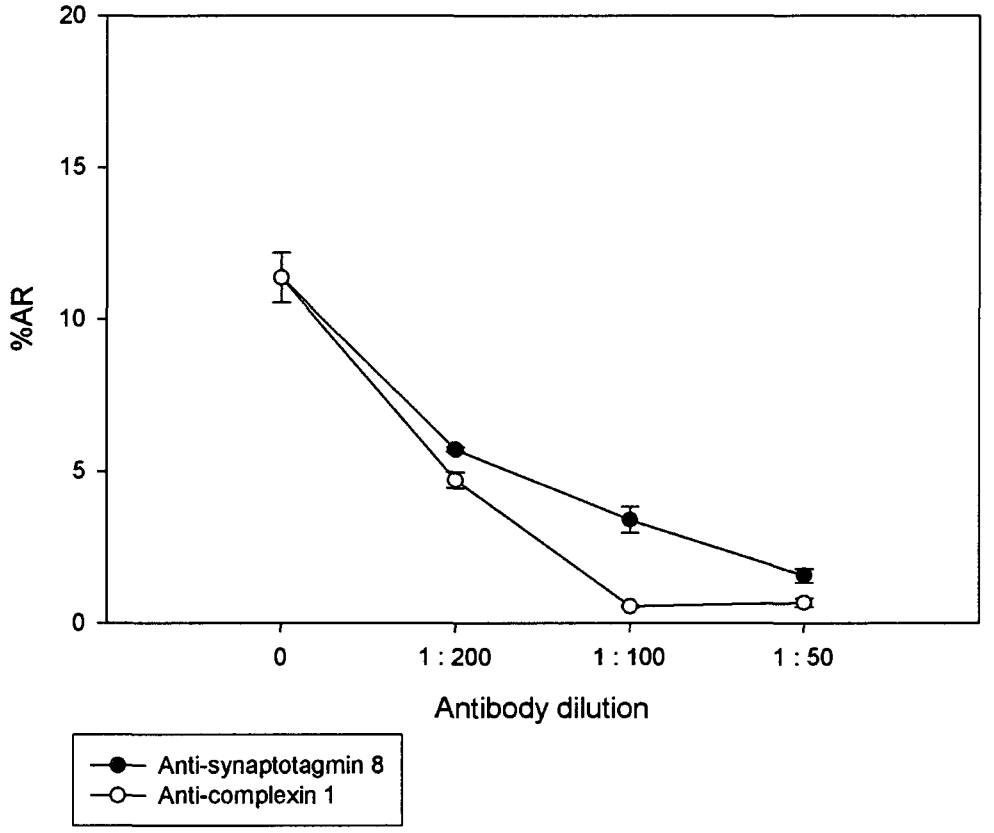

60
50
40
30
20
% AR corrected
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
[His6-cplxI], uM

Figure 15: Effect of recombinant complexin I on the percentage of AR non-capacitated and capacitated SLO-permeabilized sperm. (**A**) Non-capacitated sperm were permeabilized with SLO and increasing amounts of recombinant complexin I (0 μM to 3.1 μM) were introduced in the permeabilized sperm. Then the sperm was stimulated with 1 mM Ca^{2+}. The values are expressed as means ± SEM (n=5). (**B**) Capacitated sperm were permeabilized with SLO and increasing amounts of recombinant complexin I were introduced in the permeabilized sperm. Then the sperm was stimulated with 1 mM Ca^{2+}. The values are expressed as means ± SEM (n=5). 0.25 million spermatozoa were used for each experiment. The values are expressed as means ± SEM (n=5).

A


20
15
10
5
0
%AR
0
1 : 200
1 : 100
1 : 50
Antibody dilution
Anti-synaptotagmin 8
Anti-complexin 1

B

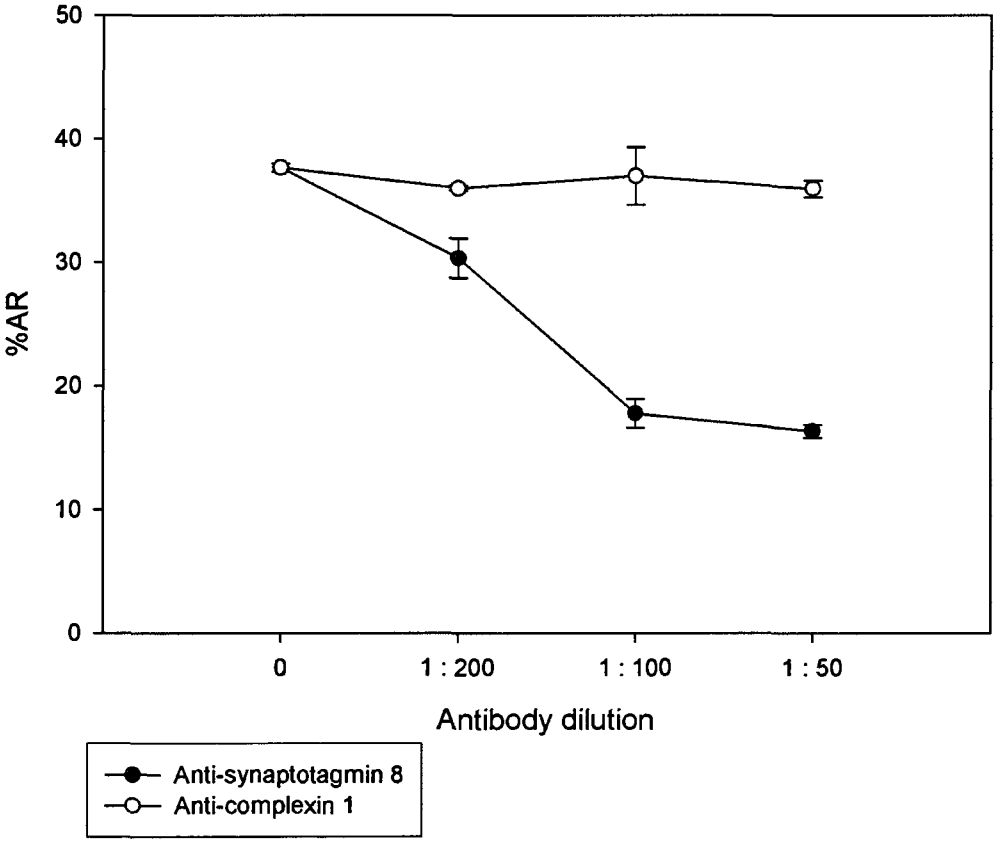

50
40
30
20
10
0
%AR
0
1 : 200
1 : 100
1 : 50
Antibody dilution
Anti-synaptotagmin 8
Anti-complexin 1

Figure 16: Effect of complexin I antibody on the percentage of AR non-capacitated and capacitated SLO-permeabilized sperm. (**A**) Non-capacitated sperm were permeabilized with SLO and different dilutions of anti-synaptotagmin VIII and anti-complexin I were introduced in the permeabilized sperm. (**B**) Capacitated sperm were permeabilized with SLO and different dilutions of anti-synaptotagmin VIII and anti-complexin I were introduced in the permeabilized sperm. Anti-synaptotagmin VIII is used as a positive control. Samples untreated and treated with non-immune rabbit serum and non-immune rabbit IgG were used as a control and to determine the percentage of spontaneous AR. 0.25 million spermatozoa were used for each experiment. The values are expressed as means $\pm$ SEM (n=5).

non-capacitated and capacitated SLO-permeabilized sperm (Appendix I). Those percentages were consistent throughout the experiments. Statistical analysis was performed by comparing the amount of acrosome-reacted sperm of the treated population to the untreated population, at each recombinant complexin I quantity, using ANOVA. There seems to be a trend toward a small increase of the percentage of acrosome-reacted spermatozoa upon addition of recombinant complexin I from 0 μM to 1.9 μM, but statistical analysis by ANOVA suggests this increase (7%) is not statistically significant ($p<0.05$) (Figure 15).

Interestingly, in capacitated sperm, the introduction of increasing amounts of recombinant complexin I did not have any effect on the percentage of spermatozoa undergoing acrosome reaction (Figure 15). This could be due to the fact that either the recombinant complexin I did not bind to the SNARE complexes available or it could not bind to the SNARE complexes because something was preventing it from doing so. In capacitated samples, the permeabilization with SLO is done only once capacitation is complete. It is possible that endogenous complexin I was already bound to the SNARE complexes, therefore adding more of this protein will not have an effect, since it will not have a binding target.

Similarly, affinity purified rabbit anti-complexin I and rabbit anti-synaptotagmin VIII (in antiserum form) antibodies were introduced into SLO-permeabilized capacitated and non-capacitated sperm, at concentrations of 1:200. 1:100 and 1:50. Dilutions were used instead of concentrations as the anti-complexin I is an affinity purified IgG fraction whereas the anti-synaptotagmin VIII is from a serum fraction. In non-capacitated permeabilized sperm, the introduction of anti-complexin I and anti-synaptotagmin VIII significantly decreased the acrosome reaction percentage to 5.6 % and 6.7 % at 1:200, 7.9 % and 10.8 % at 1:100 and

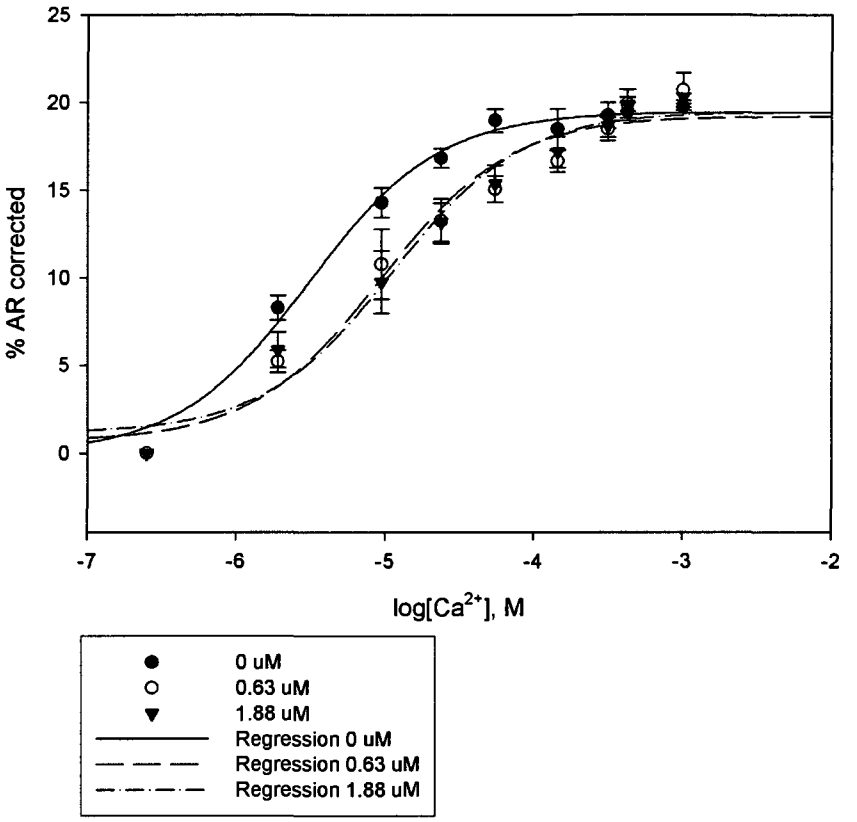
9.8 % and 10.7 % at 1:50, for anti-synaptotagmin VIII and anti-complexin I, respectively ($p<0.05$). When the antibodies were introduced in capacitated SLO-permeabilized sperm, only the anti-synaptotagmin VIII antibody significantly decreased the AR percentage by 21.3 %, ($p<0.05$) whereas the anti-complexin I antibody had close to no effect (Figure 16). This suggest that complexin I is still available for binding with the antibody in non-capacitated sperm but not in capacitated sperm. This implies two possibilities: either the antibody does not recognize complexin I or the antigen is unavailable to the antibody because the protein is complexed with other proteins, such as the SNARE complex. Furthermore, the fact that anti-synaptotagmin VIII has an effect on both non-capacitated and capacitated sperm suggests that synaptotagmin VIII only operates at a late stage of the acrosome reaction, which is why it is still sensitive to the antibody. This is in accordance with the fact that synaptotagmin has been suggested to be the putative Ca^{2+} sensor of the SNARE complex fusion machinery. Therefore, it seems as though complexin I could act upstream of synaptotagmin VIII.

Altogether, these results suggest that during capacitation, complexin I binds to a core set of proteins, the SNARE complex, to stabilize and "clamp" this complex into a fusion competent state, until the Ca^{2+} sensor, probably synaptotagmin VIII, relieves the complex from the "clamping" mechanism of complexin, knowing the properties of complexin I as well as its interplay with Synaptotagmin.

Effect of complexin I on the calcium sensitivity of non-capacitated and capacitated sperm

To determine if complexin I has an effect on the Ca^{2+} sensitivity of non-capacitated and capacitated sperm, the Ca^{2+} titration curve experiments were supplemented with

A


% AR corrected
log[Ca2+], M
0 uM
0.63 uM
1.88 uM
Regression 0 uM
Regression 0.63 uM
Regression 1.88 uM

B

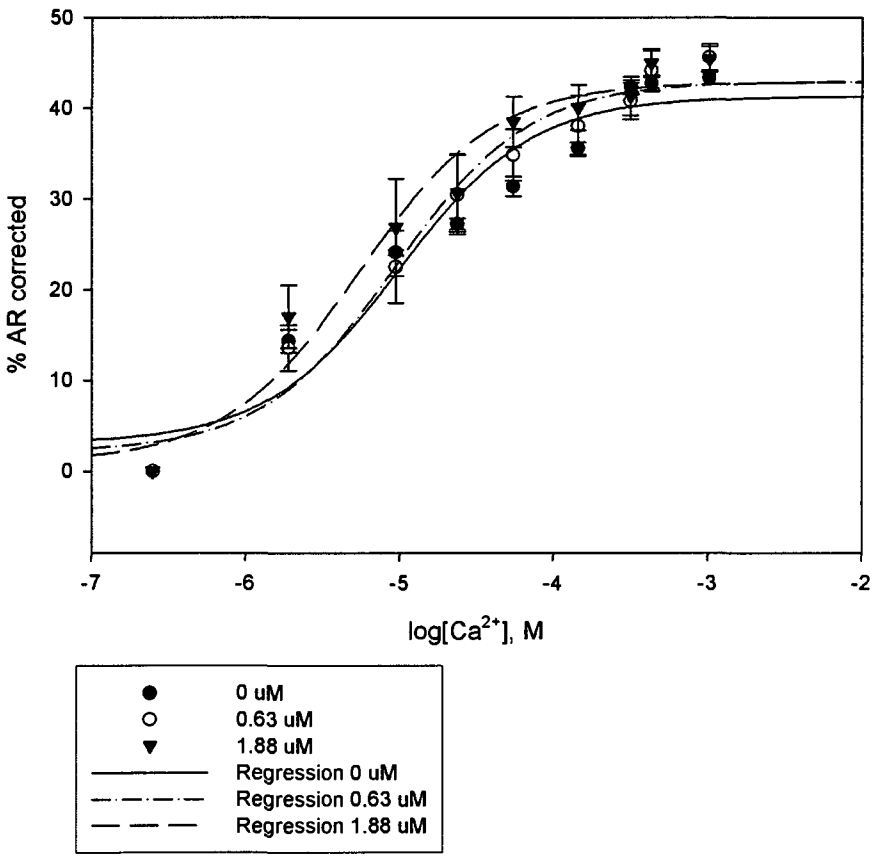
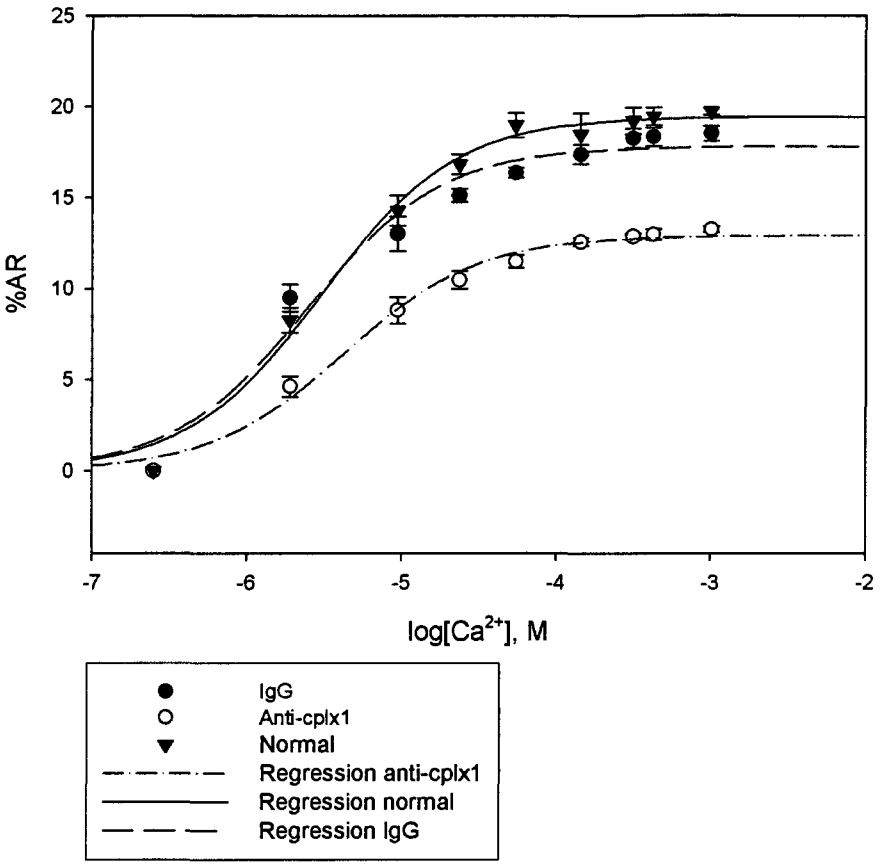

% AR corrected
log[Ca2+], M
0 uM
0.63 uM
1.88 uM
Regression 0 uM
Regression 0.63 uM
Regression 1.88 uM

Figure 17: Effect of recombinant complexin I on the calcium sensitivity of non-capacitated and capacitated SLO-permeabilized sperm. SLO-permeabilized sperm were incubated in KRB containing free Ca^{2+} ranging from 1.9 μM to1 mM together with 0.63 μM or 1.88 μM of recombinant complexin I. The percentage of AR sperm was assessed at each Ca^{2+} concentration for non-capacitated and capacitated sperm. (**A**) In non-capacitated sperm, the value for the EC_{50}s are 3.1 μM Ca^{2+}, 9.4 μM Ca^{2+} and 11.1 μM Ca^{2+}, for 0 μM, 0.6 μM and 1.9 μM respectively; (**B**) In capacitated sperm, the values for the EC_{50}s are 9.8 μM Ca^{2+}, 9.4 μM Ca^{2+} and 5.4 μM Ca^{2+}, for 0 μM, 0.63 μM and 1.88 μM recombinant complexin I, respectively. In all conditions, the values have been corrected for spontaneous acrosome reaction. The values are expressed as means ± SEM (n=5).

A


25
20
15
10
5
0
%AR
-7
-6
-5
-4
-3
-2
log[Ca2+], M
IgG
Anti-cplx1
Normal
Regression anti-cplx1
Regression normal
Regression IgG

B

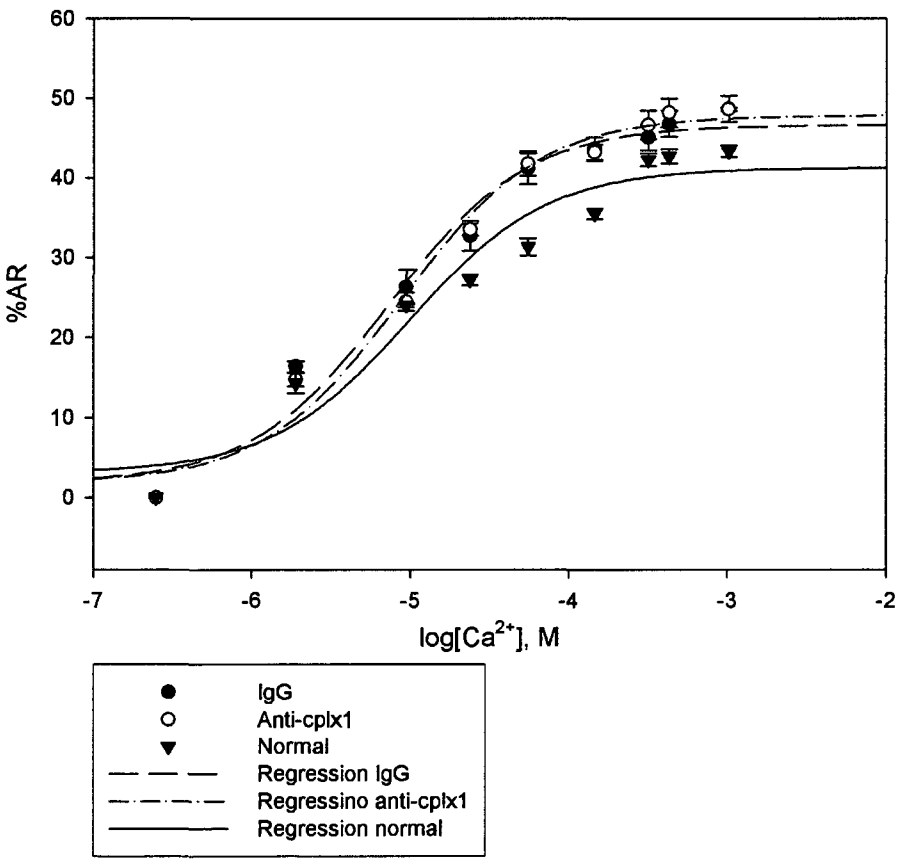

60
50
40
30
20
10
0
%AR
-7
-6
-5
-4
-3
-2
log[Ca2+], M
IgG
Anti-cplx1
Normal
Regression IgG
Regressino anti-cplx1
Regression normal

Figure 18: Effect of complexin I antibody on the calcium sensitivity of non-capacitated and capacitated SLO-permeabilized sperm. SLO-permeabilized sperm were incubated in KRB containing free Ca^{2+} ranging from 1.9 μM to 1 mM and anti-complexin I was introduced at a concentration of 150 μg/ml, for each Ca^{2+} concentration. The percentage of AR sperm was assessed at each Ca^{2+} concentration for non-capacitated and capacitated sperm. The rabbit anti-complexin I antibody being affinity purified and an IgG fraction, non-immune rabbit IgG was used as an internal control, as well as the addition of medium in the normal condition. (**A**) In non-capacitated sperm, the values of the EC_{50}s are 3.1 μM Ca^{2+}, 2.4 μM Ca^{2+} and 4.2 μM Ca^{2+}, for normal, rabbit IgG and rabbit anti-complexin I; (**B**) In capacitated sperm, the values of the EC_{50}s are 9.8 μM Ca^{2+}, 7.4 μM Ca^{2+} and 8.8 μM Ca^{2+}, for normal, IgG and anti-complexin I. For all conditions, the values have been corrected for spontaneous acrosome reaction. The values are expressed as means ± SEM (n=5)

recombinant complexin I or anti-complexin I antibody. For the recombinant complexin experiments, 0.63 μM Ca^{2+} and 1.88 μM recombinant complexin I were added to permeabilized sperm. Statistical analysis was performed by comparing the EC_{50}s of the treated population to the EC_{50}s of the normal population, with the help of ANOVA and the experiment was performed five times.

In non-capacitated sperm, the EC_{50} for both curves supplemented with 0.63 μM and 1.88 μM recombinant complexin I are 9.4 μM Ca^{2+} and 11.1 μM Ca^{2+}, respectively (Figure 17A). Compared to the control (i.e. in the absence of recombinant or antibody to complexin) EC_{50} of 3.1 μM Ca^{2+}, adding recombinant complexin I significantly shifted the EC50 to the right ($p<0.05$), which is clearly shown in Figure 17A. This suggests that upon addition of recombinant complexin I, the sperm requires more Ca^{2+} to undergo acrosome reaction and becomes less responsive to Ca^{2+}.

As for capacitated sperm, adding 0.63 μM and 1.88 μM recombinant complexin I gave an EC_{50} value of 9.4 μM Ca^{2+} and of 5.4 μM Ca^{2+} respectively. Here, the value of the EC_{50} for 0.63 μM recombinant complexin I is similar to the control (no recombinant complexin I) value of the EC50 for capacitated sperm (9.8 μM Ca^{2+}) whereas the value of the EC_{50} for 1.88 μM recombinant complexin I shifts to the left (Figure 17B). According to the statistical analysis, this shift is not significant ($p>0.05$). In the previous results shown as the effect of complexin I on the acrosome reaction of non-capacitated and capacitated sperm (Figure 15), adding more recombinant complexin I to sperm did not have an effect on the acrosome reaction percentage and the same trend is observed on the Ca^{2+} sensitivity: adding low or high amounts of complexin I, the sperm does not seem to be more or less responsive to Ca^{2+} stimulation.

Anti-complexin I antibody was also introduced in sperm to estimate how the Ca^{2+} sensitivity would change if the endogenous complexin I was sequestered or inhibited from binding to the SNARE complex. Since this antibody is an affinity purified IgG fraction, a rabbit IgG was included as a control, at the same concentrations. Normal equals to a normal condition where medium was added instead of an antibody.

In non-capacitated sperm, the Ca^{2+} titration curves supplemented with rabbit IgG and anti-complexin I gave an EC_{50} of 2.4 μM Ca^{2+} and 4.2 μM Ca^{2+}, respectively (Figure 18A). The curve of the Ca^{2+} titration supplemented with anti-complexin I clearly shows a decrease in the percentage of the acrosome reaction level of about 5 %, in accordance with my previous results. As expected, the values of the normal and the IgG EC_{50} are similar (value of normal and IgG EC_{50}s are 3.1 μM and 2.4 μM Ca^{2+}). The value of the EC_{50} of the Ca^{2+} titration supplemented with anti-complexin I is 4.2 μM Ca^{2+} and significantly shifts the EC_{50} value to the right compared to the normal EC_{50} value ($p<0.05$). This suggests that the anti-complexin I antibody lowers the sensitivity of non-capacitated sperm, therefore making the sperm less responsive to Ca^{2+} stimulation.

In the case of capacitated sperm, the EC_{50} of the Ca^{2+} titration curve of the rabbit IgG and anti-complexin I antibody are 7.4 μM Ca^{2+} and 8.8 μM Ca^{2+}, respectively, compared to the EC_{50} of the normal Ca^{2+} titration curve (9.8 μM Ca^{2+}) (Figure 18B). The values of the EC_{50} of the titrations supplemented with the rabbit IgG and the anti-complexin I antibody are not significantly different ($p>0.05$), according to ANOVA, and it suggests that the anti-complexin I antibody does not have an effect on the Ca^{2+} sensitivity of capacitated sperm. Furthermore, in accordance with my previous results, the anti-complexin I antibody did not have an effect on the percentage of the sperm having undergone AR.

PART 2: Syntaxin 2

Syntaxin 2 is one of the three SNARE proteins that form the SNARE complex, which is thought to be the basic machinery for membrane fusion [40, 45]. More specifically, it belongs to the family of t-SNARE or Q-SNARE and forms a complex with its partner SNAP-25 before binding to the v-SNARE VAMP [40, 82]. Like other proteins, syntaxin 2 activity requires regulation of its activity, especially if it participates in the process of membrane fusion where proper regulation prevents unwanted and random fusion. Epimorphin, an isoform of syntaxin 2, was shown to be cleaved in epidermal cells and to be exported in a non-classical way to become a morphogen [122]. Syntaxins were shown to be phosphorylated by serine/threonine kinases, such as casein kinase 2 [123] and dephosphorylated [133]. The uniqueness of the AR calls for a precise and efficient regulation of the proteins participating in the exocytosis of the acrosome. This could be achieved by either the intervention of a regulatory protein, such as complexin I, or regulation at the level of one of the SNARE proteins, such as syntaxin 2. Thus, complexin 1 might have a role in regulating the AR by stabilizing the SNARE complex as suggested by others. Knowing that syntaxin is the target of posttranslational modifications and that the spermatozoon presents a broad array of modifications, it can be speculated that syntaxin 2 could be modified upon capacitation to help regulate the AR.

Detection of a fast-migrating syntaxin 2 band in capacitated sperm

First, using electrophoresis, the possibility that syntaxin 2 could undergo changes associated with capacitation was assessed. To be able to detect syntaxin 2, a membrane fraction of non-capacitated and capacitated sperm was prepared by differential

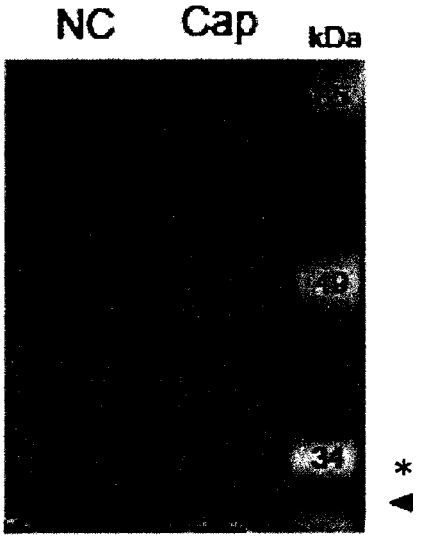


Figure 19: Detection of syntaxin 2 in non-capacitated and capacitated sperm. Lysate of non-capacitated and capacitated sperm was obtained by sonication at 30% power of the sonicator. The lysate was spun at 500 x g for 5 minutes to get rid of insoluble materials. Then the supernatant was spun at high speed (30,000 x g) for 1h and the pellets dissolved in 1X sample buffer. The intent of this procedure was to collect the membrane fraction of the spermatozoa and to detect syntaxin 2 protein in both non-capacitated and capacitated sperm. The equivalent of $20x10^6$ sperm were loaded on a 10% SDS gel. The transferred nitrocellulose blots were probed with a rabbit anti-syntaxin 2 antibody (n=8). The faster migrating syntaxin 2 is indicated by an asterisk (*) while a non-specific cross-reactive band is indicated by an arrowhead (◄).

centrifugation. This procedure was revised from Baker *et al.* (2002) [37] and the experiment was performed eight times. First, the sperm was supplemented with a protease inhibitor cocktail of leupeptin (20 μg/ml), aprotinin (20 μg/ml), PMSF (1 mM), benzamidine (200 μg/ml), was sonicated and cell debris were spun down at 500 x g for 5 minutes. Then, the supernatants were spun at 30,000 x g for 1h at 4°C and the pellets were dissolved in 1X SDS buffer. The equivalent of $20x10^6$ sperm were loaded on a 10% SDS gel. Syntaxin 2 is a transmembrane protein of 35 kDa. In this SDS gel, it migrates at approximately 38.2 kDa in the non-capacitated sperm sample. Interestingly, in capacitated sperm, the appearance of a fast-migrating band was observed, at approximately 34.4 kDa (Figure 19), as well as a band at about 37.2 kDa, compared to the non-capacitated syntaxin 2 band of 38.2 kDa. The same results were seen with sperm solubilized in 0.5% Triton X-100, but sonication was preferred to Triton X-100 solubilization because of the distortion of bands on the immunoblot caused by the Triton leftover. Furthermore, the method used in this experiment was revised from Baker *et al.* [37]. The pattern observed could be the result of posttranslational modifications, leading to the appearance of this fast-migrating band. Proteolytic cleavage and dephosphorylation could explain the pattern observed, as a similar case was observed in the context of the dephosphorylation of human methylenetetrahydrofolate (MTHF) reductase [134]. Furthermore, syntaxin 2, also known as epimorphin, was shown to be cleaved and become an extracellular morphogen, after being flipped across the plasma membrane [122].

To investigate these two possibilities, the assay *alkaline extraction* was first performed. This assay is used to determine whether a protein is an integral membrane protein, a cytosolic or peripheral plasma membrane protein. Then, the lysate of non-capacitated and capacitated sperm was treated with a general, broad range phosphatase, alkaline phosphatase, to see if the downward shift of the slow-migrating band

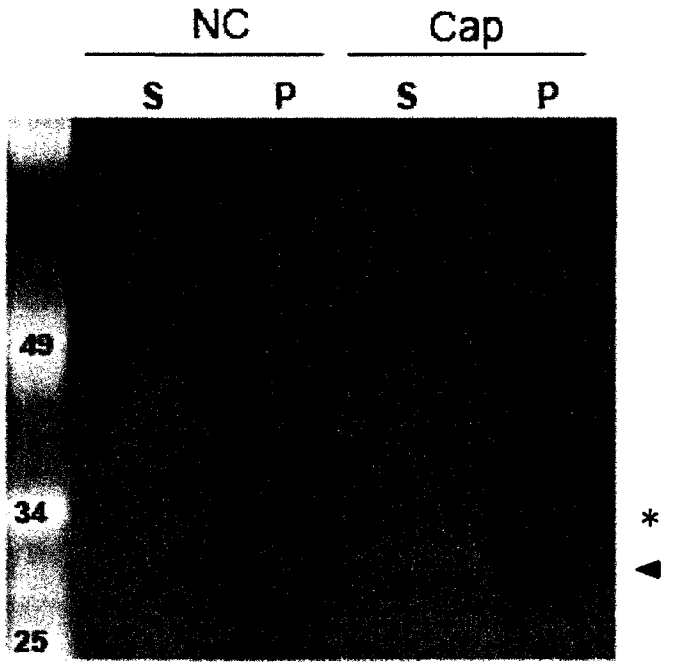


Figure 20: Alkaline extraction of non-capacitated and capacitated sperm lysate. Non-capacitated and capacitated sperm were sonicated then spun at 30,000 x g for 30 minutes to pellet down the membrane fraction. After resuspending the pellets in KRB buffer, the equivalent of $20x10^6$ sperm were treated with 0.1 M Ca^{2+} carbonate pH 11 on ice. The samples were subjected to a high speed (100,000 x g) centrifugation for 30 minutes. The supernatant (S) were TCA-precipitated and the pellet (P) dissolved in 1X sample buffer. The supernatant should contain all cytosolic and peripheral membrane proteins and the pellet, all integral membrane proteins. The equivalent of $20x10^6$ sperm were loaded on a 10% SDS gel for both the supernatant and the pellet and the transferred nitrocellulose blots were probed with a rabbit anti-syntaxin 2 antibody. The faster migrating syntaxin 2 is indicated by an asterisk (*) while a non-specific cross-reactive band is indicated by an arrowhead (◄) (n=5).

of non-capacitated sperm to the level of the fast-migrating band of capacitated sperm is due to a dephosphorylation event.

Alkaline extraction of non-capacitated and capacitated sonicated sperm lysates

The supernatant contains the cytosolic and peripheral proteins and the pellet contains the integral membrane proteins and the experiment was performed five times, at different days with different mice. Both syntaxin 2 bands were clearly detected in the pellet fractions, thus even in the fast-migrating form, syntaxin 2 seems to still be anchored in the membrane (Figure 20). This suggests that proteolytic cleavage of the cytoplasmic portion is probably not the posttranslational modification that causes the observed pattern. Since the antibody used to detect syntaxin 2 recognizes only residues 1-19, it would not be able to detect syntaxin 2 if it was to be cleaved after the residue 19. Therefore I can conclude that proteolytic cleavage is probably not the modification responsible for the observed band shift. However, this does not exclude the possibility that the cleavage could occur at the transmembrane domain and that the protein remains attached to the membrane via strong interactions, for example with SNAP-25 and VAMP.

Treatment of non-capacitated and capacitated sonicated sperm lysates with alkaline phosphatase

The other possible posttranslational modification is the dephosphorylation of syntaxin 2 during the process of capacitation. Capacitation is known to result in diverse modifications such as extensive phosphorylation (both on tyrosine and serine/threonine residues). Phosphatases have been shown to be present and to play a significant role in capacitation and the pattern observed in Figure 19 is similar to the one observed in a work published by

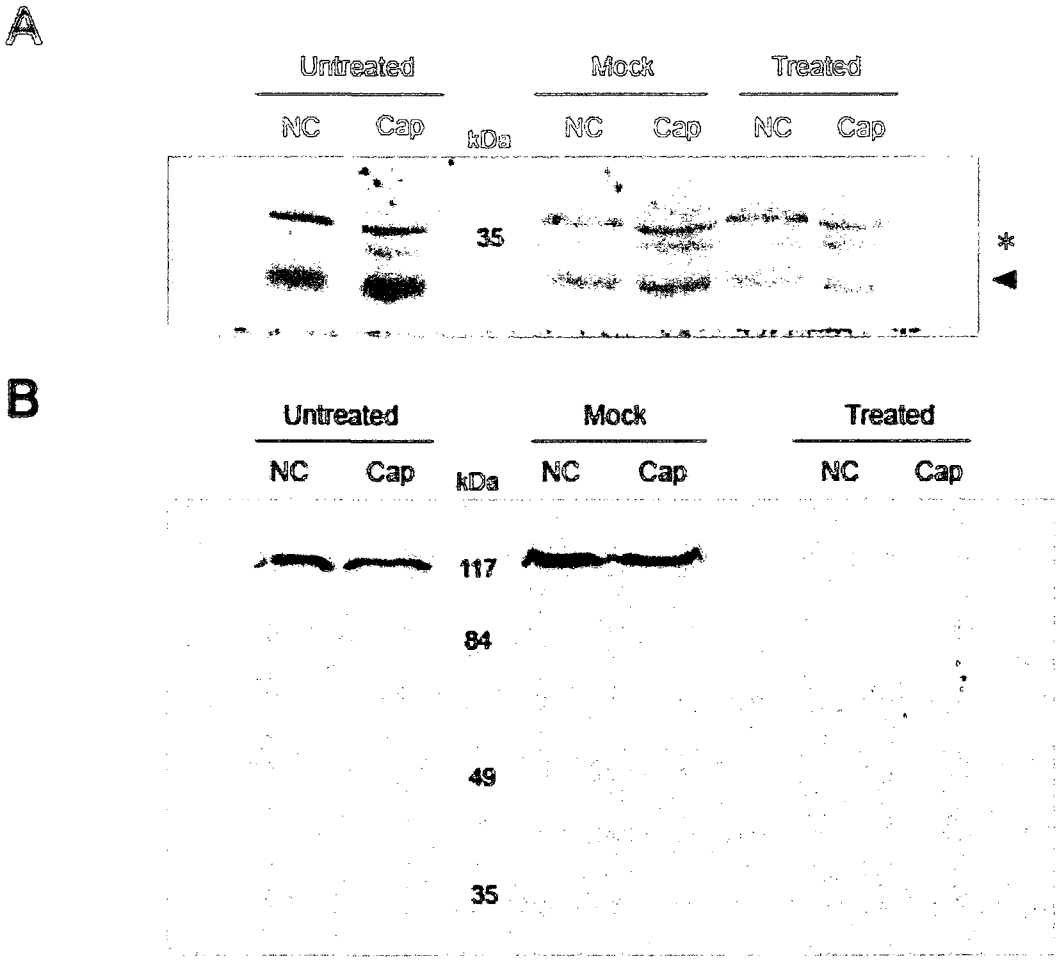


Figure 21: Treatment of non-capacitated and capacitated sperm lysate with alkaline phosphatase. (**A**) Non-capacitated and capacitated sperm were sonicated at power 30% and briefly spun at 500 x g for 5 minutes to get rid of insoluble material. The supernatant was subjected to a hard spin at 30,000 x g for 30 minutes and the pellets were resuspended in dephosphorylation buffer. After quantification with a protein assay, 100 µg of each condition were treated with 50 U of alkaline phosphatase (treated), dephosphorylation buffer (Mock) or 2X sample buffer (untreated) for 2h at 37°C. The reaction was stopped by addition of 2X sample buffer. Blots were probed with a rabbit anti-syntaxin 2 antibody, which recognizes a band around 35 kDa. (**B**) Samples were obtained in the same way as in (A) but the blot is probed with a mouse anti-phosphotyrosine100 antibody, as well, which recognizes a band around 100 kDa (n=4). The faster migrating syntaxin 2 is indicated by an asterisk (*) while a non-specific cross-reactive band is indicated by an arrowhead (◄).

Yamada *et al.*, where human methylenetetrahydrofolate reductase was found to be regulated by phosphorylation and alkaline phosphatase altered its mobility [134]. To verify if the change in band mobility is due to a phosphatase activity, sonicated samples were treated with alkaline phosphatase to assess if the non-capacitated slow-migrating band would actually shift down to the level of the faster migrating band in capacitated sperm. The blot was probed with the anti-syntaxin 2 antibody and with a mouse anti-phosphotyrosine100 antibody, as a control of the functionality of the alkaline phosphatase and the experiment was performed four times. Surprisingly, no difference was seen between the untreated, the mock and the treated samples. Alkaline phosphatase is a broad range phosphatase that can dephosphorylate tyrosine, serine and threonine residues [135]. Therefore, the fact that, even if treated, the non-capacitated band did not shift downward suggests that syntaxin 2 does not get dephosphorylated (Figure 21A). Even though in the blot showed, there seems to be a faint band in the treated non-capacitated sperm lysate, the intensity was variable amongst the repeats (n=4). Furthermore, the anti-phosphotyrosine antibody does not detect a band around 35 kDa, which is the expected size of syntaxin 2, and this implies that there is no phosphorylated tyrosine in syntaxin 2 of non-capacitated sperm. This is not surprising knowing that kinases known to phosphorylate syntaxins are serine/threonine kinases. Hence one could think that the phosphatase that would dephosphorylate syntaxin would be a serine/threonine phosphatase. The alkaline phosphatase was shown to be operational, as proven by the blot reprobed with anti-phosphotyrosine 100: the band detected by this antibody nearly disappears in the treated sample (Figure 21B). However, when the blot is probed with anti-syntaxin 2, treating the sperm lysates with alkaline phosphatase does not dephosphorylate the non-capacitated syntaxin 2. In the end, dephosphorylation may not be the modification responsible for the shift downward of the syntaxin 2 band or the

phosphorylated residues were not accessible to the enzyme at the time of treatment. One could have thought that there was some endogenous phosphatase inhibitor in sperm, but the fact that the control with phosphotyrosine antibody clearly shows that alkaline phosphatase is functional, reveals that this is likely not to be the case, even though, the binding of chaperone is not to be excluded. Overall, the post-translational modification of the 38 kDa remains unknown.

DISCUSSION

PART 1: complexin I

Regulated exocytosis is dependent on the SNARE complex which is formed by the three SNARE proteins: syntaxin, SNAP-25 and VAMP, and by regulatory components that modulate the activity of the SNARE proteins [40, 41]. The acrosome reaction is a specialized form of exocytosis in sperm, necessary for the fertilization of the egg. Exocytosis of the acrosome results in the fusion of the PM with the OAM at multiple fusion points, forming fenestrae that result in little vesicles and the loss of PM and OAM and finally the release of the acrosomal content. The SNARE proteins along with their regulatory components, such as complexin, were shown to be present in sperm and to be involved in the acrosome reaction [86, 90].

Complexin I is present in sperm, in a membrane-bound and a cytosolic form

In the first part of my project, the presence of complexin I in the sperm was confirmed in both non-capacitated and capacitated sperm. It has been shown that complexin I is present in sperm, in the acrosomal region [104, 136], but there is very little information on the localization of complexin I before and after capacitation. As expected, my results have shown that complexin I is found in the cytosolic fraction, as well as in the membrane fraction of both non-capacitated and capacitated sperm. This implies that complexin I is stably bound to a set of membrane proteins. Given that complexin I binds only assembled SNARE complexes and not monomeric SNARE proteins [94, 104], the logical binding candidates for complexin I are the SNARE proteins assembled in the SNARE complex.

The latter can help us determine the status of the SNARE proteins in non-capacitated and capacitated sperm: the SNARE proteins are assembled into a SNARE complex in non-capacitated as well as in capacitated sperm. It has been speculated that the SNARE proteins can form partially assembled *trans* (Q-SNARE and R-SNARE residing on opposite membranes) complexes, which are sensitive to the light chains of botulinum neurotoxin B, BoNT/B, but not to the light chains of tetanus toxin, TeTx [108]. Furthermore, according to Liu *et al.* (2006), it seems that complexin I can bind to the SNARE complex in different assembled states [101] but with different affinity. One possible hypothesis is that the SNARE proteins are assembling into the SNARE complex during capacitation, which would explain why complexin I is available to the antibody in the cytosolic and the membrane fraction in non-capacitated sperm. Sperm must undergo capacitation, a process needed for the acquisition of fertilizing abilities, where sperm are prepared for the encounter with the egg. Therefore a fraction of the SNARE proteins seems to be assembled into a complex in non-capacitated sperm, most likely into a partially assembled complex. In capacitated sperm, complexin I seems to be unavailable to the rabbit anti-complexin I antibody in both the cytosolic and the membrane fractions, most probably bound to the assembled SNARE complex. This was expected, since capacitation prepares the sperm for acrosome reaction.

The calcium sensitivity of non-capacitated and capacitated sperm

After permeabilizing sperm with SLO, I used this assay to determine the Ca^{2+} requirements for the AR in non-capacitated and capacitated sperm. The estimated values of the EC_{50} were 3.1 μM Ca^{2+} and 9.8 μM Ca^{2+}, for non-capacitated and capacitated sperm respectively. Darren Hutt *et al.* (2004) estimated a different EC_{50} value of about 87 μM Ca^{2+} for capacitated sperm [137]. The approximate 10 fold difference with my estimated EC_{50}

could be due to different methodological techniques or standard, as there seems to be a discrepancy amongst the different attempts of different groups to predict the calcium requirements of the AR. The EC_{50} value estimated in this project seems to be closer to physiological concentration found in different systems relying on secretory pathways, as astrocytes [137] or even sperm, where the Ca^{2+} level before and after AR were approximately 0.16 μM and 0.40 μM Ca^{2+}, by fura-2 detection [138]. Other studies have estimated the EC_{50} of capacitated sperm to be 20 μM Ca^{2+} [139]. The difference between the calcium requirements of sperm upon capacitation reflects also that there seems to be no standard value for the Ca^{2+} requirements, on top of the technical variability. One can notice that capacitated sperm have an EC_{50} three fold higher that of non-capacitated sperm which seems to indicate that non-capacitated sperm do have the necessary machinery to respond to Ca^{2+} stimulation but are not mature to adequately respond to the right Ca^{2+} concentration range. However, it is possible that a fraction of the non-capacitated sperm used were undergoing the beginning of the capacitation process, so that this population may respond to calcium stimulation in a similar way than capacitated sperm. This early activation of sperm could explain the lower Ca^{2+} requirements for the stimulated non-capacitated sperm. A way to verify this venue could be to collect sperm destined for non-capacitation in an absolute non-capacitating medium such as PBS or KRB without Ca^{2+} or bicarbonate, or to subject the non-capacitated samples to immunofluorescence for tyrosine phosphorylation, or to score the level of hyperactivated motility. Capacitation not only reorganizes the plasma membrane phospholipids and proteins, but it prepares the sperm to undergo the acrosome reaction in a precise and timely manner and increases the number of fusion-competent and Ca^{2+} sensitive SNARE complex. The putative Ca^{2+} sensor, synaptotagmin, has been shown to play a crucial role in the completion of the acrosome reaction. Two isoforms, synaptotagmin VI and

synaptotagmin VIII, have been suggested to be present in sperm and to be implicated in the Ca^{2+} detection of the AR [92]. In the small population of non-capacitated sperm that can acrosome react, the SNARE complex might be adequately assembled, but the Ca^{2+} sensor, either synaptotagmin VI or synaptotagmin VIII, might not be optimally prepared, in that it would lack regulation of its activity. The regulation and readiness of the Ca^{2+} sensor are crucial for the timing of the acrosome reaction in capacitated sperm. The Ca^{2+} sensor system being less regulated, it could react prematurely and to lesser Ca^{2+} concentrations. This could explain why the population of non-capacitated sperm detected as having acrosome react has lower Ca^{2+} sensitivity than capacitated sperm. It is possible that upon capacitation, the two isoforms form a combinatorial association for a more precise Ca^{2+} detection and for a restrictive action of synaptotagmin on the SNARE complex.

Recombinant complexin I and anti-complexin I have different effects on non-capacitated and capacitated sperm

Capacitation is also the host of plasma membrane dynamics, reorganizing phospholipids and microdomains in the plasma membrane [23, 140]. These domains have been speculated to contain proteins involved in neuronal secretion, such as the SNARE proteins [47]. Capacitation could place the SNARE proteins in an optimal environment where they could form a more stable SNARE complex, in order for complexin I to bind and lock the SNARE complex into a fusion-competent intermediate. The fact that complexin I could still be available to the antibody in non-capacitated sperm suggests that it might not be bound to a fully assembled SNARE complex. Actually, it has been suggested that complexin I can bind to partially assembled SNARE complex, but in a less stable manner than fully assembled SNARE complex [101]. This is correlated by the differential centrifugation

experiment as well as by the SLO assays where increasing amounts of recombinant complexin I and anti-complexin I antibodies were introduced in permeabilized sperm. The introduction of recombinant complexin I in non-capacitated sperm did not give a significant increase according to ANOVA statistical analysis, but this does not imply that complexin I does not have an effect on the acrosome reaction, as shown by Roggero *et al.* with the use of recombinant peptides [108] and Zhao *et al.* with the use of KO mice [104]. The apparent lack of response upon introduction of recombinant complexin I could be due to the recombinant protein not properly folded or not active. To verify these issues, the recombinant protein could have been tested in one of the many conventional system available to evaluate membrane fusion, such as liposomes or the recombinant protein could be boiled or pre-incubated with the rabbit anti-complexin I antibody and introduced in SLO-permeabilized sperm. The treated sperm would then be stained and scored for acrosome-reacted spermatozoa. Or a blank pQE9 vector could be transformed into *E.Coli* and the resulting protein preparation could be purified with Ni-NTA beads to assess whether anything other than recombinant complexin I that sticks to the column could alter the recombinant protein activity. Another way to assess the lack of response would be to try different vectors, such as PGEX, as used in the study of Roggero *et al.* (2005) [108] or to make a construct with a phosphomimetic mutation (mutation to an amino acid that resembles the original residue but in a phosphorylated form) and observe its effect on permeabilized sperm. It has been stated that in the brain, complexin I has a preferential binding affinity to the SNARE complex when it becomes phosphorylated, probably by CKII [141]. This scenario could also occur in sperm. However, neither recombinant complexin I nor anti-complexin I antibody had an effect on acrosome reaction in capacitated sperm. According to the relevant litterature [104, 108], the results presented above correlate with the findings of Roggero *et al.* and Zhao *et al.* and

suggest that in non-capacitated sperm, complexin I might still have a regulatory potential on the SNARE proteins and is largely accessible to the antibody, whereas in capacitated sperm it might be likely pre-bound to assembled SNARE complexes. One may think that because the sperm was stimulated at saturating Ca^{2+} concentration (1 mM), it could bypass the need for complexin I. However, because there is a decrease in the responsiveness of non-capacitated sperm after introducing anti-complexin I, it clearly shows that there is a need for complexin I in the process of acrosome reaction. That being said, the results seem to be suggesting that in non-capacitated sperm, complexin I recognizes and binds to partially assembled and loose SNARE complex, as it is still available to the rabbit anti-complexin I antibody. This could explain why complexin I is associated with the membrane fraction in non-capacitated sperm and why the antibody could effectively decrease the acrosome reaction level. However, in capacitated sperm, complexin I is mostly bound to SNARE complexes, as it is not available to the rabbit anti-complexin I antibody. Roggero *et al.* [108] suggested that adding anti-complexin I at an early stage after ejaculation of human sperm blocks the acrosome reaction but, at a later stage it had no effect [108]. It was concluded that complexin I seems to have an activating role up until the SNARE complex reaches this BoN/T sensitive state, probably the loose state, at which point, its inhibitory role takes over and keeps the SNARE complex into a metastable state up until the Ca^{2+} influx is triggered [108]. This early stage is comparable to the non-capacitated state of the sperm used in the above procedures. Upon capacitation, the SNARE proteins could form a stable yet loose complex, to which complexin I binds to. This binding is thought to stabilize the complexin-bound complex until the fusogenic signal is triggered, as suggested by others [108]. Increasing amounts of anti-synaptotagmin VIII, a putative Ca^{2+} sensor required for acrosome exocytosis, were also introduced in permeabilized sperm to verify their effect on non-

capacitated sperm and to determine where its site of action is compared to complexin I. Introduction of recombinant synaptotagmin VIII peptide (cytoplasmic portion) or antibodies has been shown to prevent the acrosome reaction of capacitated sperm [92, 93]. The results presented in this project show that the rabbit anti-synaptotagmin VIII antibody also decreased the acrosome reaction level in non-capacitated sperm. Similarly to my anti-complexin I results, it seems that, after capacitation, synaptotagmin VIII might act downstream of complexin I, since anti-synaptotagmin VIII inhibited acrosome reaction in capacitated sperm whereas anti-complexin I did not. Therefore, the results correlate with the recent finding that synaptotagmin and complexin act together to drive membrane fusion in fast synchronous release, where complexin bound to the SNARE complex forms a complex recognized by synaptotagmin, which then binds to the SNARE complex when calcium ions bind to its C2 domains [103, 108]. Synaptotagmin VIII dislodges complexin from the complex and at the same time relieves it from its "clamping" action [95, 99, 102, 103, 106, 108]. This has been shown in neurons [103], as well as sperm [108].

Complexin I has an effect on the calcium sensitivity of non-capacitated and capacitated sperm

Acrosomal exocytosis and neuronal exocytosis share common similarities in that they are both dependent on the proteins known to govern membrane fusion, the SNARE proteins, and they are both stimulated by a rise in the intracellular Ca^{2+}. It is well known that fusion driven by the SNARE core complex is dependent upon Ca^{2+} stimulation, at least in fast synchronous release [40, 72, 82, 142]. It has been shown that synaptotagmin's action is dependent on the binding of Ca^{2+} ions on its C2 domains and that synaptotagmin is thought to be the Ca^{2+} sensor of the SNARE-driven membrane fusion [92, 93, 143]. Furthermore,

introduction of recombinant synaptotagmin VIII or anti-synaptotagmin VIII blocked the acrosome reaction by considerably decreasing the percentage of acrosome-reacted spermatozoa [92] (Figure 16). Hence, knowing that the SNARE-mediated fusion is calcium dependent and that complexin I seems to play a regulatory role on the SNARE complex, one can ask what the effect of complexin I on the Ca^{2+} sensitivity of the SNARE complex is in the context of the acrosome exocytosis.

Recombinant complexin I has a differential effect on the calcium sensitivity of acrosomal exocytosis in capacitated and non-capacitated sperm

After determining that calcium is required for the AR of non-capacitated and capacitated sperm, the effect of complexin I on the Ca^{2+} sensitivity of non-capacitated and capacitated sperm was assessed. Although complexin I does not bind to the SNARE complex in a calcium-dependent manner, it seems to be involved in the regulation of AR which is Ca^{2+}-dependent, with the help of regulatory Ca^{2+}-sensitive proteins, such as the synaptotagmin family [105, 108]. In non-capacitated sperm, both low and high doses of recombinant complexin I decreased the Ca^{2+} sensitivity of the acrosome reaction. In this case, the additional complexin I could recruit and stabilize a higher number of loose SNARE complexes, therefore, higher amount of Ca^{2+} would be needed to activate synaptotagmin in order to displace the complexin I clamp in these "premature" sperm. In the Ca^{2+} titration curve, the level of acrosome reaction did not significantly increase upon addition of complexin I (Figure 17). However, it seems that recombinant complexin I does have an effect on the level of acrosome reaction at sub-saturating Ca^{2+} concentrations, as the percentage of spermatozoa that acrosome reacted is lower than the normal curve, where no recombinant protein is added. The decrease in acrosome reaction could be the result of

complexin I not being dislodged by synaptotagmin. If the Ca^{2+} concentrations are suboptimal, chances are that synaptotagmin could be partially or not activated, leaving the SNARE complex clamped by complexin I. Therefore, addition of higher amount of complexin I would reinforce the clamping mechanism on the SNARE complex and prevent partially-activated synaptotagmin from relieving the SNARE complex from complexin I clamp. This clamping mechanism has been shown and discussed by several groups [105], thus reinforcing the idea that the SNARE complex is under a tight regulation from complexin I but as well from synaptotagmin [95, 102]. Conversely, complexin I does not seem to have an effect on the Ca^{2+}sensitivity in capacitated sperm. The fact that capacitated sperm does not respond to lower or higher amount of complexin I suggest that complexin I could not activate more SNARE complex into a "substrate" recognizable by synaptotagmin, to complete the fusion event. Recombinant complexin I does not have an effect on the percentage of AR of capacitated sperm either, as observed in Figure 15. Therefore, the results discussed above reinforce the idea that complexin I actively participate in the acquisition and the regulation of Ca^{2+} sensitivity of non-capacitated sperm, as suggested by Roggero *et al.* and Zhao *et al.* [108]. It does so by forming a substrate with the SNARE complex that is recognizable by synaptotagmin during capacitation.

Anti-complexin I shows a differential effect on the calcium sensitivity of acrosomal exocytosis in non-capacitated and capacitated sperm

Using the same SLO assay, anti-complexin I antibodies were introduced at different dilutions in permeabilized non-capacitated and capacitated sperm to determine the effect of blocking endogenous complexin I on the AR. This will give an indication if the results seen previously in the Ca^{2+} titration experiments supplemented with recombinant complexin I can

be correlated. In non-capacitated sperm, anti-complexin I lowered the percentage of acrosome reacted sperm, as expected, and it also lowered the Ca^{2+} sensitivity. This loss of sensitivity suggests that preventing complexin I from stabilizing and recruiting competent SNARE complex lead to a lack of stabilization of those SNARE complexes and to a lack of complexes recognizable to synaptotagmin. Therefore, if synaptotagmin cannot bind to the SNARE complex and to the membrane, the SNARE complex cannot proceed to the fusion event. The fact that anti-complexin I can affect both the AR and the Ca^{2+} sensitivity confirms again the idea that, in non-capacitated sperm, complexin I could be present in an unbound form, available to the antibody. In capacitated sperm, anti-complexin I did not have an effect on the Ca^{2+} sensitivity, as revealed by the value of the EC_{50} of the control done with the rabbit IgG, which is consistent with my previous results, suggesting that complexin I might not be accessible to the antibody because it is sequestered in a complex of proteins. This could explain why adding more complexin I or anti-complexin I does not have an effect on the Ca^{2+} sensitivity, since endogenous complexin I is already bound to SNARE complex.

Complexin I seems to regulate the acrosome reaction in two steps: by activating and "clamping" the SNARE complex. It activates by recruiting properly assembled SNARE complex [95, 99] and this seems to be correlated by the results of the experiments in non-capacitated sperm. Then, complexin I locks the selected SNARE complex in place, probably after capacitation occurs, and keeps it in a fusion-competent state, until the Ca^{2+} signal is triggered and detected by synaptotagmin C2A domains. Synaptotagmin would then relieve the SNARE complex from the complexin I clamp and complete membrane fusion by bringing the two membranes in close apposition until fusion occurs. This could have been shown by introducing recombinant complexin I in permeabilized capacitated sperm and immunoblotting of the sperm lysate with a anti-complexin I antibody or by pull-down assay,

where the recombinant complexin I could be used as a bait in a column to determine if the SNARE complex could be pulled down.

PART 2: syntaxin 2

In the second part of my project, the possibility of a regulation of the SNARE complex at the level of one of the SNARE protein was explored. Syntaxin 2 was the chosen candidate, due to its presence in sperm [38]. Syntaxin 2 is one of the t-SNARE or Q-SNARE and resides on the target membrane. It forms a tight complex with SNAP-25, the other t-SNARE, and VAMP, the v-SNARE or R-SNARE. Syntaxin 2 has been shown to be the target of diverse posttranslational modifications, such as proteolytic cleavage [122], or phosphorylation/dephosphorylation events [123]. Posttranslational modifications are at the core of protein enhancement or inhibitory activity and of regulation of many pathways. Since the acrosome reaction is a single event, regulation at the level of one of the SNARE proteins would add an extra level of regulation to ensure that the acrosome exocytosis is well orchestrated.

Syntaxin 2 is modified upon capacitation

After obtaining membrane fractions via differential centrifugation of sonicated non-capacitated and capacitated sperm, the membrane fractions were run on a SDS gel and the nitrocellulose blot probed with an anti-syntaxin 2 antibody. Interestingly, the syntaxin 2 band in the capacitated sperm sample migrated faster than the one in non-capacitated sperm, exhibiting two bands, the lower one with greater intensity. The same pattern was also observed in samples solubilized in 0.5% Triton X-100. After estimating the molecular weight of the non-capacitated syntaxin 2 band (38.2 kDa) and the newly appeared fast-migrating

capacitated band (34.4 kDa) by measuring the distance of migration on the SDS gel, I evaluated the shift to be approximately 4 kDa. Such a small shift could be the consequence of few posttranslational modifications. The hallmark of this finding is the appearance of the fast-migrating band, detected by the rabbit anti-syntaxin 2 in the capacitated condition. The same pattern is observed between Figure 19, 20 and 21, as the fast-migrating band still appears in the capacitated sample, even though there is variability in the band intensity of syntaxin 2 band detected in the non-capacitated sample and the fast-migrating band in the capacitated sample. The main focus was now on the appearance of the fast-migrating band in the capacitated band and the consistency and the reproductibility of this appearance. Although the intensity of the band was variable, the appearance of the fast-migrating band in capacitated sample was consistent. The two modifications that came forward are proteolytic cleavage and phosphorylation/dephosphorylation events [122, 134], as both of these modifications have been observed and documented in syntaxin 2. Modified syntaxin 2 could enhance the formation of the SNARE complex therefore preparing the acrosome reaction more efficiently, or it could commit syntaxin 2 to the SNARE complex assembly and reaction in a more permanent manner.

Syntaxin 2 band shift after capacitation is not due to proteolytic cleavage

Proteolytic cleavage has been observed in the context of epimorphin, also termed syntaxin 2. Epimorphin is complexed with synaptotagmin and annexin and once the Ca^{2+} influx is triggered, it is detected by synaptotagmin and the complex is flipped from the intracellular to the extracellular space, by phosphatidylserine externalization [121, 122]. Epimorphin is then cleaved at the SNARE domain to become cytosolic, and exported to the extracellular space to act as a morphogen in epithelial cells [121, 122]. To verify if a similar

scenario occurred in the acrosome reaction, the samples were subjected to an alkaline extraction. If syntaxin 2 was to be cleaved in sperm, the enzyme would have to be present in the cytoplasm or in an membrane-associated form, either intracellularly or extracellularly, and cleave the cytoplasmic part of syntaxin 2 (the SNARE domain, the N-terminal domain, composed of the three helices Ha, Hb and Hc, or the linker region) or the C-terminal part of syntaxin 2. Syntaxin 2 was detected in the membrane fraction in both non-capacitated and capacitated sperm, which suggests that before and after capacitation, syntaxin 2 is still associated with the plasma membrane. Furthermore, the anti-syntaxin 2 antibody used only detects the residues 1 to 19, which corresponds to the very beginning of the N-terminal domain, just before the first helix Ha. To be detected in the membrane fraction by the anti-syntaxin 2 antibody, syntaxin 2 needs to be associated with the plasma membrane and to have an intact N-terminal Habc domain, as a loss of the N-terminal domain would result in the loss of the epitope recognized by the anti-syntaxin 2 antibody used. Therefore, cleavage of the protein would result in the loss of immunodetection of the anti-syntaxin 2 antibody in the membrane fraction. It can be concluded that syntaxin 2 is most likely not cleaved in the context of the regulation of the acrosome exocytosis. However, it is possible that, after capacitation, syntaxin 2 becomes more accessible to potential proteases and is cleaved just above the transmembrane domain so that it remains associated with the plasma membrane via strong interactions with the other SNARE proteins, SNAP-25 and VAMP [82], and is detected in the membrane fraction. With the exception of the non-classical export of epimorphin and its role in epithelial morphogenesis [122], there are no known cases of proteolytic cleavage of syntaxin 2 in other context. Therefore, even if it would render the fate of syntaxin 2 permanent in the SNARE complex, syntaxin 2 does not appear to be cleaved in the AR. It is to mention that a positive control could have been done to verify the good

extraction of a peripheral plasma membrane protein, such as the arylsulfatase A or another plasma membrane protein.

Syntaxin 2 is not dephosphorylated after capacitation

The second possibility is the dephosphorylation of syntaxin 2 upon capacitation. It has been shown that secretion depends on the phosphorylation and dephosphorylation of tyrosine and serine/threonine residues of protein directly or indirectly involved in secretion [25]. Sperm has been shown to be the host of extensive protein tyrosine, serine/threonine kinase and phosphatase activity, especially during the capacitation event [16, 17, 20, 25, 26, 29, 144]. In fact, phosphorylation is a hallmark of capacitation, where it is thought to be in part responsible for the hyperactive motility pattern of capacitated sperm and the activation of the cAMP pathways [4, 6, 16, 145]. Protein kinases and phosphatases have been implicated in the modulation of T-type Ca^{2+} channels, which are induced in signalling cascades leading to the acrosome reaction. Syntaxins have been implicated in the binding and regulation of VGCC in the brain, suggesting a role in the Ca^{2+} influx in brain or sperm [73, 124]. The syntaxin family is mostly phosphorylated in the N-terminal region, in the first 50 residues by different serine/threonine kinases [123]. Using recombinant proteins, Risinger *et al.* suggested that syntaxin 2 is phosphorylated by casein kinase II (CKII) and by calmodulin-dependent kinase (CAMK) or PKC, but at low levels [123]. Foletti *et al.* showed that phosphorylation of syntaxin 1 by CKII increases its association with SNAP-25, forming preferentially binary t-SNARE syntaxin/SNAP-25 complexes in neurons [124]. Since the phosphorylation of syntaxins seems to occur in the N-terminal part of the protein, this suggests that this phosphorylation event has a minimal impact on SNARE complex assembly and may not be involved in the fusion process itself, but more in the regulation of

interactions with SNAP-25, VGCC or synaptotagmin [74]. This being said, dephosphorylation seems to be implicated in the SNARE complex assembly. Marash *et al.* suggested that, in yeast, phosphorylation of t-SNARE proteins decreases their affinity for their other SNARE binding partner whereas dephosphorylation promotes it, so that t-SNARE proteins dephosphorylation promotes the SNARE complex assembly and exocytosis in yeast [133]. Since it has been demonstrated that the yeast SNARE complex shows high similarity to mammalian SNARE complex, this dephosphorylation event could be applicable to the sperm SNARE complex [61]. In fact, the shift in molecular weight observed between non-capacitated and capacitated sperm could be the consequence of a phosphatase. It resembles the pattern observed in the regulation of human MTHF reductase, where it was shown that dephosphorylation of the MTHF reductase with an alkaline phosphatase resulted in an alteration of the electrophoretic mobility of the MTHF reductase band, as shown by the appearance of a faster-migrating band in the treated sample [134]. In addition, if this dephosphorylation event results from the action of a sperm specific phosphatase, it is probably not a protein tyrosine phosphatase, based on the anti-phosphotyrosine100 antibody immunoreactivity, which clearly shows no band in the alkaline phosphatase treated samples. It is more likely to be a serine/threonine phosphatase, which would act on syntaxins phosphorylated by serine/threonine kinases, such as CKII or CAMK [123, 124]. However, the band patterns observed between non-capacitated and capacitated sperm do not seem to be due to a dephosphorylation event. No difference was observed between the untreated, the mock and the treated samples. This could either be because the modification of syntaxin 2 shown is not a dephosphorylation event, or because the dephosphorylation site was not accessible or was not specific to the enzyme used, the latter is highly improbable knowing that the alkaline phosphatase is a broad tyrosine and serine/threonine phosphatase [135].

Palmer *et al.* [125] stated that syntaxin 1 could be nitrosylated on a unique cysteine residue and that this post translational modification could act as a controlling switch on the binding to Munc18-1 [125], therefore regulating the SNARE complex assembly and fusion machinery. It would seem that nitric oxide regulates the syntaxin-Munc18 interaction by a local rearrangement of the syntaxin linker and H3 regions. Nitric oxide-induced protein S-nitrosylation has emerged as an important post-translational modification affecting exocytosis in a variety of cell types, including neurons [125] and sperm [125]. But the fact that this nitrosylation is a covalent addition of nitric oxide does not fit with the results: from non-capacitated to capacitated sperm, syntaxin 2 molecular weight decreases, thereby the nitric oxide group would have to be removed. Furthermore, the behavior of nitrosylated proteins in SDS gel is not predictable beforehand, since SDS also depends on the net charge. For example, to analyse nitrosylated proteins, proteins have to be subjected to a biotin switch technique, and it is those newly biotinylated proteins that are run on an SDS gel [125]. Therefore, even though S-nitrosylation has been shown to be present in sperm [125], it is highly improbable that it is the posttranslational modification responsible for the syntaxin 2 modification observed. Aside from the folding back of its N-terminal three helix Ha, Hb, Hc bundle domain, syntaxin 2 has not been found to be the target of another conformational change. Nevertheless, it is not excluded, since a conformational change could make the capacitated band migrate faster in the SDS gel.

Syntaxin 2 could also be present in multiple conformations, each conformation being favored at certain points during the capacitation process. It has been suggested that the two isoforms of synaptotagmin found in sperm, synaptotagmin VIII and VI, might have a combinatorial role in assessing the Ca^{2+} signal with precision. Therefore, it is possible that

syntaxin 2 is present in different variants forms or one with two specific conformations in non-capacitated and capacitated sperm, to modulate the SNARE complex assembly.

Other possibility: presence of a preferential syntaxin variant in non-capacitated and capacitated sperm

After capacitation, sperm exhibit a syntaxin 2 isoform different from the one found in non-capacitated sperm: it has a smaller molecular weight and two bands are detected, with the lower one having greater intensity. This change in the molecular weight is not likely due to cleavage or dephosphorylation, although a cleavage at the C-terminal domain is not to be excluded. Another possibility could be that syntaxin 2 exists in two forms, with one being preferentially used after capacitation or it might be the target of another posttranslational modification. Interestingly, Quinones *et al.* [125] determined that syntaxin 2 exhibited different splicing variants, syntaxin 2A, 2B, 2C and 2D, with different expression patterns and biochemical properties [125]. According to his research, all the variants of syntaxin 2 have identical N-terminal cytoplasmic domain but they differ in the C-terminal region structural difference is the presence (syntaxin 2A and 2B) or the absence (syntaxin 2C and 2D) of a hydrophobic domain that serves as a membrane anchor. Furthermore, he showed that the syntaxin 2 variants expressed in the testis were syntaxin 2A, 2B and 2C [125]. One idea is that these variants might be expressed in sperm, especially the integral membrane syntaxin 2A and 2B, and might participate in the acrosomal exocytosis. But what is more appealing is the fact that syntaxin 2B seems to migrate as 2 bands in an SDS gel. The difference between the two bands is about 4 kDa and resembles the pattern observed in my results. Additionally, syntaxin 2A exhibits a band of similar molecular weight than the fast-migrating band of syntaxin 2B [125]. The only difference between syntaxin 2A and 2B

resides in the sequence of their transmembrane domain and even if no difference was seen in the ability to bind to SNAP-25 and VAMP2, and in the localization in non-polarized cells, a difference was noticed when the two variants were expressed in polarized epithelial cell lines, where syntaxin 2A was present on the apical plasma membrane whereas syntaxin 2B was present on both the apical and the basolateral membranes. Since sperm are polarized cell, one has to keep in mind that the function of transmembrane domains is not only to anchor proteins in the selected membrane, but also to help the protein targeting in the membrane. For example, the transmembrane domain of influenza hemagglutinin is important for raft domain association and the transmembrane domain of the influenza virus neuramidase promotes its incorporation into detergent-insoluble lipid microdomains. Sperm has been suggested to possess those detergent-insoluble microdomains [30] and these microdomains or lipid rafts contain synaptic proteins [47], such as syntaxin [32]. Since capacitation is known to change the membrane phospholipids composition [24, 31], to alter lipid rafts composition and stability, and to be the host of multiple posttranslational modifications, there are two possibilities regarding the hypothetical presence of two isoforms of syntaxin 2 in sperm. First, sperm lacks functional apparatus for DNA transcription or protein expression, therefore the two variants syntaxin 2A and 2B would have to both be present on the sperm membrane in non-capacitated and capacitated sperm. Upon capacitation and the reorganization of the microdomains, one variant is preferred to the other to commit to the SNARE complex assembly and function, which could explain the difference in the mobility of the syntaxin 2 bands after capacitation. In capacitated sperm, a faster-migrating band appeared and was detected by the rabbit anti-syntaxin 2 antibody. It is reasonable to think that upon capacitation, the syntaxin 2 variant that might be preferred would be syntaxin 2B, since syntaxin 2B is detected as two bands. The antibody used in the study conducted by

Quinones *et al.* (1999) was a rabbit polyclonal antiserum that could recognize all variants. Before capacitation, the presence of a syntaxin 2 variant that cannot commit to the completion of the fusion of PM and OAM via the SNARE complex, ensures that the fusion machinery does not fully assemble and stabilize before due time. But, since they are similar in their protein sequence, this "non-capacitated" variant would still be able to loosely assemble to its SNARE partners, without completing the formation of the SNARE complex. After the reorganization of the plasma membrane during capacitation, the "capacitated" syntaxin 2 variant would be preferred to form the final SNARE complexes with SNAP-25 and VAMP. Since the successful completion of fusion with SNARE complexes depends as well on the interaction of the transmembrane domains, it is possible that the difference in the transmembrane domain composition could be the reason why one variant would be favored in the assembly and functionality of the SNARE complex. The other possibility is that only one variant is present and participate in the fusion between the PM and the OAM, by adopting a "non-capacitated" and a "capacitated" conformation. The most plausible variant according to the band mobility pattern observed in my results and in the study of Quinones *et al.* (1999) [125] is syntaxin 2B, which is a close match to what was observed in capacitated sperm immunoblotting results. Before capacitation, syntaxin 2B would be present under a conformation or form, which would not be suitable for the proper SNARE complex assembly and stabilization and could be detected as a single band. Therefore, it could bind to SNAP-25 and VAMP, but only in a loose manner. Consequently, the SNARE complex formed with this "non-capacitated" syntaxin 2B would not be stable and would assemble and disassemble alternatively. Upon capacitation, the syntaxin 2B variant could be the target of a posttranslational modification or a conformational change, as revealed by the detection of two bands in the capacitated sample, the fast-migrating band being the result of this

modification. This would result in a more favored and definitive binding to VAMP and SNAP-25, leading to the formation of the final SNARE complexes, which would then be stabilized and activated by complexin I. As for the sperm lacking the transcription and translation apparatus, the second possibility could be the most plausible one, as it does not involve a switch between two variants of syntaxin 2 and it involves posttranslational modifications, known to be significantly present and useful in sperm, as sperm cannot synthesize proteins along its journey to the egg. But the presence of two different variants is not to be excluded. A way to verify the presence of one or two variants of syntaxin 2 could be to lyze sperm samples and run the resulting lysate on a SDS-PAGE gel and perform immunoblotting.

CONCLUSION

Sperm acrosome reaction is a specialized secretory event, resulting in the fusion of the PM and the OAM and the release of the acrosome content. SNARE proteins have been implicated in the fusion process and the SNARE complex, formed by the three SNARE proteins via their SNARE motif, is at the core of this fusion event. Acrosome reaction is a single and unique event that needs tight regulation of the assembly and activation of the SNARE complex. Here, the regulation and stabilization of the SNARE complex is proposed to be achieved via the action of complexin I and modification of syntaxin 2. In non-capacitated sperm, the SNARE proteins are present but not in an optimal environment to form stable complexes that can be activated by complexin I. Instead, they form loose and partially assembled complexes that are still recognized by complexin I but not enough to activate them into fusion-ready metastable complexes. In addition, non-capacitated syntaxin 2, which is probably not the variant or in the conformation needed for optimal SNARE assembly, needs to be in another conformation to appropriately participate in the AR. Upon capacitation, there is reorganization of the plasma membrane as well as acquisition of fertilizing abilities of the sperm. Synaptic proteins are rearranged to optimize their field of action and the SNARE proteins can form a more stable SNARE complex. Therefore, complexin I can bind to and lock the complex into a fusion competent state. Furthermore, with the reorganization of the membrane, along with the microdomains, capacitated syntaxin 2 would be in the proper conformation to efficiently promote the assembly of the SNARE complex and to add another level of stabilization. Once Ca^{2+} is released from the Ca^{2+} stores, the putative Ca^{2+} sensor, synaptotagmin VIII or VI, will detect the signaling ion and undergo conformational changes to bind to the phospholipids and to syntaxin. It will then release the

complexin I clamp, and complete membrane fusion. A coordinated action between the different actors of the AR is proposed. Complexin I would have two roles: activating the SNARE complex, and then blocking it into a metastable complex waiting for its fusogenic signal, and forming the substrate for synaptotagmin VIII or VI binding to the complex. Syntaxin 2 would be present either in two forms, with a preferential one upon capacitation, or in a single form, that would change its conformation along with capacitation. This could potentially add another level of regulation and further control before the final Ca^{2+} signal is released.

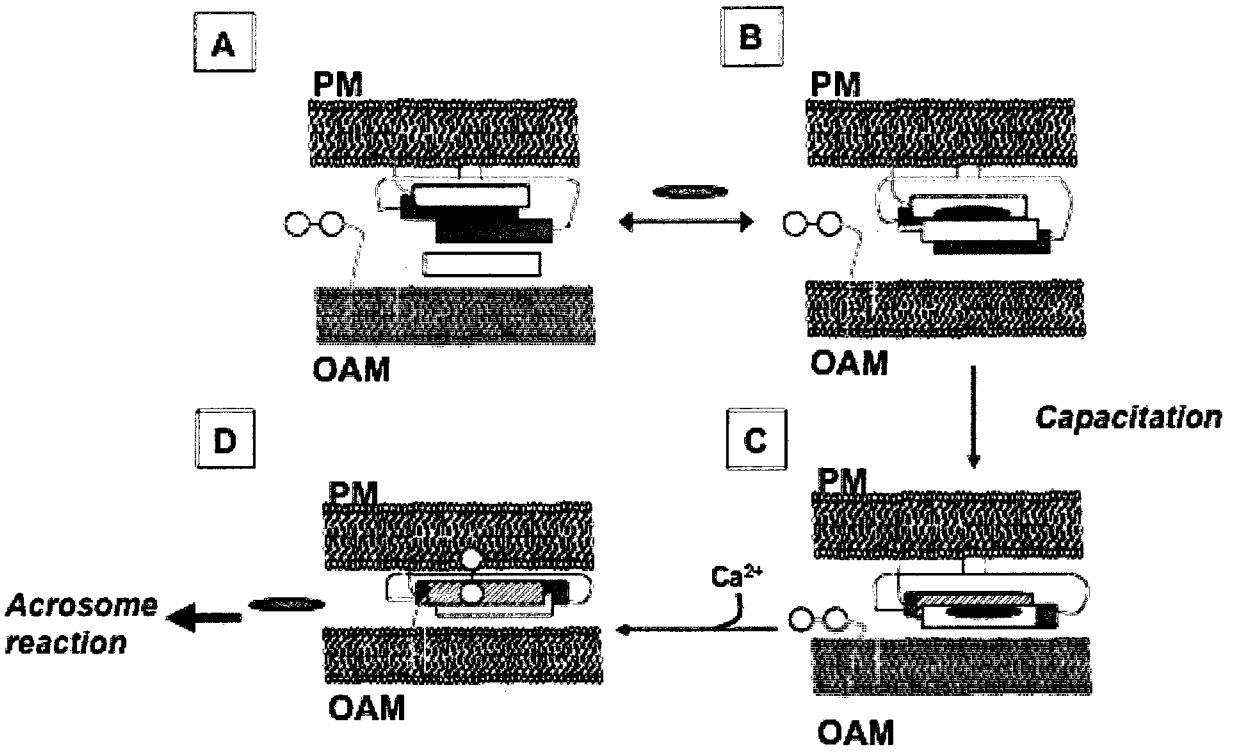


Figure 22: Suggested model for the regulation of the acrosome reaction via the action of complexin I and the t-SNARE syntaxin 2. (A) Before capacitation, the SNARE proteins are present but not in the optimal conformation to fully commit and form proper SNARE complex. However, they can still form partial, very loose and unstable complexes, that can be recognized by complexin I. (B) There is a dynamic between the monomeric proteins and the partially formed complex, so that complexin I can bind to those partially-assembled complexes. (C) Once capacitation occurs, the SNARE proteins can form a fully assembled, but loose four-helix SNARE complex. Complexin I will bind to the complex and act as a clamp to stabilize it and lock it until the fusogenic signal is released. At the same time, syntaxin 2 is either favoring a "capacitated" variant to commit to the SNARE complex or the same "uncapacitated" variant is being modified by conformational changes during capacitation. (D) Once the Ca^{2+} signal is released, it will be detected by the putative calcium sensor synaptotagmin VIII or VI, which will bind to the SNARE complex and phospholipids, dislodge complexin I of the SNARE complex and relieving the clamp of complexin I. Then, membrane fusion will occur as will the acrosome reaction. PM= Plasma membrane; OAM = Outer acrosome membrane; syntaxin 2 = red (modified or other variant = red dashed); SNAP-25 = green; VAMP = yellow; synaptotagmin VIII = purple; complexin I= blue. For simplistic reasons, syntaxin 2 was represented without its N-terminal bundle.

APPENDIX I

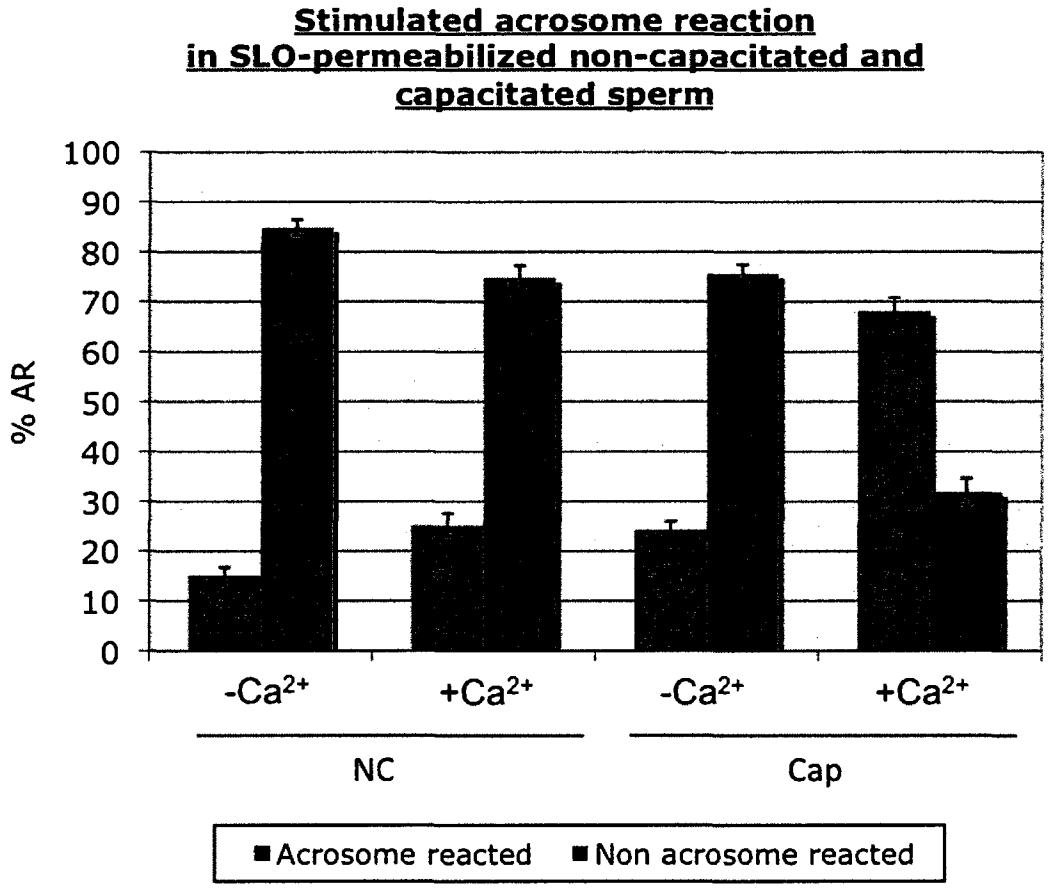


Figure 23: Acrosome reaction in SLO-permeabilized non-capacitated and capacitated sperm.

Non-capacitated and capacitated sperm were permeabilized with streptolysin O (SLO) at a concentration of 2 μg/ml at 37°C for 10 minutes. Then sperm were incubated with 1mM $CaCl_2$ at 37°C for 20 minutes. The reaction was stopped with formaldehyde and the sperm were stained with Coomassie Brilliant Blue and scored as being acrosome-intact or acrosome reacted. The levels of acrosome reacted sperm when no Ca^{2+} is present are defined as the spontaneous acrosome reaction levels. For non-capacitated sperm, the percentage of spontaneous acrosome reaction is of 15.1% and the percentage of stimulated acrosome reaction is of 25.2%. For capacitated sperm, non-stimulated sperm displayed a percentage of spontaneous acrosome reaction of 24% and stimulated sperm displayed a percentage of 68.1%. The values are expressed as mean ± SEM (n=4)

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