REGULATION OF SNARE FUNCTION DURING SPERM CAPACITATION TO
PROMOTE THE ACROSOME REACTION

BY

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DISSERTATION

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ABSTRACT

Infertility is a tragedy for millions of couples worldwide. According to the World Health Organization, the male factor alone accounts for over one third of total infertility. There are various causes of male infertility but defective acrosomal exocytosis is perhaps one of the biggest contributors towards male infertility. Unfortunately there is little information available on the most frequent molecular causes of the inability of sperm to undergo acrosomal exocytosis.

In the current study, my goal was to develop a clearer understanding of how the acrosome reaction is regulated during capacitation so that the molecular defects that cause infertility can be identified. I hypothesized that capacitation alters sperm protein phosphorylation promoting soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex formation and movement into membrane rafts in preparation for the acrosome reaction. Two t-SNAREs and a v-SNARE form a trans-SNARE complex, which is required for exocytosis. The core complex along with other regulatory proteins exerts an inward force by zippering from the N-termini. Ultimately hundreds of fusion pores are formed and hybrid vesicles are released. To test this hypothesis, I incubated mouse sperm in a capacitating (dmKRBT) or non-capacitating (dmKRBT without BSA or without HCO₃⁻) medium and detergent-extracted sperm protein. To assess phosphorylation of proteins in SNARE complexes, syntaxin and associated proteins were immunoprecipitated with a syntaxin antibody and subsequently separated by SDS-PAGE. Samples were not boiled prior to SDS-PAGE to maintain the integrity of the SNARE complex. To detect changes in total phosphorylation of syntaxin-containing (SNARE) complexes, immunoprecipitated complexes were separated by SDS-PAGE and stained with Pro-Q Diamond. I observed phosphoprotein staining in 75, 100, and 150 and 230 KD protein complexes. Immunoblotting with a syntaxin antibody demonstrated that each complex contained syntaxin
and was presumably a SNARE complex. After 30 min of capacitation, overall phosphorylation of SNARE complexes was higher in the 75, 100 and 150 KD complexes. To detect tyrosine phosphorylation of syntaxin and associated proteins, the anti-syntaxin immunoprecipitates were blotted with a phosphotyrosine antibody. Tyrosine phosphorylation of SNARE complexes decreased after 15 min of capacitation time. These data demonstrate SNARE complex phosphorylation is a dynamic process during capacitation and suggest that phosphorylation may regulate SNARE complex formation during capacitation in preparation for the acrosome reaction. Movement of SNAREs into membrane rafts was assessed using a detergent-free sucrose density gradient centrifugation method followed by immunoblotting with syntaxin and synaptobrevin antibodies. I observed movement of syntaxin and synaptobrevin into rafts in sperm incubated with capacitating dmKRBT and was delayed in dmKRBT lacking bicarbonate. No movement of syntaxin and synaptobrevin in sperm incubated with non-capacitating dmKRBT or dmKRBT lacking BSA was observed. Taken together, these data show that during capacitation, SNARE complex serine-threonine phosphorylation decreases whereas tyrosine phosphorylation increases. Syntaxin and synaptobrevin moved from non-raft fractions into rafts, and this movement was dependent on BSA and delayed in HCO$_3^-$ free medium. These results suggest that capacitation shifts SNAREs into rafts, allowing trans-SNARE complex formation, which may ultimately promote membrane fusion at those sites. Results of these fundamental studies may be helpful to develop more accurate laboratory fertility assessments, to reduce fertility, when contraception is desired, and to diagnose infertility and develop corrective therapies, when conception is needed.
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# TABLE OF CONTENTS

LIST OF FIGURES .......................................................................................................................... VIII

LIST OF TABLES ............................................................................................................................... X

CHAPTER 1: INTRODUCTION ............................................................................................................ 1

1.1. PROPOSED MODEL ..................................................................................................................... 2

1.2. AIM 1: DOES PHOSPHORYLATION STATUS OF SPERM PROTEIN CHANGE DURING CAPACITATION IN PREPARATION FOR THE ACROSOME REACTION? (SEE MODEL STEP 3) ................................................................. 3

1.3. AIM 2: DOES CAPACITATION PROMOTE SNARE COMPLEX FORMATION AND MOVEMENT INTO RAFFTS IN PREPARATION FOR THE ACROSOME REACTION? (SEE MODEL STEP 4) ................................................................................................. 4

1.4. SUMMARY ................................................................................................................................. 5

1.5. FIGURES .................................................................................................................................. 6

1.6. REFERENCES ............................................................................................................................. 7

CHAPTER 2: LITERATURE REVIEW ............................................................................................... 11

2.1. MALE INFERTILITY .................................................................................................................. 11

2.2. OVERVIEW OF FERTILIZATION ............................................................................................ 12

2.3. ACROSOME REACTION IN MAMMALIAN SPERM .................................................................. 13

2.4. EXOCYTOSIS IN SOMATIC CELLS .......................................................................................... 14

2.5. DIFFERENCES AND SIMILARITIES BETWEEN NEURONAL AND ACROSOMAL EXOCYTOSIS ................................................................. 14

2.6. REGULATION OF SNARE FUNCTION ....................................................................................... 16

2.7. REGULATION OF SNARE FUNCTION BY PHOSPHORYLATION CHANGES ........................... 17

2.8. MEMBRANE LIPID RAFFTS ................................................................................................. 20

2.9. RAFFTS IN SPERM MEMBRANES ............................................................................................ 22

2.10. SUMMARY .............................................................................................................................. 24

2.11. FIGURES AND TABLES ........................................................................................................... 25

2.12. REFERENCES ......................................................................................................................... 45

CHAPTER 3: SNARE PROTEINS ARE RE-LOCALIZED INTO RAFFTS DURING MOUSE SPERM CAPACITATION IN A BSA DEPENDENT MANNER ............................................................................................................. 63

3.1. ABSTRACT ............................................................................................................................... 63

3.2. INTRODUCTION ....................................................................................................................... 64

3.3. MATERIALS AND METHODS ................................................................................................. 68

3.4. RESULTS ................................................................................................................................. 72
CHAPTER 4: SNARE PHOSPHORYLATION IS ALTERED PRIOR TO MOUSE SPERM ACROSOMAL EXOCYTOSIS ................................................................................................................................ 94

4.1. ABSTRACT ................................................................................................................................. 94
4.2. INTRODUCTION ........................................................................................................................ 95
4.3. MATERIAL AND METHODS ....................................................................................................... 98
4.4. RESULTS .................................................................................................................................. 103
4.5. DISCUSSION .............................................................................................................................. 106
4.6. FIGURES ................................................................................................................................... 109
4.7. REFERENCES ............................................................................................................................ 117

CHAPTER 5: CAPACITATION PROMOTES THE FORMATION OF SDS-RESISTANT HIGHER MOLECULAR WEIGHT SNARE COMPLEXES IN MOUSE SPERM ................................................................................. 124

5.1. ABSTRACT .................................................................................................................................. 124
5.2. INTRODUCTION ........................................................................................................................ 125
5.3. MATERIAL AND METHODS ....................................................................................................... 128
5.4. RESULTS .................................................................................................................................. 133
5.5. DISCUSSION .............................................................................................................................. 136
5.6. FIGURES ................................................................................................................................... 139
5.7. REFERENCES ............................................................................................................................ 144

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS ............................................................................. 148

6.1. CONCLUSIONS ........................................................................................................................... 148
6.2. FUTURE STUDIES ...................................................................................................................... 151
6.3. FIGURES ................................................................................................................................... 154
6.4. REFERENCES ............................................................................................................................ 155

APPENDIX A: LIST OF ABBREVIATIONS ......................................................................................... 157

APPENDIX B: STATISTICAL ANALYSES ........................................................................................ 159
LIST OF FIGURES

Figure 1.1. Proposed model showing steps that may be involved in capacitation to prepare sperm for the acrosome reaction ................................................................. 6

Figure 2.1. Mechanism of Egg-Sperm Interaction. ............................................................. 25

Figure 2.2. Overview of mammalian fertilization. ........................................................... 26

Figure 2.3. Molecular basis of signaling events associated with sperm capacitation .......... 27

Figure 2.4. A Model for the acrosome reaction .............................................................. 28

Figure 2.5. Differences between neuronal and acrosomal exocytosis ................................ 29

Figure 2.6. A model of regulated exocytosis .................................................................. 30

Figure 2.7. A model of membranes fusion mediated by SNARE proteins ...................... 31

Figure 2.8. SNARE proteins and the core SNARE complex ............................................ 32

Figure 2.9. Protein tyrosine phosphorylation during capacitation and the acrosome reaction ...... 33

Figure 2.10. Regulation of exocytosis by phosphorylation ............................................. 34

Figure 2.11. Components of membrane lipid rafts and caveolae .................................... 35

Figure 2.12. History of the cell membrane raft concept .................................................. 36

Figure 2.13. Mechanism of activation of receptors, kinases, and calcium channels within lipid rafts of cells ........................................................................................................ 37

Figure 3.1. Detection of SNARE proteins in mouse sperm ............................................. 78

Figure 3.2. Syntaxin reorganization into sperm membrane rafts during capacitation ............ 79

Figure 3.3. Synaptobrevin reorganization into sperm membrane rafts during capacitation ......... 80

Figure 3.4. The proportion of caveolin in sperm membrane rafts does not change during capacitation .................................................................................................................. 81

Figure 3.5. Reorganization of syntaxin into rafts is dependent on PKA activity .................... 82
Figure 3.6. Proposed mechanism of SNARE movement into membrane rafts during mouse sperm capacitation ................................................................. 83

Figure 4.1. Changes in total phosphorylation during capacitation ................................................................. 109

Figure 4.2. Total phosphorylation decreases during capacitation at 90 min of incubation. ........ 110

Figure 4.3. Decrease of total phosphorylation during capacitation is initiated at 30 min of incubation. .............................................................................................................................................................................. 111

Figure 4.4. Total phosphorylation increases in SNAREs during capacitation. ................................................... 112

Figure 4.5. After an initial increase at 15 min, tyrosine phosphorylation of syntaxin-containing complexes decreases during capacitation ................................................................................................................... 113

Figure 4.6. Tyrosine phosphorylation of syntaxin-containing SNARE complexes during capacitation was similar at 0 and 90 min of incubation. .............................................................................................................................. 114

Figure 4.7. Total phosphorylation increases in SNAREs during capacitation is dependent on BSA and bicarbonate ...................................................................................................................................................... 115

Figure 4.8. Proposed model of SNAREs phosphorylation changes during mouse sperm capacitation .............................................................................................................................................................................. 116

Figure 5.1. Detection of SNARE complexes in capacitated and non-capacitated sperm. .................... 139

Figure 5.2. Proportion of syntaxin in higher molecular weight complexes in capacitated and non-capacitated sperm ............................................................................................................................................................................. 140

Figure 5.3. SNARE complexes formation during capacitation is dependent on BSA and bicarbonate .............................................................................................................................................................................. 141

Figure 5.4. SNARE complex formation during capacitation is dependent on the activity of PKA. 142

Figure 5.5. Proposed Model for SNARE complex formation .................................................................................................................................................................................................................. 143

Figure 6.1. Proposed model showing various steps that may be involved in capacitation to prepare the sperm for the acrosome reaction ...................................................................................................................................... 154
LIST OF TABLES

Table 2.1. An incomplete list of phosphorylation sites of SNAREs and associated proteins ..........38
Table 2.2. Classification of membrane rafts. ..............................................................................43
Table 2.3. Techniques to study membrane rafts. ........................................................................44
Table B.1. Results of statistical analysis for syntaxin reorganization into rafts (Fig. 3.2)............159
Table B.2. Results of statistical analysis for synaptobrevin reorganization into rafts (Fig. 3.3).....160
Table B.3. Results of statistical analysis for overall phosphorylation changes in total sperm
protein during capacitation (Fig. 4.2).........................................................................................161
Table B.4. Results of statistical analysis for overall phosphorylation changes in total sperm protein
during capacitation (Fig. 4.3).................................................................................................162
Table B.5. Results of statistical analysis for total phosphorylation changes in SNARE proteins
during capacitation (Fig. 4.4).................................................................................................163
Table B.6. Results of statistical analysis for tyrosine phosphorylation changes in SNARE proteins
during capacitation (Fig. 4.5).................................................................................................164
Table B.7. Results of statistical analysis for tyrosine phosphorylation changes in SNARE proteins
during capacitation (Fig. 4.6).................................................................................................165
Table B.8. Results of statistical analysis for SNARE complex formation (Fig. 5.1)..................166
Table B.9. Results of statistical analysis for SNARE complex formation (Fig. 5.2)..................167
Table B.10. Results of statistical analysis for SNARE complex formation (Fig. 5.3)..............168
Table B.11. Results of statistical analysis for SNARE complex formation (Fig. 5.4)..............169
CHAPTER 1

INTRODUCTION

Infertility is a tragedy for millions of couples throughout the world, causing social, cultural, psychological and emotional problems. Male factors are responsible for over 30% of total infertility (Daar and Merali, 2002; WHO, 2002; Boivin et al., 2007; Ombelet et al., 2008). The inability to undergo acrosomal exocytosis appears to be one of the most common defects causing infertility in human males with normal sperm count and morphology. About 25-30% of men with idiopathic infertility produce sperm that, in response to zona pellucida binding, rarely undergo acrosomal exocytosis (Liu et al., 2001; Liu and Baker, 2003). Unfortunately, there is little information on the most frequent molecular causes of sperm inability to undergo acrosomal exocytosis. Sperm capacitation prepares sperm to undergo acrosomal exocytosis by altering protein phosphorylation and removing cholesterol. Sperm capacitation can be accomplished in bicarbonate-based medium including calcium and a cholesterol acceptor (Gadella and Van Gestel, 2004; Bou Khalil et al., 2006; Tanphaichitr et al., 2007). The newly ejaculated sperm encounter a change in $\text{HCO}_3^-$ concentration when it enters the female reproductive tract. In addition to changing the pH, and the resting membrane potential (Em), $\text{HCO}_3^-$ also regulates the cAMP pathway in the presence of calcium through the stimulation of soluble adenylate cyclase (SACY) (Garbers et al., 1982; Okamura et al., 1985; Visconti et al., 2011). Although the precise role of SACY is unclear, SACY knockout mice are sterile and their sperm do not develop hyper-activated motility. The cause of this sterility phenotype may be a failure in overall capacitation, of which hyperactivated motility is a part (Xie et al., 2006). The overall capacitation pathway is hypothesized to be modulated by cholesterol removal from sperm plasma membrane (Garbers et
Cholesterol is an abundant component of membrane sub-domains, often referred to as rafts, which are enriched in sterols and sphingolipids. These rafts may provide a platform for sperm proteins important for binding to the egg and exocytosis of the acrosome (Shadan et al., 2004; Cross, 2004a; van Gestel et al., 2005; Tanphaichitr et al., 2007). In somatic cells, several signaling pathways involving tyrosine kinases, G protein and others are activated by cholesterol-binding reagents such as beta-cyclodextrins (Brown and London, 1998; Kabouridis et al., 2000). Therefore, cholesterol removal may have a profound effect on sperm protein phosphorylation, SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) re-localization to rafts and hence on capacitation. Based on an understanding of the localization and changes in phosphorylation status of SNAREs and other regulatory proteins, the current study was designed to provide an in-depth molecular understanding of male infertility associated with capacitation and the acrosome reaction. The major goal of this project was to identify molecular events responsible for preparing sperm for release of the acrosome. The results of this basic study will lay the foundation for investigations that will ultimately help in diagnosing and treating male infertility, when conception is desired, and preventing fertility when contraception is needed.

1.1. Proposed Model

Based on my preliminary data and other studies, I have developed a model for SNAREs function and regulation of acrosomal exocytosis. I hypothesized that capacitation alters sperm protein phosphorylation promoting SNARE complex formation and movement into membrane rafts in preparation for the acrosome reaction. Some regulatory proteins like complexin I and others stabilize that complex by binding SNAREs. An increase in cytosolic Ca$^{2+}$ is detected by synaptotagmin, which drives SNARE-mediated membrane fusion at hundreds of points between
the outer acrosome and plasma membrane, releasing acrosomal contents. I tested parts of this model in two specific aims as described on the following pages.

1.2. **Aim 1: Does phosphorylation status of sperm protein change during capacitation in preparation for the acrosome reaction? (See model step 3)**

During capacitation, activation of kinases and phosphatases alters phosphorylation of many sperm proteins (Dube et al., 2005a; Jha et al., 2006; Salicioni et al., 2007a). In somatic cells, SNAREs and other regulatory proteins are usually phosphorylated on serine/threonine residues. This phosphorylation process helps control membrane fusion (Nagy et al., 2004; Morgan et al., 2005) as discussed in the literature review. However the role of SNARE phosphorylation in sperm is unclear.

In Aim 1, I determined if capacitation alters phosphorylation/dephosphorylation status of sperm proteins, especially SNAREs. I hypothesized that the phosphorylation status of some SNAREs and other regulatory proteins (syntaxin, synaptotagmin and complexin) changes during capacitation, and this change in protein phosphorylation is required for the acrosome reaction. In this aim, I investigated the following five questions;

i. Does total phosphorylation of sperm protein change during capacitation?

ii. Does total phosphorylation of SNAREs change during capacitation?

iii. Does tyrosine phosphorylation change in SNARE complexes during capacitation?

iv. Are phosphorylation changes in SNAREs mediated by specific kinases (tyrosine or serine/threonine) and phosphatases required for capacitation?

v. Is membrane cholesterol depletion or activation of soluble adenylate cyclase required for protein phosphorylation changes during capacitation?
1.3. Aim 2: Does capacitation promote SNARE complex formation and movement into rafts in preparation for the acrosome reaction? (See model step 4)

Sperm possess unusual lipids and are believed to contain membrane rafts although, based on the ganglioside GM1 localization, the rafts may be very large, perhaps micron scale (Cross, 2004b; Tanphaichitr et al., 2007; Asano et al., 2009; Selvaraj et al., 2009). Because cholesterol is a major component of rafts, the loss of cholesterol from the sperm membrane has a profound effect on raft function and protein composition (Travis et al., 2001; Sleight et al., 2005; Tanphaichitr et al., 2007). Importantly, capacitation can be induced by incubation with a cholesterol acceptor in a bicarbonate-based medium. However, it is not certain if the loss in cholesterol during sperm capacitation is from raft or non-raft fractions, which might de-stabilize or stabilize rafts (Cross, 2004; van Gestel et al., 2005; Bou Khalil et al., 2006). Capacitation also induces a reorganization of rafts, as detected by cholera toxin-B binding to GM1 (Selvaraj et al., 2007), which is consistent with my hypothesis illustrated in Figure 1.1. There are reports that syntaxin and synaptobrevin are found in rafts from sperm and my research has confirmed that syntaxin is in sperm rafts as shown in Figure 3.1 (Travis et al., 2001; Tsai et al., 2007). At least conceptually, sperm capacitation may have similarities to vesicle docking and/or priming in neurons and may shift SNAREs to specific sites in the plasma membrane in preparation for fusion. As these rafts are reorganized, the protein composition of rafts may also change correspondingly. My research findings are in agreement with other studies showing that, as sperm capacitate, some proteins including SNAREs shift into or out of membrane rafts and this shift could allow SNARE complexes to form, promoting membrane fusion at the site of rafts (Chamberlain et al., 2001; Chintagari et al., 2006; Puri and Roche, 2006). However, it is unclear if membrane fusion occurs at sites of rafts containing SNAREs. I hypothesized that capacitation
promotes SNARE complex formation and its movement into lipid rafts in preparation for the acrosome reaction. To test this hypothesis, in Aim 2, I determined if capacitation promotes SNARE complex formation and shifts SNAREs into rafts present on the plasma and outer acrosomal membranes. Using biochemical approaches, I expected to find that capacitation is responsible for promoting the movement of SNAREs into rafts, allowing the acrosomal exocytosis to occur. In this aim, I investigated four questions;

i. Does capacitation shift SNAREs into sperm rafts?

ii. Does capacitation promote SNARE complex formation?

iii. Are phosphorylation changes in SNAREs mediated by specific kinases (tyrosine or serine/threonine) and phosphatases required for SNARE complex movement into rafts?

iv. Is membrane cholesterol depletion or activation of soluble adenylate cyclase sufficient for SNARE protein relocation into membrane rafts?

1.4. Summary

Our lab and others have found that, despite differences in the number of fusion sites, exocytosis speed, and recycling, acrosomal exocytosis shares some molecular features with the well-studied neuronal exocytosis. Based on our lab’s data and other published data, I have developed a model of how SNARE function and acrosomal exocytosis are regulated. I proposed that capacitation alters SNARE phosphorylation and complex formation. SNAREs move into membrane rafts in preparation for the acrosome reaction. I tested parts of this model in two Specific Aims (see specific aims). These fundamental studies of acrosome regulation may provide a foundation for development of new contraceptives, more accurate diagnostic tests of male fertility, and therapies that are more effective.
1.5. Figures

Figure 1.1. Proposed model showing steps that may be involved in capacitation to prepare sperm for the acrosome reaction. Please note that the order of number 2 and 3 is unclear.
1.6. References


CHAPTER 2

LITERATURE REVIEW

2.1. Male Infertility

According to the World Health Organization, more than eighty million people are infertile and male factors contribute towards one third of infertility worldwide (WHO, 1991; WHO, 2002; Clarke et al., 2006; Harper et al., 2008). Recent studies have shown that men with unknown infertility often have sperm that are defective in the acrosome reaction (Liu et al., 2001; Liu de and Baker, 2007). Successful mammalian fertilization requires a perfectly timed and regulated acrosomal exocytosis (Saling and Storey, 1979; Florman and Storey, 1982; Yanagimachi, 1994a).

The acrosome is formed during spermiogenesis; a final maturation step of sperm formation and is maintained near the plasma membrane until it is released (Fig. 2.1) when sperm are near or at the zona pellucida (Toshimori, 2009; Jin et al., 2011). Sperm that undergo a premature acrosome reaction seem to be unable to fertilize the egg. Similarly sperm are unable to penetrate the tough extracellular matrix around the egg if they do not acrosome react near or on the zona pellucida (Yanagimachi, 1994b; Serin et al., 2011). Little information is available on the most frequent molecular causes of the inability of sperm to undergo acrosomal exocytosis. Men with unexplained infertility often resort to assisted reproductive technologies (ART) such as IVF or ICSI, but there are known and unknown problems associated with these technologies (Schieve et al., 2002; Hansen et al., 2002). Therefore, it is important to identify and treat the underlying causes of infertility. This requires a thorough understanding of the fertilization process. This study aimed to develop a clearer understanding of the fundamentals of acrosomal exocytosis that may help in the identification of molecular defects leading to infertility.
The process of exocytosis (i.e. the release of material from the cell through fusion of internal vesicles with the plasma membrane) is a highly conserved and an essential one for different cellular functions. The regulation of sperm acrosomal exocytosis is not well understood but may be similar to neuronal/somatic cell vesicle exocytosis, which has been studied extensively. The minimal protein complex that is formed during neuronal exocytosis is the SNARE complex (van den Bogaart and Jahn, 2011). Considering neuronal exocytosis as a model, my aim was to understand acrosomal exocytosis at the molecular level.

2.2. Overview of Fertilization

All species must reproduce either sexually or asexually to survive. Sexual reproduction in most animals including humans is accomplished through a series of complex processes leading to fertilization (Fig. 2.2). Fertilization itself is not a single event, but a complex sequence of specific and regulated cell-cell interaction, adhesion, signaling, and exocytotic steps, studied in the greatest detail in mice (Wassarman et al., 2001; Nixon et al., 2007; Clark, 2011). Fertilization starts with the fusion of two haploid cells (i.e. a sperm and an egg cell) and ends with the formation of a diploid zygote. For a successful fertilization, sperm must have matured through a final maturation step called capacitation (Fig. 2.3). Capacitation involves changes induced by the female reproductive tract (oviduct) making sperm competent to go through the acrosome reaction and ultimately fertilize an egg (Waberski et al., 2005). The acrosome is a single large membrane-bounded organelle containing numerous hydrolytic enzymes. The acrosome reaction is triggered either in the cumulus cells surrounding the egg or by interaction with the specialized egg coat called the zona pellucida (ZP) (Florman et al., 1998; Jungnickel et al., 2001; Evans and Florman, 2002). The zona pellucida in mice consists of three glycoproteins called ZP1, ZP2, and ZP3 (Swanson and Vacquier, 2002). The sperm plasma membrane overlying the acrosome binds ZP3
inducing the acrosome reaction (Fig. 2.4). But at least in mice, the acrosome reaction of most fertilizing sperm occurs in the cumulus mass before contact with the zona pellucida (Jin et al., 2011). The acrosome reaction enables sperm to penetrate the zona pellucida and fuse with the oocyte. Progesterone, prostaglandins, calcium ionophore, and diacylglycerol are also known to induce the acrosome reaction in vitro (Breitbart et al., 1997).

2.3. Acrosome Reaction in Mammalian Sperm

For years, the predominant model for fertilization was that the fertilizing sperm completed capacitation in the female reproductive tract, and bound to the zona pellucida inducing the acrosomal exocytosis (Wassarman et al., 2001). Early experiments found that only one zona pellucida glycoprotein, ZP3, could bind sperm and induce the acrosome reaction (Dietl and Rauth, 1989; Wassarman, 1999; Serrano and Garcia-Suarez, 2001).

More recent studies suggest that not only ZP3 but a complex of zona proteins binds sperm (the “zona scaffold” model) and this binding ultimately promotes the acrosome reaction mechanically (Dean, 2004; Baibakov et al., 2007). And in the last couple of years, the use of GFP to detect acrosomal proteins on live sperm has suggested that the acrosome reaction begins in the cumulus cells outside of the zona pellucida (Jin et al., 2011). So the physiological trigger of the acrosome reaction is controversial. There are also contrasting reports about the receptors on sperm that bind the zona pellucida. There are several candidates for plasma membrane receptors that have affinity for the zona pellucida (Miller et al., 2002; Carmona et al., 2002; Weerachatyanukul et al., 2003; Ensslin and Shur, 2003). The acrosome also contains adhesive proteins that bind the zona pellucida. The adhesive role of these proteins occurs after exposure of the acrosomal contents but before completion of acrosomal exocytosis (Evans and Florman, 2002; Bi et al., 2003). Acrosome exocytosis shares many features of receptor-mediated
exocytosis where Ca$^{2+}$ plays a central role. Comparable to many receptor-mediated processes, an initial transient rise in cytosolic Ca$^{2+}$, probably mediated by T-type Ca$^{2+}$ channels, followed by a sustained increase in cytosolic Ca$^{2+}$ is necessary for the acrosome reaction (Darszon et al., 2005). The initial rise in Ca$^{2+}$ may activate phospholipase C (PLC), generating IP$_3$ and mobilizing Ca$^{2+}$ from the acrosome. The secondary increase in Ca$^{2+}$ may be accomplished by activation of a transient receptor channel protein (TRPC2), a putative component of a store operated Ca$^{2+}$ channel (Jungnickel et al., 2001). This larger sustained increase in cytosolic Ca$^{2+}$ triggers the acrosomal exocytosis.

2.4. Exocytosis in Somatic Cells

In recent years, membrane fusion and neuronal exocytosis have received considerable attention because of their central roles in several important biological events such as cell signaling, viral infection, hormone secretion and fertilization. The initial contact between secretory vesicles and the target membrane is made by the event known as docking where the vesicle docks or tethers on the target membrane. After docking, the vesicles undergo a “priming” step (Fig. 2.6), allowing them to move into the slowly releasable and then readily releasable pool of vesicles (Becherer and Rettig, 2006). Primed vesicles respond to the increased cytosolic Ca$^{2+}$ and are released in <0.3 ms (Jahn et al., 2003).

2.5. Differences and Similarities between Neuronal and Acrosomal Exocytosis

In general, exocytosis is exquisitely regulated to ensure that it happens at only a proper time and place. This characteristic of exocytosis is true for neuronal as well as acrosomal exocytosis. Although the three molecules that make up the core SNARE complex are sufficient for membrane fusion in vitro, in vivo SNARE complex formation is controlled by a number of
regulatory molecules to ensure proper timing of exocytosis (DeBello and O'Connor, 1995; Mochida, 2000). There are a number of similarities between membrane fusion in sperm acrosomal exocytosis and synaptic vesicle exocytosis, including the requirements of increased intracellular Ca\(^{2+}\) and SNARE complex formation (Jungnickel et al., 2001; Sollner, 2003; Mayorga et al., 2007). However, the acrosome is much larger than a typical secretory vesicle and the outer membrane of this single vesicle fuses at hundreds of points with the sperm plasma membrane during exocytosis compared to single fusion pores during synaptic vesicle exocytosis (Flaherty and Olson, 1991; Nolan and Hammerstedt, 1997). After release of hybrid vesicles containing plasma membrane and outer acrosomal membrane, the inner acrosomal membrane remains on the sperm and is continuous with the remaining plasma membrane; hence, in contrast to exocytosis at synapses, membranes are lost and there is no vesicle recycling (Russell et al., 1979; Yudin et al., 1988). Acrosomal exocytosis also proceeds much more slowly than does neuronal exocytosis (Lopez-Gonzalez et al., 2001; Rettig and Neher, 2002). This slowness may be related to the larger size and the slower dispersion of some of the acrosomal proteins found within the acrosomal matrix (Kim and Gerton, 2003). Several SNARE proteins, including various syntaxin isoforms, synaptobrevin (VAMP-2) and SNAP-25, have been detected in the sperm acrosomal region (Katafuchi et al., 2000; Tomes et al., 2002; Tsai et al., 2007).

Antibodies to syntaxin and synaptobrevin inhibited the ionophore-induced acrosome reaction in bovine sperm (Ramalho-Santos et al., 2000). However, in this experiment, it is unclear how the antibodies gained access to SNAREs to inhibit the acrosome reaction. As an alternative to adding SNARE antibodies to live sperm, a model system was developed in which proteins were added to streptolysin-O-permeabilized human sperm and the acrosome reaction was induced with Ca\(^{2+}\). Using this model, Botulinum toxins (that cleave SNARE proteins) inhibit
the human sperm acrosome reaction. Antibodies to synaptobrevin and syntaxins 1A, 1B, 4, and 6, SNAP-25 and SNAP-23 block the human sperm acrosome reaction in this system (Tomes et al., 2002). Together, these data suggest SNARE complex assembly is required for the acrosome reaction.

2.6. Regulation of SNARE Function

In the last 20 years, the molecular components that allow fusion to occur have been identified. In neurons, neuroendocrine cells, mast cells and pancreatic islet cells, the formation of a highly stable protein complex known as the SNARE complex brings the vesicle and plasma membranes together to facilitate fusion (Sheu et al., 2003; Puri et al., 2003). SNAREs constitute a large family of more than 35 proteins that contain a ~60 amino acid sequence known as the SNARE motif (Jahn et al., 2003). The core SNARE complex in neuronal cells is the best-studied SNARE complex, consisting of three proteins that form a stable complex: plasma membrane-associated syntaxin and SNAP-25 (synaptosomal-associated protein of 25 kDa), and vesicle-associated membrane protein (VAMP or synaptobrevin). How the three proteins interact is shown in Figures 2.7 and 2.8. SNAREs found on transport vesicles are often grouped as v-SNAREs and those on the target (the plasma membrane) are classified as t-SNAREs. The t-SNAREs syntaxin and SNAP-25 are believed to interact first, allowing the v-SNARE synaptobrevin to bind prior to fusion (Weninger et al., 2008). The SNARE core complex is very stable. It is not denatured by SDS (unless boiled) and, once formed, is not cleaved by clostridial neurotoxin (Hayashi et al., 1994). The core complex is formed by four SNARE motifs consisting of two SNAP-25, one synaptobrevin and one syntaxin 1 motifs (Fig. 2.8). Although the core neuronal SNARE complex is composed of synaptobrevin, syntaxin 1 and SNAP-25, SNAREs function in exocytosis in non-neuronal tissues including mast cells, endocrine cells and sperm.
There are a few examples of non-neural cells expressing “neural” SNAREs. For example, sperm contain SNAP-25 (Burkin and Miller, unpublished), but it has a fairly limited tissue distribution. On the other hand, a homologue, SNAP-23 (59% identical to SNAP-25) is found in a wide variety of tissues. Various isoforms of syntaxin and synaptobrevin are expressed in many tissues where they can function in vesicle transport and/or exocytosis (Kavalali, 2002; Tomes et al., 2002; Sollner, 2003). The minimal protein complex that is formed during neuronal exocytosis is the core SNARE complex that contains syntaxins, SNAP-25, and VAMP/synaptobrevin. Studies from several laboratories have found that human sperm express syntaxins, SNAP-25, VAMP/synaptobrevin and regulatory proteins such as NSF, αSNAP, synaptotagmin and complexins (De Blas et al., 2005; Lopez et al., 2007; Zarelli et al., 2009; Zanetti and Mayorga, 2009) The speed of synaptic exocytosis and the dependence on Ca^{2+} ions require additional proteins that act on SNAREs to provide these features. In neuronal exocytosis, the Ca^{2+} sensitive step is at the point at which the “primed” secretory vesicles actually fuse with the plasma membrane. Synaptotagmin I appear to provide the Ca^{2+} sensitivity for fast neurotransmitter release (Chapman, 2002). Genetic studies in mice demonstrated that the loss of synaptotagmin I eliminates the fast synchronous vesicle release. Studies of sperm function could not be performed because synaptotagmin I deficient mice died at birth. Synaptotagmins may also act as a Ca^{2+} sensor for the sperm acrosome reaction although there is debate about which isoform(s) are important (Hutt et al., 2005).

2.7. Regulation of SNARE Function by Phosphorylation Changes

It is well established that regulated exocytosis in somatic cells is modulated at various steps by protein phosphorylation as shown in Figure 2.10 (Turner et al., 1999; Lin and Scheller, 2000; Klenchin and Martin, 2000; Morgan et al., 2005). Protein kinase A inhibitors and
phosphatase inhibitors affect chromaffin cell exocytosis at the vesicle “priming” step (Nagy et al., 2004). A number of SNAREs and SNARE regulatory proteins can be phosphorylated and, at least in some cases, phosphorylation alters their biological activity. Table 2.1 retrieved from three online phosphorylation data bases, Phospho.ELM (Dinkel et al., 2011), PhosphoSitePlus (Hornbeck et al., 2012) and PHOSIDA (Gnad et al., 2011), provides a partial list of phosphorylation sites of SNAREs and associated protein that may have a role in membrane fusion and signal transduction. Because syntaxin and complexin regulate SNARE formation and stabilization, their phosphorylation may be most relevant for capacitation. It is known that complexin I/II phosphorylation increased their affinity for the SNARE complex (Shata et al., 2007).

Syntaxin phosphorylation affects its ability to bind SNAP-25/SNAREs. Whether binding is increased or decreased depends on the amino acid that is phosphorylated (Foster et al., 1998; Foletti et al., 2000; Risinger and Bennett, 2002; Dubois et al., 2002). Since synaptotagmin is likely a Ca\(^{2+}\) sensor, its phosphorylation may control acrosomal exocytosis. Interestingly, when recombinant synaptotagmin VI was phosphorylated by protein kinase C, it became unable to inhibit acrosomal exocytosis when added to permeabilized human sperm, suggesting that PKC-catalyzed phosphorylation may regulate synaptotagmin function in sperm (Michaut et al., 2001; Roggero et al., 2007). Although there is considerable evidence that protein phosphorylation is important for sperm capacitation and acrosomal exocytosis (Fig. 2.9), it is not known whether sperm SNAREs are phosphorylated and, if so, when phosphorylation status changes (during capacitation or exocytosis) and how it affects their function. In view of the role of SNARE phosphorylation in regulating the “priming” stage of neuronal (Nagy et al., 2004; Hepp et al., 2005), capacitation may be viewed as slower “priming” for acrosomal exocytosis.
There is considerable precedent in somatic cells for the hypothesis that phosphorylation of SNAREs and SNARE regulatory proteins affect formation of a SNARE complex and regulate exocytosis (Dubois et al., 2002; Tian et al., 2003; Boczan et al., 2004). A change in protein phosphorylation is a major outcome of sperm capacitation but many of the sperm phosphoproteins are unknown. Sperm capacitation can be accomplished in bicarbonate-based medium including calcium and a cholesterol acceptor (Gadella and Van Gestel, 2004; Bour Khalil et al., 2006; Tanphaichitr et al., 2007); therefore bicarbonate-induce activation of SACY and cholesterol depletion are linked to protein phosphorylation changes. Human and mouse sperm proteins undergo tyrosine phosphorylation during capacitation (Osheroff et al., 1999; Seshagiri et al., 2007). The exocytotic function of SNAREs during neuronal and non-neuronal exocytosis is also regulated by phosphorylation (Turner et al., 1999; Chen et al., 2011). Protein kinases and phosphatases act on a number of proteins in synaptic vesicles to regulate several steps including vesicle priming and recycling. All three core SNARE proteins and some SNARE regulatory proteins are phosphoproteins including synaptotagmins, syntaxins, synapsin and others (Burgoyne and Morgan, 2003). In most cases, the functional consequences of phosphorylation are not clear; however, in some instances there is evidence that phosphorylation alters the activity of the phosphoprotein. For example, phosphorylation of synaptotagmin I by Ca^{2+}/calmodulin-dependent kinase II (CamKII) promotes its interaction with syntaxin and SNAP-25 (Verona et al., 2000). Synaptobrevin can also be phosphorylated by CamKII. Phosphorylation on some sites in syntaxin 1 promote synaptotagmin I binding but on other sites reduces SNARE complex formation (Sakisaka et al., 2004). Phosphorylation of SNAP-23 and SNAP-25 regulates exocytosis, likely by promoting binding to SNAREs and affecting vesicle priming (Hepp et al., 2005; Suzuki and Verma, 2008) and/or altering the activity of voltage-
gated Ca\textsuperscript{2+} channels (Snyder et al., 2006; Yang et al., 2007). The regulation of neuronal exocytosis by phosphorylation is of interest in view of the well-known changes in protein tyrosine phosphorylation during sperm capacitation, which can be envisioned as a kind of “priming” for acrosomal exocytosis (Osheroff et al., 1999; Dube et al., 2005; Jha et al., 2006; Salicioni et al., 2007).

Whether, in fact, priming occurs in sperm as it does in secretory cells is uncertain but it appears that sperm must undergo priming steps that have some resemblance to those in neurons (Tomes et al., 2005). Of course, in neurons, altered phosphorylation occurs very near the time of exocytosis but priming may be longer in sperm, consistent with longer exocytosis. Tyrosine phosphorylation appears to be downstream of the activation of the serine-threonine kinase, Protein Kinase A (Salicioni et al., 2007; Visconti et al., 2011) so both groups of kinases may be activated during sperm capacitation. Morphologically, it is possible that the evagination of the outer acrosomal membrane towards the plasma membrane to form fusion pores reflects priming (Fig. 2.5). Unfortunately, all that is known to date about SNARE phosphorylation in sperm is that synaptotagmin VI phosphorylation by protein kinase C affects its ability to regulate membrane fusion when added to permeabilized sperm. The kinetics of this phosphorylation is uncertain because permeabilized sperm acrosome reacts without prior capacitation (Snyder et al., 2006; Roggero et al., 2007).

2.8. Membrane Lipid Rafts

In somatic cells, SNARE are sometimes found in laterally organized dynamic 10-200 nm membrane specializations known as rafts. Rafts are heterogeneous microdomains as shown in Figure 2.11(Razani et al., 2002), enriched in sterol and sphingolipids that act as platforms for trafficking or docking molecules (Munro, 2003; Pike, 2004; Pike, 2006). The lipid composition
of these domains and the tight packing of the acyl lipid chains make these fractions resistant to detergents or NaCO₃ solubilization. Resistance to solubilization and buoyant density has been used to purify rafts. Beginning in 1970s, the raft concept evolved gradually with the advancement of techniques to study rafts as shown in Figure 2.12 (Lingwood and Simons, 2010). Based on organization of components (Simons and Toomre, 2000), rafts are traditionally classified into 4 categories as described in Table 2.2.

Rafts are classically defined by their relative insolubility in mild non-ionic detergents (i.e. Triton X-100) at cold temperatures. They have the ability to sequester specific proteins and lipids and exclude others. They are thought to play vital roles in various important biological events, such as membrane fusion (Liu et al., 2001; Liu and Baker, 2003), protein trafficking (Yanagimachi, 1994b; De Blas et al., 2005; Dube et al., 2005b), signal transduction (Retzloff and Hornstein, 2003; Sleight et al., 2005; Gamboa and Ramalho-Santos, 2005; Seshagiri et al., 2007), lipid sorting (Wassarman et al., 2001; Miller et al., 2002), and organization of the cytoskeleton (Gadella and Van Gestel, 2004; Dean, 2004). Figure 2.13 is a model for the mechanism of how receptors, kinases, and calcium channels may be within lipid rafts of cells (George and Wu, 2012). Recent studies have found that syntaxin, synaptobrevin, SNAP-25 and SNAP-23 partition into Triton X-100 insoluble raft fractions and can form an SDS-resistant ternary complex. Some syntaxins probably associate with rafts because they bind SNAP-23 or SNAP-25, palmitoylated proteins whose acyl group may localize them to rafts (Chamberlain and Gould, 2002; Salaun et al., 2005; Gil et al., 2006; Puri and Roche, 2006). It has been proposed that rafts organize exocytotic proteins and ion channels to coordinate their functions in secretion (Xia et al., 2004; Martens et al., 2004; Xia et al., 2007; Weaver et al., 2007). Interestingly, it is also known that syntaxins cluster at docking and fusion sites for vesicle secretion (Lang et al.,
This finding about syntaxins is consistent with the membrane fusion model in which trans-SNARE complexes form (SNAREpins) and zip together to overcome the energy barrier and promote membrane fusion at these sites.

However, most data supporting the SNARE’s zippering hypothesis comes from experiments using recombinant proteins reconstituted in liposomes (Weber et al., 2000), from in vitro studies of fusion among yeast vacuoles genetically manipulated to prevent formation of cis complexes (Ungermann et al., 1998) and from fusion of cells expressing “flipped” SNAREs on the outer surface of the plasma membrane (Giraudo et al., 2006). A very attractive model is that membrane rafts serve to organize syntaxins, SNAP-25 and other regulatory proteins together at sites of future membrane fusion. This protein complex could then form trans-SNARE complexes with VAMP/synaptobrevin in vesicles (e.g. the acrosome). Miller’s group and others have shown that as sperm capacitate, proteins including some SNARE reorganize into or out of membrane rafts and reorganization could allow SNAREpin complexes to form promoting membrane fusion at the site of rafts. However, it is unclear whether docking and fusion begin at sites of membrane rafts (Lang et al., 2001; Ikonen, 2001; Ohara-Imaizumi et al., 2004; San-Juan-Vergara et al., 2012) and if rafts contain SNAREs at the time of fusion. With the advancement of technology, we now have better ways to study the rafts, raft association and signalings (Simons and Toomre, 2000; Sonnino and Prinetti, 2013) associated with rafts as discussed in Table 2.3.

2.9. Rafts in Sperm Membranes

Sperm possess unusual lipids and are believed to contain membrane rafts although, based on the ganglioside GM1 localization, the rafts may be very large, perhaps micron scale (Trevino et al., 2001; Shadan et al., 2004; Cross, 2004; Selvaraj et al., 2006; Selvaraj et al., 2007; Tanphaichitr et al., 2007; Selvaraj et al., 2009; Asano et al., 2009). Nonetheless, the loss of
cholesterol, a major component of rafts, has a profound effect on their function and the protein composition of rafts (Travis et al., 2001; Sleight et al., 2005; Tanphaichitr et al., 2007). Importantly, capacitation can be induced by incubation with a cholesterol acceptor in bicarbonate-based medium. However, it is not certain if the loss in cholesterol is from raft or non-raft fractions, which might de-stabilize or stabilize rafts, respectively (Cross, 2004; Sleight et al., 2005; van Gestel et al., 2005; Bou Khalil et al., 2006; Tanphaichitr et al., 2007).

Capacitation also induces a reorganization of rafts, as detected by cholera toxin-B binding to GM1 (Selvaraj et al., 2007), which is consistent with our hypothesis (Fig 1-1). There are reports that the SNAREs syntaxin and synaptobrevin are found in rafts from sperm (Travis et al., 2001; Tsai et al., 2007) and our research has confirmed that syntaxin is in rafts of capacitated sperm. At least conceptually, sperm capacitation may have similarities to vesicle docking and/or priming in neurons and may shift SNAREs to specific sites in the plasma membrane in preparation for fusion. As these rafts are reorganized, the protein composition of rafts may also change correspondingly. Most studies use Triton X-100 to extract non-raft proteins, but it is becoming increasingly clear that rafts are heterogeneous and different subclasses of rafts are extracted by Triton X-100 compared to another mild non-ionic detergent such as BRIJ96 (Ikonen, 2001; Pike, 2004; Pike, 2006). This heterogeneity was recently demonstrated in sperm rafts (Asano et al., 2009).

One recent article concluded that there was a modest change in localization and raft partitioning of syntaxins 1 and 2 and synaptobrevin as boar sperm capacitate (Tsai et al., 2007). The authors used 1% Triton X-100 to isolate rafts which solubilize all but the most stable rafts. Using only the Triton X-100 extraction could easily overlook protein movement into and out of intermediate stability rafts identified by BRIJ96 resistance. Finally several additional papers were
published describing the behavior of rafts in sperm, including one revealing raft heterogeneity and another demonstrating that acrosomal membranes contain a raft marker (Asano et al., 2009; Selvaraj et al., 2009). Membrane rafts have been identified in both human and mouse sperm, as well as sperm from other species, based on biochemical fractionation, cholera toxin B binding of ganglioside GM1, and perfringolysin O binding of sterols (Cross, 2004; Gamboa and Ramalho-Santos, 2005; Bou Khalil et al., 2006; Tsai et al., 2007; Tanphaichitr et al., 2007; Asano et al., 2009; Selvaraj et al., 2009).

2.10. Summary

Infertility is a major health problem affecting about eighty million people worldwide. Recent reports have found that men with unexplained infertility often have sperm that are defective in the acrosome reaction. The ability to undergo acrosomal exocytosis appears to be one of the most common defects causing infertility in human males with normal sperm count and morphology. Acrosomal exocytosis has some characteristics of traditional secretory vesicle exocytosis (e.g. triggered by an increase in intracellular Ca²⁺, change in phosphorylation status) but it is also unique. In comparison to secretory vesicles, the acrosome is a single large vesicle that is released slowly and not recycled. Rather than a single membrane fusion pore, the release of acrosomal contents is accomplished by hundreds of fusion pores.
2.11. Figures and Tables

Figure 2.1. **Mechanism of Egg-Sperm Interaction.** Fresh sperm (A) completes capacitation in the oviduct and penetrates the cumulus cell mass (B). The capacitated sperm acrosome reacts either before or at contact with the zona pellucida (C) allowing sperm to penetrate through the zona, bind to the oocyte membrane (D), and finally fuse with and activate the oocyte (E). PVS, perivitelline space; ZP, zona pellucida (Ikawa et al., 2010).
Figure 2.2. **Overview of mammalian fertilization.**

**A.** Mammalian Sperm;  
**B.** Mammalian Egg;  
**C.** Adhesion to Zona Pellucida;  
**D.** The Acrosome Reaction;  
**E.** Cell-Membrane Fusion (sperm and egg plasma membranes fusion);  
**F.** Destruction of sperm receptors on the ZP (Swanson and Vacquier, 2002).
Figure 2.3. **Molecular basis of signaling events associated with sperm capacitation.**

HCO₃⁻ and Ca²⁺ from the seminal fluid enter newly ejaculated sperm through a Na⁺ /HCO₃⁻ cotransporter (NBC), and calcium channels (e.g., CatSper) respectively, activating soluble adenylyl cyclase (SACY). The SACY, through the activation of protein tyrosine kinases and/or the inhibition of protein phosphatases, activates protein kinase A (PKA), which allows the activation of flagellar movement and prepares sperm for the acrosome reaction. The overall pathway is hypothesized to begin by the removal of cholesterol from the sperm plasma membrane. All these events are necessary for the sperm to acquire fertilizing capacity (BSA bovine serum albumin, PTK nonreceptor tyrosine kinases, PPs protein phosphatases, pHᵢ internal pH) (Signorelli et al., 2012).
Figure 2.4. A Model for the acrosome reaction. TRPC2–ZP3 receptors in the sperm head are activated when they adhere to the zona pellucida of the egg resulting in calcium entry through T-type channels, causing a transient increase of cytosolic calcium concentration and activation of PLC through a Gi1 and/or Gi2 protein-mediated pathway. This pathway mediates the production of IP3 and DAG. The transient calcium rise and PLC function together start a persistent calcium entry through a TRPC2 channel that triggers the acrosome reaction i.e. fusion of the outer acrosome membrane with the plasma membrane releasing acrosomal contents (Evans and Florman, 2002).
Figure 2.5. Differences between neuronal and acrosomal exocytosis. **A.** The pathway of synaptic vesicles in the nerve terminal divided into various stages (Jahn and Fasshauer, 2012). Please note the difference in two systems as discussed (Exocytosis in Somatic Cells). **B.** Temporal characteristics of the acrosome reaction in human sperm using a two-probe technique (Harper et al., 2008). IAM, Inner Acrosomal Membrane.
Figure 2.6. A model of regulated exocytosis. The critical aspect of this model is that priming might occur in two steps that involve partial and full assembly of the core complex. Only the fully assembled state, which is stabilized by a regulatory protein, complexin, can support fast, Ca\textsuperscript{2+}-triggered neurotransmitter release. SNAP-25, synaptosomal-associated protein of 25 KDa (Rizo and Südhof, 2002).
Figure 2.7. **A model of membranes fusion mediated by SNARE proteins.** Lipid membranes do not fuse with each other because of the presence of highly repulsive forces between them. The SNARE proteins are hypothesized to provide the energy that facilitates the lipid reorganizations in membranes necessary for fusion. Combining of the SNAREs brings two membranes close to each other forming a lipid stalk by the fusion of the proximal leaflet of the membranes. A hemifusion diaphragm can be developed by the expansion of the lipid stalk. The SNARE motifs are thought to provide the energy required for the fusion. The weakest points are considered to be at the brink of the hemifusion diaphragm. Finally the fusion process of membrane starts in the distal leaflets when membrane is ruptured at one of these weak points. Arrowheads in the stalk indicate regions of negative lipid curvature (Palfreyman and Jorgensen, 2008).
Figure 2.8. **SNARE proteins and the core SNARE complex.**

**A.** The structure of the core SNARE complex showing the helices from VAMP (synaptobrevin), syntaxin and SNAP-25. **B.** The structure of a trans SNARE complex (a model for minimal fusion machinery at a synapse) having two t-SNAREs, syntaxin and SNAP-25 found on the plasma membrane and a v-SNARE, synaptobrevin, present on the synaptic vesicle membrane (Huang et al., 2008).
Figure 2.9. **Protein tyrosine phosphorylation during capacitation and the acrosome reaction.** The above illustrations signify the possible protein tyrosine phosphorylation events associated with the sperm capacitation (A) and the acrosome reaction (B) (Ijiri et al., 2012).
Figure 2.10. **Regulation of exocytosis by phosphorylation.** The protein kinases that have been demonstrated to phosphorylate key proteins involved in regulated exocytosis are indicated. Where data are available for in vivo phosphorylation, the kinase is indicated with an asterisk (Burgoyne and Morgan, 2003).
Figure 2.11. **Components of membrane lipid rafts and caveolae.** In lipid rafts, the liquid-ordered phase is mainly composed of cholesterol and sphingolipids and the liquid-disordered phase is enriched in phospholipids (A), whereas in caveolae, the liquid-ordered domains form small cave-like invaginations called caveolae. Fourteen to sixteen individual caveolin monomers aggregate into discrete caveolin homo-oligomers. The structural meshwork for caveolae invagination is thought to be provided by the side by side packing of adjacent homo-oligomers within the caveolae. The caveolin oligomerization domain is blue whereas the caveolin oligomers are shown red (B) (Razani et al., 2002).
Figure 2.12. **History of the cell membrane raft concept.** This illustration shows how the rafts concept for sub-compartmentalization in cell membranes evolved through years with the advancement of technology to study rafts (Lingwood and Simons, 2010).
Figure 2.13. **Mechanism of activation of receptors, kinases, and calcium channels within lipid rafts of cells.** Activated raft components may already be present in native lipid rafts (A). Those molecules may move into lipid rafts after being activated by some signals (B, C). Smaller lipid rafts may aggregate to form a larger lipid raft containing activated components (D) (George and Wu, 2012).
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* retrieved from Phospho.ELM (Dinkel et al., 2011), PhosphoSitePlus (Hornbeck et al., 2012) and PHOSIDA (Gnad et al., 2011).
Table 2.2. Classification of membrane rafts.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Selected References</th>
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| Rafts      | • Native rafts found in living cells  
• Contain cholesterol, glycosphingolipids, and proteins (lipid-modified and trans membrane)  
• Are mobile having liquid-ordered phase with a diameter of 50 nanometers  | (Simons and Van Meer, 1988; Simons and Ikonen, 1997; Brown and London, 1998) |
| Clustered rafts | • Rafts clustered by lectins, antibodies, physiological crosslinking proteins and adjacent cell proteins  
• These are large in size, usually hundreds of nanometers to micrometers.  
• Clustering is used to activate signaling cascades of various biological functions | (Varma and Mayor, 1998; Friedrichson and Kurzchalia, 1998; Kenworthy, 2007) |
| DRM's      | • Rafts remained insoluble after treating with detergents such as NP-40, Triton X-100, and Brij-58  
• Demonstrate low density floatation in sucrose density gradients  
• Show variable characteristics depending on cell & detergent type and lipid to detergent ratio | (Simons and Ikonen, 1997; Brown and London, 1998; Kurzchalia and Partan, 1999; Hooper, 1999) |
| Caveolae   | • A highly specialized raft subcategory containing raft proteins, lipid and caveolins  
• Have ‘cave-like’ invaginations on exterior of the cell hence called caveolae | (Yamada, 1955; Parton, 1996; Anderson, 1998; Waugh et al., 1999; Smart et al., 1999) |
Table 2.3. Techniques to study membrane rafts.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Description</th>
<th>Selected References</th>
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| Fluorescence resonance energy transfer (FRET) | • A powerful approach which uses transfer of energy between an excited donor fluorophore and an acceptor molecule to determine proximity of the donor-acceptor in a live cell  
  • Can identify whether the components of two rafts are spatially close to each other or not | (Varma and Mayor, 1998; Pralle et al., 2000; Kenworthy et al., 2000; Rao and Mayor, 2005; Loua et al., 2009; de Almeida et al., 2009) |
| Photonic force microscopy | • A very informative method which determines the diffusion constant, size and dynamics of individual raft in live cells  
  • Requires specific technical skills and extremely specialized equipment for the analysis and acquisition of data | (Pralle et al., 2000) |
| Single-particle tracking (SPT)/Single-particle fluorescence tracking (SPFT)/Single fluorophore tracking microscopy (SFTM) | • An advanced method which uses translational trajectories of membrane molecules to monitor the diffusion and dynamics of individual raft proteins or lipids in a live cell  
  • Requires highly specialized tracking microscopy and skilled personnel | (Jacobson et al., 1995; Sheets et al., 1997; Saxton and Jacobson, 1997; Saxton, 1997; Jacobson and Dietrich, 1999; Schütz et al., 2000; Jacobson et al., 2007; Chen et al., 2007) |
| Detergent resistant membranes (DRMs) density gradient floatation | • A commonly used method which identifies raft proteins and putative rafts associations involved in signaling in non-living cells  
  • Has limitation as this method cannot detect weak association with rafts and can have possible artifacts | (Simons and Ikonen, 1997; Brown and London, 1998; Hooper, 1999) |
| Antibody patching & immunofluorescence microscopy (APIM) | • An easy to do commonly use approach which identifies putative raft association in non-living cells  
  • Better than density gradient floatation method as it can detect weak rafts association but quantification is a challenge because of cell-cell variability | (Harder et al., 1998; Janes et al., 1999) |
| Immuno-electron microscopy (IEM) | • Uses advanced immunological and electron microscopic techniques to determine location of raft components in non-living cells  
  • Require technical expertise to obtain promising results | (Fujimoto, 1996; Kurzchalia and Partan, 1999; Wilson et al., 2000) |
| Chemical crosslinking complexes (CCC) | • A straightforward method which identifies native raft proteins in live cells by using chemical crosslinking approach  
  • Selection of appropriate reaction and other experimental conditions is critical in this method | (Friedrichson and Kurzchalia, 1998) |
| Fluorescence correlation spectroscopy (FCS) Fluorescence recovery after photobleaching (FRAP), | • Relies on the translational mobility of a fluorophore to study rafts components  
  • Requires technical expertise | (Varma and Mayor, 1998; Rao and Mayor, 2005; Kenworthy, 2007; Kabayama et al., 2007) |
| Stimulated emission depletion (STED) microscopy | • A highly advanced method which relies on a single molecule diffusion of a fluorescence-labeled probe at the nano-scale to study raft and its associations  
  • Require technical expertise to obtain promising results | (Eggeling et al., 2009; Iwabuchi et al., 2012) |
2.12. References


Fujimoto, T. 1996. GPI-anchored proteins, glycosphingolipids, and sphingomyelin are sequestered to caveolae only after crosslinking. *J.Histochem.Cytochem.* 44:929-941.


Weaver, A.K., M.L. Olsen, M.B. McFerrin, and H. Sontheimer. 2007. BK channels are linked to inositol 1,4,5-triphosphate receptors via lipid rafts: a novel mechanism for coupling [Ca(2+)](i) to ion channel activation. *J.Biol.Chem.* 282:31558-31568.


CHAPTER 3

SNARE PROTEINS ARE RE-LOCALIZED INTO RAFTS DURING MOUSE SPERM CAPACITATION IN A BSA DEPENDENT MANNER

3.1. Abstract

Rafts are heterogeneous micro-domains enriched in sterol and sphingolipids that act as platforms for trafficking or docking molecules. Studies have shown that as sperm capacitate; proteins including some SNAREs (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) shift into or out of membrane rafts. This movement could allow SNAREpin complexes to form, promoting membrane fusion at the site of rafts. However, it is unclear if membrane fusion occurs at rafts containing SNAREs. Importantly, capacitation can be induced by incubation with bovine serum albumin (BSA) (cholesterol acceptor) in a bicarbonate-based medium; however, it is not certain if the loss in cholesterol is from raft or non-raft fractions, which might de-stabilize or stabilize rafts, respectively. I hypothesized that capacitation shifts SNAREs into membrane rafts to form trans-SNARE complexes, which are ultimately the site of membrane fusion. This change is BSA dependent. To test this hypothesis, sperm from wild type (CD-1) male mice were incubated in dmKRBT(capacitating), dmKRBT without BSA and HCO$_3^-$ (non-capacitating), dmKRBT without BSA or dmKRBT without HCO$_3^-$ for 0, 30 and 60 min. Sperm membrane fractions were extracted using a detergent-free method, subjected to a sucrose density gradient ultracentrifugation and were analyzed by gradient SDS-PAGE. Subsequently SNAREs in different fractions were detected by immunoblotting with antibodies to synaptobrevin and syntaxin. Caveolin served as a raft marker for rafts. My results show that during capacitation syntaxin and synaptobrevin move from non-raft fractions into rafts, and this movement was dependent on BSA, not on HCO$_3^-$.
PKA inhibitor, H8, the SNARE re-localization was completely blocked. These results suggest that capacitation re-localizes SNAREs into rafts in a BSA and PKA dependent manner, allowing trans-SNARE complex formation, which may ultimately, promotes membrane fusion to occur at these sites.

3.2. Introduction

In somatic cells, SNAREs are sometimes found in laterally organized dynamic 10-200 nm membrane specializations known as rafts. Rafts are heterogeneous microdomains (Razani et al., 2002), enriched in sterol and sphingolipids that act as platforms for trafficking or docking molecules (Munro, 2003; Pike, 2004; Pike, 2006). The lipid composition of these domains and the tight packing of the acyl lipid chains make these fractions resistant to detergents or NaCO₃ solubilization. Resistance to solubilization and buoyant density has been used to purify rafts. Beginning in 1970s, the raft concept evolved gradually with the advancement of techniques to study rafts (Lingwood and Simons, 2010). Based on organization of components, rafts are traditionally classified into four categories; rafts, clustered rafts, detergent resistant membranes (DRMs) and caveolae (Simons and Toomre, 2000).

Rafts are classically defined by their relative insolubility in mild non-ionic detergents (i.e. Triton X-100) at cold temperatures. They have the ability to sequester specific proteins and lipids and exclude others. Rafts are thought to play vital roles in various important biological events such as membrane fusion (Liu et al., 2001; Liu and Baker, 2003), protein trafficking (Yanagimachi, 1994b; De Blas et al., 2005; Dube et al., 2005b), signal transduction (Retzloff and Hornstein, 2003; Sleight et al., 2005; Gamboa and Ramalho-Santos, 2005; Seshagiri et al., 2007), lipid sorting (Wassarman et al., 2001; Miller et al., 2002), and organization of the cytoskeleton (Gadella and Van Gestel, 2004; Dean, 2004). Recent studies have found that
syntaxin, synaptobrevin, SNAP-25 and SNAP-23 partition into Triton X-100 insoluble raft fractions and can form an SDS-resistant ternary complex (Chamberlain and Gould, 2002; Salaun et al., 2005; Gil et al., 2006; Puri and Roche, 2006). Some syntaxins probably associate with rafts because they bind SNAP-23 or SNAP-25, palmitoylated proteins whose acyl group may localize them to rafts (Chamberlain and Gould, 2002; Salaun et al., 2005; Gil et al., 2006; Puri and Roche, 2006). It has been proposed that rafts organize exocytotic proteins and ion channels to coordinate their functions in secretion (Xia et al., 2004; Martens et al., 2004; Xia et al., 2007; Weaver et al., 2007). Interestingly, it is also known that syntaxins cluster at docking and fusion sites for vesicle secretion (Lang et al., 2001). This finding about syntaxins is consistent with the membrane fusion model in which trans-SNARE complexes (SNAREpins) form and zip together to overcome the energy barrier and promote membrane fusion at these sites. However, most data supporting the SNARE’s zipper hypothesis comes from experiments using recombinant proteins reconstituted in liposomes (Weber et al., 2000), from in vitro studies of fusion among yeast vacuoles genetically manipulated to prevent formation of cis complexes (Ungermann et al., 1998), and from fusion of cells expressing “flipped” SNAREs on the outer surface of the plasma membrane (Giraudo et al., 2006).

A very attractive model is that membrane rafts serve to organize syntaxins, SNAP-25 and other regulatory proteins together at sites of future membrane fusion. This protein complex could then form trans-SNARE complex with VAMP/synaptobrevin in vesicles (e.g. the acrosome). Research with boar sperm has shown that as sperm capacitate, proteins including some SNAREs re-localize into or out of detergent resistant membranes (Tsai et al., 2007). Re-localization could allow SNAREpin complexes to form, promoting membrane fusion at the site of rafts. However, it is unclear whether docking and fusion begin at sites of membrane rafts (Lang et al., 2001;
Ikonen, 2001; Ohara-Imaizumi et al., 2004) and if these rafts contain SNAREs at the time of fusion. With the advancement of temporal and spatial resolution technology, we now have better ways to study the rafts, raft association and signaling associated with rafts (Simons and Toomre, 2000; Simons and Gerl, 2010; Sonnino and Prinetti, 2013).

Sperm possess unusual lipids and are believed to contain membrane rafts although, based on the ganglioside GM1 localization, the rafts may be very large, perhaps micron scale (Trevino et al., 2001; Shadan et al., 2004; Cross, 2004; Selvaraj et al., 2006; Selvaraj et al., 2007; Tanphaichitr et al., 2007; Selvaraj et al., 2009; Asano et al., 2009). Nonetheless, the loss of cholesterol, a major component of rafts, has a profound effect on their function and the protein composition of rafts (Travis et al., 2001; Sleight et al., 2005; Tanphaichitr et al., 2007). Importantly, capacitation can be induced by incubation with a cholesterol acceptor in bicarbonate-based medium. However, it is not certain if the loss in cholesterol is from raft or non-raft fractions, which might de-stabilize or stabilize rafts, respectively (Cross, 2004; Sleight et al., 2005; van Gestel et al., 2005; Bou Khalil et al., 2006; Tanphaichitr et al., 2007). Capacitation also induces a re-localization of rafts, as detected by cholera toxin-B binding to GM1 (Selvaraj et al., 2007), which is consistent with my hypothesis.

The reports that syntaxin and synaptobrevin are found in rafts from sperm (Travis et al., 2001; Tsai et al., 2007) suggest a model in which, at least conceptually, sperm capacitation may have similarities to vesicle docking and/or priming in neurons and may shift SNAREs to specific sites in the plasma membrane in preparation for fusion (Tsai et al., 2010; Tsai et al., 2012). As these rafts are re-localized or modified, the protein composition of rafts may also change correspondingly. One report concluded that there was a modest change in localization and raft partitioning of syntaxins 1 and 2 and synaptobrevin as boar sperm capacitate (Tsai et al., 2007).
The researchers used 1% Triton X-100 to isolate rafts. The field of lipid rafts has been very controversial (Calder and Yaqoob, 2007) mainly because of pitfalls present in methods to study rafts. Despite being widely used and instrumental in rafts discovery, the detergent extraction method (Simons and Ikonen, 1997; Brown and London, 1998; Hooper, 1999) has its own limitations (Shogomori and Brown, 2003). There are serious concerns about dynamics, size and properties of rafts studied by this method as this involves breaking up the membranes (Lai, 2003; Pike, 2003; Pike, 2004; Pike, 2006). Thus, the detergent extraction method remains a controversial method to isolate rafts. Yet most studies of sperm membrane rafts, to date, have used this method. Recently many new approaches to study rafts have been developed that use intact cells (Simons and Gerl, 2010) but many are not amenable to sperm or do not allow analysis of the protein components of rafts (Eggeling et al., 2009; Iwabuchi et al., 2012). These modern techniques include fluorescence resonance energy transfer (FRET) (Varma and Mayor, 1998; Pralle et al., 2000; Kenworthy et al., 2000; Rao and Mayor, 2005; Loua et al., 2009; de Almeida et al., 2009), photonic force microscopy (Pralle et al., 2000), single-particle tracking (SPT)/ single-particle fluorescence tracking (SPFT)/ single fluorophore tracking microscopy(SFTM) (Jacobson et al., 1995; Sheets et al., 1997; Saxton and Jacobson, 1997; Saxton, 1997; Jacobson and Dietrich, 1999; Jacobson et al., 2007; Chen et al., 2007), fluorescence correlation spectroscopy (FCS)/ fluorescence recovery after photobleaching (FRAP) (Varma and Mayor, 1998; Rao and Mayor, 2005; Kenworthy, 2007; Kabayama et al., 2007) and stimulated emission depletion (STED) microscopy (Eggeling et al., 2009; Iwabuchi et al., 2012).

The detergent-free extraction method is relatively artifact free and has been successfully applied to study rafts in somatic as well as sperm cells (Shah and Sehgal, 2007; Persaud-Sawin et
al., 2009; Sanchez-Wandelmer et al., 2010; Casado et al., 2012). This method is suitable to study sperm as a large number of sperm can be used that gives sufficient amount of raft components (proteins etc.) to study their properties.

Membrane rafts have been identified in both human and mouse sperm, as well as sperm from other species, based on biochemical fractionation, cholera toxin B binding of ganglioside GM1, and perfringolysin O binding of sterols (Cross, 2004; Gamboa and Ramalho-Santos, 2005; Bou Khalil et al., 2006; Tsai et al., 2007; Tanphaichitr et al., 2007; Asano et al., 2009; Selvaraj et al., 2009). However little is known about the organization and protein associations of rafts during capacitation. The purpose of the current study was to determine whether SNARE proteins move into rafts during capacitation and how this re-localization is regulated. The findings of this study demonstrate that two SNARE proteins, syntaxin and synaptobrevin move into membrane rafts during mouse sperm capacitation. Our data further demonstrate that this movement of SNAREs is BSA dependent and requires protein kinase A.

3.3. Materials and Methods

3.3.1. Reagents and Animals

Wild type CD-1 mice were purchased from Harlan Sprague-Dawley, Inc. and transgenic (Syb-KI) mice were obtained from Jens Rettig in Hamburg, Germany. Antibodies to Syntaxin 2 and Synaptobrevin 2 were purchased from Synaptic System, Germany. Caveolin antibody was from BD Biosciences, Franklin Lakes, NJ and Synaptic Systems, Goettingen, Germany. Phosphotyrosine and GAPDH antibodies were purchased from Millipore, Billerica, MA and Sigma-Aldrich, St. Louis, MO respectively. Protein markers and precast gels were from Bio-Rad, Hercules, CA. HRP -secondary antibody and ECL substrate was purchased from GE
Healthcare, Little Chalfont, UK. The proteinase inhibitor cocktail (Cat# S8830) was purchased from Sigma-Aldrich, Saint Louis MO) and the phosphatase inhibitor cocktail (Cat# 78428) was purchased from Thermo Scientific, Pittsburgh, PA. The inhibitors, H8, SU6656 and okadaic acid were obtained from Calbiochem (now EMD Millipore, Darmstadt, Germany).

3.3.2. Preparation of Mouse Sperm

To obtain sperm, at least 10 mature male mice were euthanized by CO₂ asphyxiation in accordance with Institutional Animal Care and Use Committee guidelines and the cauda epididymides were isolated. The University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee approved experimental protocols. Sperm were collected from the epididymis by piercing it with a 23-gauge needle and squeezing the sperm into a 60 mm petri dish containing 3ml dmKRBT. The petri dish was placed into a 37 °C incubator for 10 minutes to allow sperm to swim out. Sperm concentration and motility were checked under a microscope using a hematocytometer. Samples having at least 1.5x10⁶ sperm/ml and 65% motility were considered for further experiments. The sperm sample was divided into appropriate aliquots and incubated either in capacitating (dmKRBT: 120mM NaCl, 2mM KCl, 2mM CaCl², 10mM NaHCO₃, 1.2mM MgSO₄, 0.36mM NaH₂PO₄, 5.6mM glucose, 1.1mM pyruvic acid, 25mM TAPSO, 18.5mM sucrose, 0.6% BSA, 10units/ml penicillin, and 10μg/ml streptomycin (pH 7.3)) or non-capacitating (dmKRBT without sodium bicarbonate and BSA) medium depending on the experiment. Sperm were then processed through a Percoll cushion to get viable and debris-free sperm (Furimsky et al., 2005) Briefly 0.5 mL of 45% percoll was layered on 0.5 mL of 90% percoll in an eppendorf tube. The gradient was centrifuged at 650 × g for 30 min at 25°C. This allowed immotile sperm to layer at interface between the two Percoll layers and motile sperm to sediment as a pellet. The supernatant containing immotile sperm and percoll
solutions were removed. The pellet was suspended in dmKRBT and centrifuged at 450 × g for 10 min at 25°C. The supernatant was removed and the final pellet containing sperm was further processed according to a specific protocol depending on the experiment.

### 3.3.3. Lipid Rafts Extraction and Protein Quantification

Samples were thawed on ice and homogenized in 500 μL of detergent-free lysis buffer (1× TBS:150 mM NaCl, 10 mM Tris pH8, 1% proteinase inhibitor cocktail, 1 mM PMSF, 5 mM NaF, 1 mM Na Orthovanadate, 1 mM CaCl₂, and 1 mM MgCl₂)(Macdonald and Pike, 2005; Shah and Sehgal, 2007; Persaud-Sawin et al., 2009). The homogenate was sheared through a 23-gauge needle with 20 complete passes, subsequently centrifuged at 1,000 × g for 10 min at 4°C. The postnuclear supernatant (PNS) was removed and maintained on ice. The same procedure was repeated on the pellet and the final pellet was discarded. The supernatants from both shearings were mixed and stored either at −80°C for later use or directly subjected to a sucrose density gradient ultracentrifugation using a 5 mL thin wall, Ultra-Clear™ tube and SW 55 Ti rotor (Beckman Coulter). A 250 μL volume of the pooled supernatant was placed in a pre-cooled ultracentrifuge tube on ice and mixed with 225 μL of 85% sucrose/TBS with gentle pipetting to prevent the formation of bubbles. To this mixture, 3.0 mL of 35% sucrose/TBS was overlayed, followed by 675 μL 5% sucrose/TBS. The tubes were centrifuged (Beckman Coulter) at 200,000 × g for 18 h at 4°C with the deceleration rate set to zero. Finally, eight sequential fractions of 260 μL from the top of the tube were gently removed and aliquoted separately. The protein profile of the sperm lysate was determined by bicinchoninic acid protein assay using BSA as a standard (Pierce, Rockford, IL).
3.3.4. SDS-PAGE Electrophoresis

To detect SNARE proteins, the sperm lysate or membrane fraction samples were treated for 30 min at RT in a standard Laemmli sample buffer, or were denatured by boiling for 5 min in reducing Laemmli buffer (Laemmli, 1970). 20 μg of sperm protein or 50 μL of fraction samples were loaded on precast 4–16% or 4-20% linear gradient gel (Bio-Rad Laboratories, Inc. Hercules, CA ).

3.3.5. Western Blot Analysis

For western blots, proteins on gels were transferred to a nitrocellulose membrane. The membranes were then blocked with 5% non-fat milk or 3% BSA in Tris-buffered saline (pH 7.6) with 0.1% Tween-20 (TBST) for 1 h at RT or overnight at 4 °C with gentle agitation. After blocking, the membranes were washed three times (10 min each) with TBST and then incubated with primary antibody. After incubation for overnight with a 1:1000 dilution of primary antibody, membranes were washed six times (5 min each), and then incubated at ~25°C for 1 h with HRP-conjugated secondary antibodies at a 1:5,000 dilution. All secondary antibody incubations were with 2.5 % Milk in TBST. The membrane was rinsed briefly with water and then HRP activity was visualized with ECL chemiluminescence substrate and data collected using an ImageQuant 4010 (GE HealthCare) imaging system.

3.3.6. Data Analysis

All experiments were conducted with pooled semen samples obtained from at least 10 mature male mice and each was repeated for a minimum of three times unless otherwise stated. Bands intensities were measured using ImageQuant TL software from GE HealthCare. Combined bands intensity in low density raft fractions (1-4) was normalized as the percentage of
overall syntaxin bands intensities in all 8 fractions for each incubation time. The correlation among biological replicates was assessed by the Pearson Correlation Coefficient test. Statistical significance was determined using an analysis of variance (ANOVA) and specific comparisons were made by writing contrasts in proc glm procedure using SAS software. Significant difference between specific comparisons was concluded if p < 0.05.

3.4. Results

3.4.1. Detection of SNARE proteins in mouse sperm

Homologues of the t-SNARE syntaxin and the v-SNARE VAMP/synaptobrevin that are proposed to be critical for the acrosome reaction are present in mouse sperm (Ramalho-Santos et al., 2000). My first goal was to confirm that syntaxin and synaptobrevin were present sperm from wild type CD-1 and mRFP-synaptobrevin knock-in mice, respectively. Syntaxin was detected in sperm from wild type mice by western blot (Fig. 3.1B). Detecting synaptobrevin from wild type mouse sperm is difficult, so I used sperm from a knock-in mouse line. In this transgenic mouse line, mRFP was fused to the synaptobrevin C-terminus. The mice are fertile, neural tissues from the mRFP line have normal electrophysiology and normal localization of the fusion protein. Synaptobrevin from mRFP-synaptobrevin knock-in mouse sperm was detected by western blot using an mRFP antibody (Fig. 3.1C). GAPDH served as loading control for western blot experiments (Fig. 3.1). Thus I was able to detect two “neuronal” SNAREs in mouse sperm.

3.4.2. Capacitation shifts SNAREs into sperm rafts

To test if SNARE proteins move into lipid rafts during capacitation, capacitated sperm and sperm not incubated in capacitating conditions were extracted using a detergent-free method (Macdonald and Pike, 2005; Shah and Sehgal, 2007; Persaud-Sawin et al., 2009). Rafts
were separated by sucrose density gradient ultra-centrifugation. Proteins in different fractions were separated by SDS-PAGE. The transferred proteins were immunoblotted with syntaxin antibody to detect the presence of SNAREs in raft fractions. In sperm that were not capacitated, syntaxin was found mostly in the high density fractions (not observed in low-density fractions 1-4) and in the insoluble pellet (Fig. 3.2). In contrast, after 30 min of capacitation, more syntaxin was found in the low density fractions that contain sperm rafts. After 60 min of time for capacitation, at which time most sperm have completed capacitation; most syntaxin was found in the low density rafts. The same experiment was repeated to detect synaptobrevin in different fractions. Prior to capacitation, most synaptobrevin was found in high density fractions but after capacitation, some synaptobrevin had shifted into the low density fractions (Fig. 3.3). This change in syntaxin and synaptobrevin was not simply due to incubation time because syntaxin did not move to low density raft fractions in a mock incubation in which the medium lacked BSA and bicarbonate (Fig.3.2 & 3.3). The results of these experiment show that capacitation promote re-localization of syntaxin and synaptobrevin into low-density lipid rafts although synaptobrevin re-localization was less abundant as compared to syntaxin.

3.4.3. Caveolin does not re-localize during capacitation

Caveolin is believed to be associated with membrane rafts although in sperm it does not form caveolae (Thaler et al., 2006; Brown and Jacobson, 2008; Baltiérrez-Hoyos et al., 2012). In order to test whether caveolin is re-localized during capacitation, we ascertained if caveolin was present in low density membrane fractions from sperm. Caveolin was found in low-density fractions (Fraction 1-3) under all condition suggesting that capacitation does not re-localize or re-organize caveolin in membrane rafts (Fig. 3.4). Hence caveolin served as a marker for rafts in our re-localization experiments.
3.4.4. Membrane cholesterol depletion is sufficient for SNARE proteins relocation into lipid rafts

In these experiments I tested whether cholesterol depletion or activation of soluble adenylate cyclase is needed for SNAREs movement to the lipid rafts in preparation for the acrosome reaction. I incubated sperm in capacitating medium with no BSA (cholesterol accepter) or with no bicarbonate (soluble adenylate cyclase activator). The capacitating medium lacking BSA did not induce syntaxin re-localization (Fig. 3.2) or synaptobrevin re-localization (Fig 3.3). Syntaxin and synaptobrevin from sperm in medium lacking bicarbonate were delayed in their movement into low density raft fractions and a lower proportion of the SNAREs shifted into rafts (Fig 3.2 and 3.3). The raft marker caveolin did not move into lower density fractions in any condition. These data demonstrate that cholesterol depletion by BSA and to a lesser degree bicarbonate are necessary for SNARE movement into sperm membrane rafts.

3.4.5. Phosphorylation changes in SNAREs mediated by PKA are required for SNAREs movement into rafts

Bicarbonate activates a soluble adenylate cyclase in sperm that, in turn, produces cAMP and activated Protein Kinase A and a variety of protein phosphorylation steps that are a hallmark of capacitation (Visconti et al., 1995; Jha and Shivaji, 2002). Because bicarbonate-free medium were less effective at promoting SNARE movement into rafts, I tested the effect of the cell permeable Protein Kinase A inhibitor (H8) on SNARE re-localization during capacitation. To test the effect of the inhibitor, sperm were pre-incubated with inhibitors in non-capacitating medium for 15 min prior to dilution in capacitating medium and the beginning of the capacitating period. Controls were incubated with vehicle. A negative “incubation control” was
performed in medium lacking BSA and bicarbonate that does not allow sperm capacitation. After 60 min incubation, the sperm membrane fractions were extracted and processed for the detection of SNAREs. In this experiment, the low density and high density fractions were combined prior to the western blot. Results showed that syntaxin and synaptobrevin movement into low density fractions was blocked by H8 but not by the tyrosine kinase inhibitor SU6656 or okadaic acid (inhibitors of type 1 and 2A serine/threonine phosphatases). These results demonstrate that sperm protein kinase A is necessary for the movement of some SNARE proteins into rafts suggest a novel method for regulation of membrane raft composition (Fig. 3.5).

3.5. Discussion

The main findings of this study are that during capacitation two SNARE proteins (syntaxin and synaptobrevin) move into rafts and this movement is dependent on cholesterol depletion but only partially on SACY. The PKA pathway is required in this re-localization. However capacitation does not seem to have a gross effect on membrane rafts because the amount of caveolin in low density rafts is unaffected by capacitation. My results show that syntaxin and synaptobrevin proteins are re-localized into low-density raft fractions prepared without detergent in a BSA dependent manner. In contrast, there was no movement of caveolin observed from high to low density raft fractions. There are controversies in the literature about whether caveolin moves into sperm rafts during capacitation (Travis et al., 2001; Gamboa and Ramalho-Santos, 2005; Thaler et al., 2006; Kabayama et al., 2007; Baltiérrez-Hoyos et al., 2012). Some reports show that caveolin is reorganized during capacitation (Sleight et al., 2005; Thaler et al., 2006; Baltiérrez-Hoyos et al., 2012) whereas others results agree with our findings (van Gestel et al., 2005). Our results suggest that caveolin
does not move but remains in raft fractions during capacitation. The differences in the literature may be attributed to the extraction methods used.

Sperm capacitation is modulated by removal of cholesterol from the sperm plasma membrane (Garbers et al., 1982; Okamura et al., 1985; Visconti et al., 2011). Rafts may provide a platform for SNAREs and other sperm proteins important for binding to the egg and exocytosis of the acrosome (Shadan et al., 2004; Cross, 2004; van Gestel et al., 2005; Tanphaichitr et al., 2007). In somatic cells, several signaling pathways involving tyrosine kinases, G protein, etc. are activated by cholesterol-binding reagents such as beta-cyclodextrins (Brown and London, 1998; Kabouridis et al., 2000). Bicarbonate is also an important component for capacitation that regulates PKA and Ca^{2+} pathways through SACY pathway. My findings suggest that the removal of cholesterol from the membrane but not bicarbonate (SACY) was critical for SNAREs re-localization into rafts.

Based on published data (Tsai et al., 2007; Salicioni et al., 2007; Visconti, 2009; Xia and Ren, 2009; Visconti et al., 2011) and my results, I propose a model for SNAREs re-localization into rafts (Fig. 3.6). BSA present in the capacitating media regulates SNAREs re-localization pathway as it does with the capacitation pathway. It removes cholesterol from the sperm membrane, which in turn is believed to activate PKA pathways and Ca^{2+} influx through cat sper calcium channels by an unknown mechanism. The increase in Ca^{2+} and activation of PKA ultimately activates tyrosine kinases and hence tyrosine phosphorylation of sperm proteins which is a prerequisite of capacitation. Tyrosine phosphorylation/capacitation finally promotes SNARE’s re-localization into membrane rafts to prepare sperm for the acrosome reaction. These SNAREs may form a core SNARE complex, a necessary step for most of the exocytosis processes including the acrosome reaction. In my pharmacological experiment, I noticed that
SNAREs re-localization into rafts was blocked in the capacitating medium containing H8, a specific PKA inhibitor. Whereas media lacking bicarbonate did not stop re-localization of SNAREs into rafts, suggesting that this process is regulated by PKA pathway that is independent of bicarbonate. I speculate that re-location of SNAREs into membrane rafts follows the PKA and Ca\(^{2+}\) pathways that may be regulated by BSA (cholesterol removal) as the media lacking BSA blocked re-location of SNAREs.

This is the first study showing that capacitation promotes SNARE re-localization into membrane rafts in preparation for the acrosome reaction in mouse sperm. This re-localization was dependent on cholesterol removal from the membrane and partially dependent on bicarbonate, an activator of SACY. Blocking PKA with H8 prevented SNARE protein movement into rafts. Re-localization of SNAREs into rafts suggest that rafts may provide SNAREs a platform for core SNARE complex formation and priming for fusion, which are hallmarks of membrane fusion.
3.6. Figures

A

B

C

Figure 3.1. **Detection of SNARE proteins in mouse sperm.** A. A model showing localization of synaptobrevin in acrosome tagged with mRFP at the C terminal in mRFP-synaptobrevin knock-in mice. B. Detection of syntaxin from wild type mouse sperm and brain samples by blotting with anti-syntaxin antibody. C. Detection of synaptobrevin from mRFP-synaptobrevin knock-in mouse sperm and brain by blotting with anti-mRFP antibody. The molecular weights of the bands are provided.
Figure 3.2. Syntaxin reorganization into sperm membrane rafts during capacitation. **A.** Compilation of representative western blot images showing the reorganization of syntaxin. Lipid raft (1-4) and nonraft (5-8) fractions were separated by centrifugation using the detergent-free sucrose gradient method from sperm incubated for 0, 30 and 60 min in capacitating dmKRBT (C), non-capacitating dmKRBT (N) or capacitating dmKRBT lacking BSA (-B) or bicarbonate (-H), separated by SDS-PAGE and subsequently immunoblotted for syntaxin. **B.** A graph showing the relative change in combined intensity of bands for syntaxin in 4 low-density (rafts) fractions, where at time zero the net change in syntaxin intensity in all low density fractions was zero in all treatment groups. Syntaxin shifted into raft fractions in capacitating medium. BSA and to a lesser degree, HCO$_3^-$ were required for this shift. The values in the graph show the average from three independent experiments. Combined syntaxin bands intensity in low density raft fractions (1-4) was normalized as the percentage of overall syntaxin bands intensities in all 8 fractions for each incubation time. For the graph, the sum of all signals in the low density (raft) fractions was divided by the total signal in all 8 fractions. The graph shows the net change from 0 min for each treatment. A value of 100% means that all signal is in the low density fractions. Different letters indicate significant difference between treatment groups and incubation times.
Figure 3.3. Synaptobrevin reorganization into sperm membrane rafts during capacitation. A.
Compilation of representative western blot images showing the reorganization of synaptobrevin. Lipid raft (1-4) and nonraft (5-8) fractions were separated by centrifugation using the detergent-free sucrose gradient method from sperm incubated for 0, 30 and 60 min in capacitating dmKRBT (C), non-capacitating dmKRBT (N) or capacitating dmKRBT lacking BSA (-B) or bicarbonate (-H), separated by SDS-PAGE and subsequently immunoblotted for synaptobrevin. B. A graph showing the relative change in combined intensity of bands for synaptobrevin in 4 low-density (rafts) fractions, where at time zero the net change in synaptobrevin intensity in all low density fractions was zero in all treatment groups. Synaptobrevin shifted into raft fractions in capacitating medium. BSA and to a lesser degree, HCO₃⁻ were required for this shift. The values in the graph show the average from three independent experiments. Combined syntaxin bands intensity in low density raft fractions (1-4) was normalized as the percentage of overall syntaxin bands intensities in all 8 fractions for each incubation time. For the graph, the sum of all signals in the low density (raft) fractions was divided by the total signal in all 8 fractions. The graph shows the net change from 0 min for each treatment. A value of 100% means that all signal is in the low density fractions. Different letters indicate significant difference between treatment groups and incubation times.
Figure 3.4. The proportion of caveolin in sperm membrane rafts does not change during capacitation. A. A compilation representative western blot images showing the reorganization of caveolin. Lipid raft fractions were separated by ultracentrifugation using the detergent-free sucrose gradient method from sperm incubated for 0, 30 and 60 min in capacitating dmKRBT (C), non-capacitating dmKRBT (N) or dmKRBT lacking BSA (-B) or bicarbonate (-H), separated by SDS-PAGE and subsequently immunoblotted for caveolin. B. A graph showing the relative change in combined intensity of bands for caveolin in 4 low-density (rafts) fractions, where at time zero the net change in caveolin intensity in all low density fractions was zero in all treatment groups. The values in the graph show the average from three independent experiments. Combined caveolin bands intensity in low density raft fractions (1-4) was normalized as the percentage of overall caveolin bands intensities in all 8 fractions for each incubation time. For the graph, the sum of all signals in the low density (raft) fractions was divided by the total signal in all 8 fractions. The graph shows the net change from 0 min for each treatment. A value of 100% means that all signal is in the low density fractions.
Figure 3.5. **Reorganization of syntaxin into rafts is dependent on PKA activity.** A. A representative western blot showing the reorganization of syntaxin. Sperm were incubated for 60 min in capacitating dmKRBT (C-60), non-capacitating dmKRBT (N-60) or capacitating dmKRBT with H8 inhibitor (H-60) medium, separated by SDS-PAGE and immunoblotted for caveolin and syntaxin. In this experiment, the high density (HDF) and low density (LDF) fractions were combined prior to western blotting. B. A Scatter plot of two experiments showing bands intensity for syntaxin and caveolin in low density fraction (LDF) from Figure 3.5- A. Syntaxin showed re-localization from the high density fraction (HDF) to LDF when in capacitating medium but this movement was blocked by H8 (a specific PKA inhibitor). Bands intensity for syntaxin was similar to that of non-capacitating and control bands in LDF. A Pearson coefficient of .95 showed a high consistency between two biological replicates.
Figure 3.6. Proposed mechanism of SNARE movement into membrane rafts during mouse sperm capacitation. Bicarbonate present in capacitating medium enters sperm through NBC and activates SACY which ultimately activates PKA pathways. Whereas BSA removes cholesterol from the sperm membrane and is believed to activate PKA pathways and Ca^{2+} influx through CatSper by an unknown mechanism, it consequently increases tyrosine phosphorylation of sperm proteins. Tyrosine phosphorylation ultimately leads to sperm capacitation and promotes the movement of SNARE into membrane rafts to prepare sperm for the acrosome reaction. A specific PKA inhibitor, H8, blocked re-localization whereas media lacking bicarbonate only partially reduced movement of SNAREs into rafts. SNARE movement requires PKA and perhaps Ca^{2+}-activated pathways that may be regulated by membrane cholesterol removal by BSA. Broken arrows indicate that there might be multiple unknown steps from start to the target point. NBC, sodium bicarbonate co-transporter; SACY, soluble adenylate cyclase; PKA, Protein kinase A
3.7. References


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CHAPTER 4

SNARE PHOSPHORYLATION IS ALTERED PRIOR TO MOUSE SPERM ACROSOMAL EXOCYTOSIS

4.1. Abstract

During sperm capacitation, protein kinases and phosphatases are activated that may alter phosphorylation of all three core SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins and other SNARE regulatory proteins. I hypothesized that during capacitation, SNARE protein phosphorylation is altered to promote SNARE complex formation and the acrosome reaction. To test this hypothesis, mouse sperm were incubated in a capacitating (dmKRBT) or non-capacitating (dmKRBT without BSA and HCO₃⁻) medium. To isolate formed SNARE complexes, sperm detergent extracts were immunoprecipitated with a syntaxin antibody and subsequently separated by SDS-PAGE. Samples were not boiled prior to SDS-PAGE to maintain the integrity of the SNARE complex. Immunoprecipitated SNARE complexes were separated by SDS-PAGE and stained with Pro-Q Diamond to detect changes in total phosphorylation. Phosphoproteins were detected in 75, 100, and 150 and 230 KD protein complexes. Immunoblotting with a syntaxin antibody demonstrated that each complex contained syntaxin and was presumably a SNARE complex. Quantitation of SNARE complex phosphorylation showed that after 30 min of capacitation, overall phosphorylation was higher in the 75, 100 and 150 KD bands. To detect phosphorylation on tyrosine residues of syntaxin-containing protein complexes, the syntaxin immunoprecipitates were probed with a phosphotyrosine antibody. Tyrosine phosphorylation of SNARE complexes decreased 15 min after the initiation of capacitation. These data demonstrate SNARE complex phosphorylation is a
dynamic process during capacitation and suggest that phosphorylation may regulate SNARE complex formation during capacitation in preparation for the acrosome reaction.

4.2. Introduction

It is well established that regulated exocytosis in somatic cells is modulated at various steps by protein phosphorylation (Turner et al., 1999; Lin and Scheller, 2000; Klenchin and Martin, 2000; Morgan et al., 2005). Protein kinase A inhibitors and phosphatase inhibitors affect chromaffin cell exocytosis at the vesicle “priming” step (Nagy et al., 2004). A number of SNAREs and SNARE regulatory proteins can be phosphorylated and, at least in some cases, phosphorylation alters their biological activity. Because syntaxin and complexin regulate SNARE formation and stabilization, their phosphorylation may be most relevant for capacitation. It is known that complexin I/II phosphorylation increases their affinity for the SNARE complex (Shata et al., 2007). Syntaxin phosphorylation affects its ability to bind SNAP-25/SNAREs and whether binding is increased or decreased depends on the amino acid that is phosphorylated (Foster et al., 1998; Foletti et al., 2000; Risinger and Bennett, 2002; Dubois et al., 2002). Because synaptotagmin is likely a Ca$^{2+}$ sensor, its phosphorylation may control acrosomal exocytosis. Interestingly, when recombinant synaptotagmin VI was phosphorylated by protein kinase C, it became unable to inhibit acrosomal exocytosis when added to permeabilized human sperm, suggesting that PKC-catalyzed phosphorylation may regulate synaptotagmin function in sperm (Michaut et al., 2001; Roggero et al., 2007). Although there is considerable evidence that protein phosphorylation is important for sperm capacitation and acrosomal exocytosis, it is not known whether sperm SNAREs are phosphorylated and, if so, when phosphorylation status changes (during capacitation or exocytosis) and how it affects function of SNAREs. In view of the role of SNARE phosphorylation in regulating the “priming” stage of neuronal exocytosis
(Nagy et al., 2004; Hepp et al., 2005), capacitation may be viewed as slower “priming” for acrosomal exocytosis.

The regulation of neuronal exocytosis by phosphorylation is of interest in view of the well-known changes in protein tyrosine phosphorylation during sperm capacitation (Osheroff et al., 1999; Dube et al., 2005; Jha et al., 2006; Salicioni et al., 2007). Whether, in fact, priming occurs in sperm as it does in secretory cells is uncertain but it appears that sperm must undergo priming steps that have some resemblance to those in neurons (Tomes et al., 2005). Of course, in neurons, altered phosphorylation occurs very near the time of exocytosis but priming may be longer in sperm, consistent with longer exocytosis.

Capacitation of sperm can be achieved by incubating sperm in a calcium and bicarbonate-containing medium supplemented with a cholesterol-acceptor such as albumin (Larson and Miller, 1999). The newly ejaculated sperm encounter a change in HCO₃⁻ concentration when it enters female reproductive tract (Lishko et al., 2012). In addition to changing the pHᵢ and the resting membrane potential (Em), HCO₃⁻ also regulates the cAMP pathway in the presence of calcium through the stimulation of soluble adenylate cyclase (SACY)(Garbers et al., 1982; Okamura et al., 1985; Visconti et al., 2011; Battistone et al., 2013). Cholesterol is an abundant component of membrane sub-domains, often referred to as rafts, which are enriched in sterols and sphingolipids. These may provide a platform for sperm proteins important for binding to the egg and exocytosis of the acrosome (Shadan et al., 2004; Cross, 2004; van Gestel et al., 2005; Tanphaichitr et al., 2007). In somatic cells, several signaling pathways involving tyrosine kinases, G protein, etc. are activated by cholesterol-binding reagents such as beta-cyclodextrins (Brown and London, 1998; Kabouridis et al., 2000). The precise role of cholesterol removal and SACY during capacitation is unclear. They may have a profound effect on sperm protein
phosphorylation prompting SNARE complex formation, re-localization to rafts and hence capacitation.

There is considerable evidence in somatic cells for the hypothesis that phosphorylation of SNAREs and SNARE regulatory proteins affect formation of a SNARE complex and regulate exocytosis in neuronal and non-neuronal cells (Dubois et al., 2002; Tian et al., 2003; Boczan et al., 2004; Turner et al., 1999; Klenchin and Martin, 2000; Lin and Scheller, 2000; Morgan et al., 2005). Protein kinases and phosphatases act on a number of proteins in synaptic vesicles to regulate several steps including vesicle priming and recycling. All three core SNARE proteins and some SNARE regulatory proteins are phosphoproteins including synaptotagmins, syntaxins, synapsin and others. In most cases, the functional consequences of phosphorylation are not clear; however, in some instances there is evidence that phosphorylation alters the activity of the phosphoprotein. For example, phosphorylation of synaptotagmin I by Ca\(^{2+}\)/calmodulin-dependent kinase II (CamKII) promotes its interaction with syntaxin and SNAP-25 (Verona et al., 2000). VAMP/Synaptobrevin can also be phosphorylated by CamKII (Nielander et al., 1995). Phosphorylation on some sites in syntaxin 1 promotes synaptotagmin I binding but on other sites reduces SNARE complex formation (Risinger and Bennett, 2002; Sakisaka et al., 2004). Phosphorylation of SNAP-23 and SNAP-25 regulates exocytosis, likely by promoting binding to SNAREs and affecting vesicle priming (Nagy et al., 2004; Hepp et al., 2005) and/or altering the activity of voltage-gated Ca\(^{2+}\) channels (Snyder et al., 2006; Yang et al., 2007; Pozzi et al., 2008). The regulation of neuronal exocytosis by phosphorylation is of interest in view of the well-known changes in protein tyrosine phosphorylation during sperm capacitation, which can be envisioned as a kind of “priming” for acrosomal exocytosis (Osheroff et al., 1999; Dube et al., 2005b; Jha et al., 2006; Salicioni et al., 2007b). Whether, in fact, priming occurs in sperm as it
does in secretory cells is uncertain but it appears that sperm must undergo priming steps that have some resemblance to those in neurons (Tomes et al., 2005). Of course, in neurons, altered phosphorylation occurs very near the time of exocytosis but priming may be longer in sperm, consistent with longer exocytosis. Tyrosine phosphorylation appears to be downstream of the activation of the serine-threonine kinase, Protein Kinase A (Visconti and Kopf, 1998; Salicioni et al., 2007) so both groups of kinases are activated during sperm capacitation. Morphologically, it is possible that the evagination of the outer acrosomal membrane towards the plasma membrane to form fusion pores reflects priming. Unfortunately, all that is known to date about SNARE phosphorylation in sperm is that synaptotagmin VI phosphorylation by protein kinase C affects its ability to regulate membrane fusion when added to permeabilized sperm. The kinetics of this phosphorylation are uncertain because permeabilized sperm acrosome react without prior capacitation (Roggero et al., 2007).

The objective of the current study was to determine if phosphorylation status of SNARE complex proteins changes during capacitation. The findings of this study demonstrate that SNARE complex proteins are phosphorylated on both tyrosine and serine-threonine residues dynamically during mouse sperm capacitation. This phosphorylation may regulate the ability of SNARE proteins to form a trans-SNARE complex and ultimately promote membrane fusion during the acrosome reaction.

4.3. Material and Methods

4.3.1. Reagents and Animals

Wild type CD-1 mice were purchased from Harlan Sprague-Dawley, Inc. and transgenic (Syb-KI) mice were obtained from Jens Rettig in Hamburg, Germany. Antibodies to Syntaxin 2
and Synaptobrevin 2 were purchased from Synaptic System, Germany. Caveolin antibody was from BD Biosciences, Franklin Lakes, NJ and Synaptic Systems, Goettingen, Germany. Phosphotyrosine and GAPDH antibodies were purchased from Millipore, Billerica, MA and Sigma-Aldrich, St. Louis, MO respectively. Protein markers and precast gels were from Bio-Rad, Hercules, CA. HRP-secondary antibody and ECL substrate was purchased from GE Healthcare, Little Chalfont, UK. The proteinase inhibitor cocktail (Cat# S8830) was purchased from Sigma-Aldrich, Saint Louis MO) and the phosphatase inhibitor cocktail (Cat# 78428) was purchased from Thermo Scientific, Pittsburgh, PA. The inhibitors, H8, SU6656 and okadaic acid were obtained from Calbiochem (now EMD Millipore, Darmstadt, Germany). Pro-Q Diamond and SYPRO Ruby protein gel stains were purchased from Molecular Probes, Inc., Eugene, OR.

4.3.2. Preparation of Mouse Sperm

To obtain sperm, at least 3 mature male mice were euthanized by CO2 asphyxiation in accordance with Institutional Animal Care and Use Committee guidelines and the cauda epididymides were isolated. The University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee approved experimental protocols. Sperm were collected from the epididymis by piercing it with a 23-gauge needle and squeezing the sperm into a 60 mm petri dish containing 3ml dmKRBT. The petri dish was placed into a 37 °C incubator for 10 minutes to allow sperm to swim out. Sperm concentration and motility were checked under a microscope using a hematocytometer. Samples having at least 1.5x10^6 sperm/ml and 65% motility were considered for further experiments. The sperm sample was divided into appropriate aliquots and incubated either in capacitating (dmKRBT: 120mM NaCl, 2mM KCl, 2mM CaCl₂, 10mM NaHCO₃, 1.2mM MgSO₄, 0.36mM NaH₂PO₄, 5.6mM glucose, 1.1mM pyruvic acid, 25mM TAPSO, 18.5mM sucrose, 0.6% BSA, 10units/ml penicillin, and 10μg/ml streptomycin (pH
7.3)) or non-capacitating (dmKRBT without sodium bicarbonate and BSA) medium depending on the experiment. Sperm were then processed through a Percoll cushion to get viable and debris-free sperm (Furimsky et al., 2005) Briefly 0.5 mL of 45% percoll was layered on 0.5 mL of 90% percoll in an eppendorf tube. The gradient was centrifuged at 650 × g for 30 min at 25°C. This allowed immotile sperm to layer at interface between the two Percoll layers and motile sperm to sediment as a pellet. The supernatant containing immotile sperm and percoll solutions were removed. The pellet was suspended in dmKRBT and centrifuged at 450 × g for 10 min at 25°C. The supernatant was removed and the final pellet containing sperm was further processed according to a specific protocol depending on the experiment.

4.3.3. Protein Extraction and Quantification

Sperm were concentrated by centrifugation at 10,000 xg for 3 min in an Eppendorf centrifuge (centrifuge 5810R), suspended in RIPA buffer containing 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, as well as 7 ug/ml each of leupeptin and pepstatin; vortexed for 10 sec at maximum setting using a Fisher vortex (Genie 2™) and placed on ice for 30 min. Finally, the lysate was centrifuged again and the supernatant containing the protein was collected. The protein concentration of the sperm lysate was determined by bicinchoninic acid protein assay using BSA as a standard (Pierce, Rockford, IL).

4.3.4. Immunoprecipitation

To isolate syntaxin and associated proteins, the sperm lysate was pre-cleared using a non-specific antibody (anti-GAPDH). The pellet was discarded and supernatant was processed for immunoprecipitation. SNARE complex and other associated proteins were immunoprecipitated with a syntaxin antibody and isolated using Dynabeads. Briefly 50 uL of suspended Dynabeads
were transferred to a tube. The tube was placed on the magnet to separate beads from the solution. The supernatant was discarded and the tube was removed from the magnet. 10 ug of anti-syntaxin antibody diluted in 200 uL of PBS with Tween 20 were added to the tube and incubated for 10 min with gentle rotation at 21°C. After incubation, the tube was placed on the magnet and supernatant was removed. After adding 100 uL of pre-cleared sample, the tube was gently pipetted to suspend the Dynabeads-Antibody (Dyn-Ab) complex. The tube was incubated with gentle agitation for 30 min at room temperature to allow the syntaxin and associated proteins (Syn) to bind to the Dyn-Ab complex. After 30 min, the tube was placed on the magnet and the supernatant was removed. The Dyn-Ab-Syn complex was washed three times in 200 uL. The supernatant was removed between each wash on the magnet. The final Dyn-Ab-Syn complex was suspended in 100 ul PBS and transferred to a clean tube. The tube was placed on the magnet and the supernatant was removed. 20 ul of elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS) were added by gentle pipetting to resuspend the Dyn-Ab-Syn complex and resulting suspension was incubated with gentle rotation at room temperature for 5 min to dissociate the complex. The tube was then placed on the magnet and the supernatant containing eluates was transferred to a clean tube. Finally the precipitated protein was either frozen or run directly on an SDS-PAGE.

4.3.5. SDS-PAGE Electrophoresis

To detect SNARE proteins, the sperm lysate or membrane fraction samples were treated for 30 min at RT in a standard Laemmli sample buffer, or were denatured by boiling for 5 min in reducing Laemmli buffer (Laemmli, 1970). 20 μg of sperm protein or 50 μL of fraction samples were loaded on precast 4–16% or 4-20% linear gradient gel (Bio-Rad Laboratories, Inc. Hercules, CA ).
4.3.6. Detection of Total Phosphorylation

After separating the proteins, gels were incubated with Pro-Q diamond to detect total phosphorylation in sperm protein. Briefly gels were immersed in ~100 mL of fix solution and incubated at room temperature with gentle agitation for at least 30 minutes. The fixation step was repeated once more to ensure that all of the SDS was washed out of the gel. After fixation, the gel was washed three times in ~100 mL of ultrapure water with gentle agitation for 10 minutes each time. The washed gels were incubated in ~100 mL of Pro-Q Diamond phosphoprotein gel stain with gentle agitation in the dark for 60–90 minutes. The gels were then incubated in 80–100 mL of destain solution with gentle agitation for 30 minutes at room temperature in the dark. The destaining step was repeated three times. Finally the gels were washed twice with ultrapure water at room temperature for 5 minutes per wash before imaging by ImageQuant 4010. These gels were subsequently stained with SYPRO Ruby to measure total proteins and to normalize the amount of protein in the Pro-Q Diamond bands. Briefly, the gels were incubated overnight in 60 mL of SYPRO Ruby gel stain with gentle agitation. Then the gels were transferred to a clean container and washed with 100 mL of wash solution for 30 minutes. Before imaging the gels were rinsed twice in ultrapure water for 5 min.

4.3.7. Western Blot Analysis

For western blots, proteins on gels were transferred to a nitrocellulose membrane. The membranes were then blocked with 5% non-fat milk or 3% BSA in Tris-buffered saline (pH 7.6) with 0.1% Tween-20 (TBST) for 1 h at RT or overnight at 4°C with gentle agitation. After blocking, the membranes were washed three times (10 min each) with TBST and then incubated with primary antibody. After incubation for overnight with a 1:1000 dilution of primary antibody, membranes were washed six times (5 min each), and then incubated at ~25°C for 1 h.
with HRP-conjugated secondary antibodies at a 1:5,000 dilution. All secondary antibody incubations were with 2.5 % Milk in TBST. The membrane was rinsed briefly with water and then HRP activity was visualized with ECL chemiluminescence substrate and data collected using an ImageQuant 4010 (GE HealthCare) imaging system.

4.3.8. Data Analysis

All experiments were conducted with pooled semen samples obtained from at least 3 mature male mice and each experiment was replicated a minimum of three times unless otherwise stated. Bands intensities were measured using ImageQuant TL software from GE HealthCare, compared/normalized with the control and results were shown as a percent change. The band intensity was normalized with detecting the blots with antibodies to either GAPDH or syntaxin after separating boiled proteins by SDS-PAGE. Statistical significance was determined using an analysis of variance (ANOVA) and specific comparisons were made using SAS software. Significance was concluded if p < 0.05.

4.4. Results

4.4.1. Overall phosphorylation of sperm protein decreases during capacitation

Most protein phosphorylation in cells is on ser/thr residues (Aivaliotis et al., 2009; Ham, 2013) however this process in sperm has not been studied as thoroughly as tyrosine phosphorylation of sperm proteins due to technical difficulties in assessing ser/thr phosphorylation. Therefore my first goal was to determine if overall sperm protein phosphorylation changes during capacitation using Pro-Q Diamond staining for the detection of total phosphorylation (Steinberg, 2009; Monasky et al., 2010). Pro-Q Diamond detects 1-8 ng of phosphoproteins, depending on the phosphorylation state. Total protein in the same gel was
measured sequentially with SYPRO Ruby (Fig. 4.1) (Schulenberg et al., 2003; Schulenberg et al., 2004; Schilling et al., 2005). Total phosphorylation of sperm proteins decreased during a 90 min incubation under capacitating conditions (Fig. 4.2). However total phosphorylation of sperm proteins incubated in non-capacitating medium for 0 and 90 min was unchanged. To determine more precisely when phosphorylation changed during the 90 min capacitation time, total protein phosphorylation capacitated and non-capacitated sperm was assessed at 0, 30, 60 and 90 min. Total phosphorylation of sperm protein incubated in capacitating medium started decreasing at 30 min but no decrease was observed in total phosphorylation of sperm protein incubated in non-capacitating medium throughout incubation period (Fig. 4.3).

4.4.2. Total phosphorylation of SNAREs increases during capacitation.

All three core SNARE proteins and some SNARE regulatory proteins are phosphoproteins including synaptotagmins, syntaxins, synapsin and others (Burgoyne and Morgan, 2003). In most cases, the functional consequences of phosphorylation are not clear; however, in some instances there is evidence that phosphorylation alters the activity of the phosphoprotein. In these experiments the goal was to determine if phosphorylation status of SNARE proteins in mouse sperm changes during capacitation.

Protein from capacitated and non-capacitated sperm was prepared as described in Material and Methods. To assess phosphorylation of formed SNARE complexes, syntaxin and associated proteins were precipitated with a syntaxin antibody and the precipitated complexes were analyzed by western blot. A duplicate gel loaded with the precipitated syntaxin complexes (SNAREs) was stained with Pro-Q Diamond to assess total phosphorylation. Sizes of phosphoproteins were compared to sizes of SNARE proteins to provide clues to their identity. To confirm that the syntaxin complexes contained other SNAREs, immunoprecipitated samples
were not boiled prior to SDS-PAGE to avoid dissociating the complexes and the precipitated sperm protein was immunoblotted with an antibody to mRFP that detected the mRFP-synaptobrevin fusion protein in knock-in mice. I found that total phosphorylation of syntaxin complexes that migrated at 35, 100, and 150 kDa increased (Fig. 4.4).

4.4.3. Tyrosine phosphorylation changes dynamically in SNARE complexes during capacitation

Sperm proteins undergo tyrosine phosphorylation during capacitation (Osheroff et al., 1999; Seshagiri et al., 2007). The exocytotic function of SNAREs during neuronal and non-neuronal exocytosis is also regulated by phosphorylation (Turner et al., 1999; Chen et al., 2011). Proteins from capacitated and non-capacitated sperm were prepared as described in Materials and Methods. To assess tyrosine phosphorylation of formed syntaxin complexes, syntaxin complexes were immunoprecipitated with a syntaxin antibody, separated by SDS-PAGE (without boiling), and transferred to nitrocellulose and immunoblotted with phosphotyrosine antibody. Tyrosine phosphorylation of SNARE proteins appears to decrease after 15 min of capacitation time. In experiments with sperm incubated in capacitated or non-capacitated medium for 15 and 30 min, the syntaxin precipitates are phosphorylated at tyrosine at 15 min of capacitation with reduced phosphorylation at 30 min (Fig. 4.5). In follow-up experiments, I tested changes in the phosphorylation of syntaxin immune precipitates in capacitated sperm incubated for 0 and 90 min. No difference in tyrosine phosphorylation of syntaxin precipitates was observed between sperm capacitated at 0 and 90 min (Fig. 4.6). Figures 4.5 and 4.6 are the results of similar experiments except they have different times of incubation.
4.4.4. **SNAREs phosphorylation changes during capacitation are dependent on membrane cholesterol depletion and activation of soluble adenylate cyclase**

Capacitation is modulated by the activation of bicarbonate and removal of cholesterol from the sperm plasma membrane (Gadella and Van Gestel, 2004; Bou Khalil et al., 2006; Tanphaichitr et al., 2007). Therefore in these experiments, I tested whether cholesterol depletion or activation of soluble adenylate cyclase are needed to change phosphorylation status of SNAREs and other sperm proteins in preparation for the acrosome reaction. To test these effects on total phosphorylation and tyrosine phosphorylation of SNAREs, I used incubation medium with no BSA (the cholesterol accepter) or bicarbonate (soluble adenylate cyclase activator). Overall phosphorylation changes were assessed using Pro-Q Diamond staining and tyrosine phosphorylation detected with a phosphotyrosine antibody. A negative “incubation control” was performed in medium lacking BSA and bicarbonate that does not allow sperm capacitation (Larson and Miller, 1999). After a 90 min incubation under capacitating or non-capacitating conditions, sperm proteins were subjected to immunoprecipitation with a syntaxin antibody. The immunoprecipitated syntaxin complex was separated by SDS-PAGE and the gel was stained with Pro-Q Diamond to assess overall phosphorylation of SNARE complexes (both without boiling in sample buffer). Overall phosphorylation of syntaxin-associated protein was decreased when either BSA or bicarbonate were absent from the incubation medium and their absence reduced protein phosphorylation to the same amount as found in sperm that were mock-incubated (Fig. 4.7).

4.5. **Discussion**

The major findings of this study are that during mouse sperm capacitation 1) overall phosphorylation of sperm protein decreases, 2) overall phosphorylation of SNARE proteins
increases, 3) tyrosine phosphorylation of SNARE proteins changes dynamically, and 4) changes in SNARE protein phosphorylation are dependent on cholesterol removal from sperm membrane and bicarbonate-induced activation of SACY.

To date, only a few of the proteins that are phosphorylated during capacitation have been identified (Ficarro et al., 2003; Xiao et al., 2011). In general, the biological significance of altered protein phosphorylation during capacitation is not clear, but a reasonable hypothesis is that the alteration in phosphorylation during capacitation may serve to “prime” sperm to respond to zona binding with exocytosis. During sperm capacitation, a number of sperm proteins become phosphorylated on tyrosine residues (Osheroff et al., 1999; Tardif et al., 2001; Jha et al., 2006; Salicioni et al., 2007). Activation of tyrosine kinase activity depends on prior activation of a serine/threonine kinase, protein kinase A, so both kinases are active in sperm (Jha et al., 2006; Salicioni et al., 2007). Even though both families of kinases are activated, tyrosine phosphorylation of a number of proteins have been studied in great detail due to the availability of a specific probe, antibodies to phosphotyrosine. Because most protein phosphorylation is on serine and threonine residues, my first goal was to determine whether the overall phosphorylation status of sperm protein is changed during capacitation. My results show that overall phosphorylation of sperm protein decreased during capacitation. Because of the abundance of ser/thr phosphorylation, this suggests that sperm proteins were de-phosphorylated on ser/thr residues during capacitation.

Sperm capacitation can be accomplished in bicarbonate-based medium including calcium and a cholesterol acceptor (Gadella and Van Gestel, 2004; Bou Khalil et al., 2006; Tanphaichitr et al., 2007). BSA usually serves as a cholesterol acceptor in capacitating medium and I found that BSA was required for the increase in SNARE complex protein phosphorylation. Bicarbonate
activates soluble adenylate cyclase in sperm, which subsequently activates protein kinase A, a ser/thr kinase. If bicarbonate was absent, one would not expect any change in adenylate cyclase activity and that resulted in no change to SNARE complex protein phosphorylation.

Based on these data and data from others, I have developed a model of how SNARE function and acrosomal exocytosis are regulated by phosphorylation (Fig. 4.8). I propose that capacitation alters SNARE phosphorylation and complex formation. SNAREs move into membrane rafts at the sites of future membrane fusion. SNAREs regulatory proteins such as complexin 1 stabilize that complex by binding to SNAREs. An increase in cytosolic Ca^{2+} is detected by synaptotagmin, which drives SNARE-mediated membrane fusion at hundreds of points between the outer acrosome/plasma membrane, releasing acrosomal contents.

Novel findings of the current study have elucidated a major gap in our knowledge that how capacitation and protein phosphorylation prepare sperm for membrane fusion, likely by regulating SNAREs. It will be important to identify the particular proteins whose phosphorylation status changes and their role in capacitation and membrane fusion. These fundamental studies of fertilization may provide a foundation for development of new contraceptives, more accurate diagnostic tests of male fertility, and more effective therapies.
4.6. Figures

Figure 4.1. **Changes in total phosphorylation during capacitation.** A representative SDS-PAGE gel of capacitated and non-capacitated sperm proteins at 0 and 90 min, stained with Pro-Q Diamond for total phosphorylation. Lane M, Protein marker; Lane P, mouse brain protein gel stained with Pro-Q Diamond showing total phosphorylated proteins; Lane S, the same gel (Lane P) subsequently stained with SYPRO Ruby Stain showing total protein (phosphorylated and non-phosphorylated); Lane C-0, Phosphorylated proteins of sperm incubated in capacitating medium for 0 min and detected with Pro-Q Diamond; Lane C-90, Phosphorylated proteins of sperm incubated in capacitating medium for 90 min and detected with Pro-Q Diamond; Lane NC-0, Phosphorylated proteins of sperm incubated in non-capacitating medium for 0 min and detected with Pro-Q Diamond; Lane NC-90, Phosphorylated proteins of sperm incubated in non-capacitating medium for 90 min and detected with Pro-Q Diamond.
Figure 4.2. **Total phosphorylation decreases during capacitation at 90 min of incubation.** A. A representative SDS-PAGE gel of capacitated sperm proteins at 0 and 90 min and stained with Pro-Q Diamond for total phosphorylation with corresponding band intensities on left. B. Quantitation of total phosphorylation in capacitated and non-capacitated sperm proteins at 0 and 90 min. Each value represents a normalized value of total phosphorylation to total protein in the same lane. Values are expressed as arbitrary units per mg protein and are the mean±SEM of five independent experiments. C-0, Phosphorylated proteins of sperm incubated in capacitating medium for 0 min; C-90, Phosphorylated proteins of sperm incubated in capacitating medium for 90 min; NC-0, Phosphorylated proteins of sperm incubated in non-capacitating medium for 0 min; NC-90, Phosphorylated proteins of sperm incubated in non-capacitating medium for 90 min.
Figure 4.3. **Decrease of total phosphorylation during capacitation is initiated at 30 min of incubation.**

A. A representative SDS-PAGE gel of capacitated and non-capacitated sperm proteins at 0, 30, 60 and 90 min and stained with Pro-Q Diamond for total phosphorylation (left) and with SYPRO Ruby for total protein (right). 

B. Quantitation of total phosphorylation of capacitated and non-capacitated sperm proteins at 0, 30, 60 and 90 min. Each value represents a normalized value of total phosphorylation to total protein in the same lane (n=4). C-0, Proteins of sperm incubated in capacitating medium for 0 min; C-30, Proteins of sperm incubated in capacitating medium for 30 min; C-60, Proteins of sperm incubated in capacitating medium for 60 min; C-90, Proteins of sperm incubated in capacitating medium for 90 min; NC-0, Proteins of sperm incubated in non-capacitating medium for 0 min; NC-30, Proteins of sperm incubated in non-capacitating medium for 30 min; NC-60, Proteins of sperm incubated in non-capacitating medium for 60 min; NC-90, Proteins of sperm incubated in non-capacitating medium for 90 min.
Figure 4.4. **Total phosphorylation increases in SNAREs during capacitation.**

**A.** A representative SDS-PAGE gel of proteins from capacitated sperm at 0 (IP-C0) and 90 (IP-C90) min of capacitation, immunoprecipitated with anti-syntaxin antibody and then stained with Pro-Q Diamond for total phosphorylation with corresponding band intensities on left. **B.** Quantitation of total phosphorylation of SNARE proteins of capacitated and non-capacitated sperm at 0 and 90 min. Part B includes averages from 3 experiments. Each value represents a normalized value of total phosphorylation to total protein in the gel with respective treatment group using SYRO Ruby on the same gel.
Figure 4.5. **After an initial increase at 15 min, tyrosine phosphorylation of syntaxin-containing complexes decreases during capacitation.** A. A representative blot of capacitated and non-capacitated sperm proteins at different incubation time, as indicated, immunoprecipitated with anti-syntaxin antibody and then blotted with anti-phosphotyrosine antibody for tyrosine phosphorylation. B. Quantitation of tyrosine phosphorylation of syntaxin complexes of capacitated and non-capacitated sperm shown in 5A. Tyrosine phosphorylated syntaxin complexes of 75, 100, 150 and 230KD were observed in both capacitated and non-capacitated groups. Tyrosine phosphorylation decreases during capacitation. Data are averages of 3 experiments. Values shown are normalized intensities with syntaxin complex bands of same molecular weight run in a separate gel loaded with same volume of sample under same conditions.
Figure 4.6. **Tyrosine phosphorylation of syntaxin-containing SNARE complexes during capacitation was similar at 0 and 90 min of incubation.** A. A representative blot of capacitated sperm proteins at 0 and 90 min, immunoprecipitated with anti-syntaxin antibody and then blotted with anti-phosphotyrosine antibody for tyrosine phosphorylation with corresponding band intensities on left. 25 KD and 50 KD bands shown are for IgG light and heavy chains respectively. B. Quantitation of tyrosine phosphorylation of SNARE proteins of capacitated and non-capacitated sperm at 0 and 90 min. There was no difference in tyrosine phosphorylation between C-0 and C-90 groups. Tyrosine phosphorylated SNARE complexes of 75, 100, 150 and 230KD were observed in both groups with same band intensities. Part B includes averages from 3 experiments. Values shown are normalized intensities with syntaxin complex bands of same molecular weight run in a separate gel loaded with same volume of sample under same conditions.
Figure 4.7. **Total phosphorylation increases in SNAREs during capacitation is dependent on BSA and bicarbonate.** A. A representative SDS-PAGE gel of proteins from sperm incubated for 90 min in capacitating, capacitating without BSA, capacitating without HCO₃⁻ or non-capacitating medium, immunoprecipitated with anti-syntaxin antibody and then stained with Pro-Q Diamond (left) for total phosphorylation and subsequently with SYPRO Ruby (right) for total protein. B. Quantitation of total phosphorylation of SNARE proteins as indicated in A. Part B includes averages from 3 experiments. Each value represents a normalized value of total phosphorylation to total protein in the gel with respective treatment group using SYRO Ruby on the same gel.
Figure 4.8. Proposed model of SNAREs phosphorylation changes during mouse sperm capacitation. Bicarbonate present in capacitating medium enters sperm through NBC and activates SACY which ultimately activates PKA pathways. Whereas BSA removes cholesterol from the sperm membrane and is believed to activate PKA pathways and Ca\(^{2+}\) influx through CatSper by unknown mechanisms, it consequently increases tyrosine phosphorylation of sperm proteins. Tyrosine phosphorylation ultimately capacitates sperm and may promote the re-localization of SNAREs into membrane rafts to prepare sperm for the acrosome reaction. Media lacking bicarbonate and BSA did not induce changes in SNARE complex protein phosphorylation, suggesting that this process is regulated by the PKA pathway and cholesterol depletion. Broken arrows indicate that there might be multiple unknown steps from start to the target point. NBC, sodium bicarbonate co-transporter; SACY, soluble adenylate cyclase; PKA, Protein kinase A (chapter 3 & 5).
4.7. References


CHAPTER 5
CAPACITATION PROMOTES THE FORMATION OF SDS-RESISTANT HIGHER MOLECULAR WEIGHT SNARE COMPLEXES IN MOUSE SPERM

5.1. Abstract

Membrane fusion plays a central role in several important biological functions, including cell signaling, viral infections, hormone secretion, neuronal exocytosis and fertilization. Neuronal and somatic cell exocytosis has been studied in detail but there is little information available on the exocytosis events occurring in germ cells. At least three major membrane fusion events occur during the course of fertilization i.e. acrosome reaction in sperm, fusion of sperm and egg plasma membranes, and cortical granule reaction. In the last 20 years, many molecular components that allow fusion to occur have been identified. Intracellular membrane fusion of secretory vesicles to the plasma membrane in somatic cells is dependent on the formation a highly stable protein complex known as the core SNARE complex. The core SNARE proteins have also been found in sperm. Based on the universality of the fusion process, I hypothesized that capacitation in mouse sperm promotes the formation of the SNARE complex in preparation for the acrosome reaction. The core SNARE complex is very stable and is not denatured by SDS unless boiled. I assessed the SNARE complex formation by using a syntaxin antibody to immunoprecipitate syntaxin and associated proteins. Proteins of capacitated and un-capacitated sperm were boiled at 95°C for 5 min or heated to 37°C for 30 min and immunoblotted with specific SNARE antibodies. My results show that capacitation promotes the formation of higher molecular weight complexes that contain syntaxin. SNARE complex formation was blocked in medium lacking BSA or HCO$_3^-$- . Moreover a specific PKA inhibitor (H8) also inhibited the
formation of the SNARE complex whereas a tyrosine kinase inhibitor (SU6656) and okadaic acid (inhibitors of type 1 and 2A serine/threonine phosphatases) did not affect SNARE complex formation. Taken together these data suggest that although some SNARE complexes are present in non-capacitated sperm, capacitation promotes formation of additional higher molecular weight SNARE complexes that may have a role in the acrosome reaction of mouse sperm.

5.2. Introduction

Membrane fusion plays pivotal roles in several important biological events such as cell signaling, viral infection, protein secretion and fertilization. During secretion, the initial contact between secretory vesicles and the plasma membrane is made through an event known as docking where the vesicle docks or tethers to the target membrane. After docking, the vesicles undergo a “priming” step, allowing them to move into the slowly releasable and then readily releasable pool of vesicles (Becherer and Rettig, 2006). Primed vesicles respond to the increased cytosolic Ca\(^{2+}\) and are released in <0.3 ms (Jahn et al., 2003).

In the past 20 years, the molecular components that allow fusion to occur have been identified. In neurons, neuroendocrine cells, mast cells and pancreatic islet cells, the formation of a highly stable protein complex known as the SNARE complex brings the vesicle and plasma membranes together to facilitate fusion (Sheu et al., 2003; Puri et al., 2003). SNAREs constitute a large family of more than 35 proteins that contain a ~60 amino acid sequence known as the SNARE motif (Jahn et al., 2003). The core SNARE complex in neuronal cells is the best-studied SNARE complex, consisting of three proteins that form a stable complex: plasma membrane-associated syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25), and vesicle-associated membrane protein (VAMP or synaptobrevin). How the three proteins interact is shown in Figures 2.7 and 2.8 (Chapter 2). SNAREs found on transport vesicles are often grouped
as v-SNAREs and those on the target (the plasma membrane) are classified as t-SNAREs. The t-SNAREs syntaxin and SNAP-25 are believed to interact first, allowing the v-SNARE synaptobrevin to bind prior to fusion (Weninger et al., 2008).

The SNARE core complex is very stable. It is not denatured by SDS (unless boiled) and, once formed, is not cleaved by clostridial neurotoxin (Hayashi et al., 1994). The core complex is formed by four SNARE motifs consisting of two SNAP-25, one synaptobrevin and one syntaxin 1 motifs (Fig. 2.8). Although the core neuronal SNARE complex is composed of synaptobrevin, syntaxin 1 and SNAP-25, SNAREs function in exocytosis in non-neuronal tissues including mast cells, endocrine cells and sperm. There are a few examples of non-neural cells expressing “neural” SNAREs. For example, sperm contain SNAP-25 (Burkin and Miller, unpublished), but it has a fairly limited tissue distribution. On the other hand, a homologue, SNAP-23 (59% identical to SNAP-25) is found in a wide variety of tissues. Various isoforms of syntaxin and synaptobrevin are expressed in many tissues where they can function in vesicle transport and/or exocytosis (Kavalali, 2002; Tomes et al., 2002; Sollner, 2003).

The speed of synaptic exocytosis and the dependence on Ca$^{2+}$ ions require additional proteins that act on SNAREs to provide these features. In neuronal exocytosis, the Ca$^{2+}$ sensitive step is at the point at which the “primed” secretory vesicles actually fuse with the plasma membrane. Synaptotagmin I appear to provide the Ca$^{2+}$ sensitivity for fast neurotransmitter release (Chapman, 2002). Genetic studies in mice demonstrated that the loss of synaptotagmin I eliminates the fast synchronous vesicle release. Studies of sperm function could not be performed because synaptotagmin I deficient mice died at birth. Synaptotagmins may also act as a Ca$^{2+}$ sensor for the sperm acrosome reaction although there is a debate about which isoform(s) is/are important (Hutt et al., 2005).
In general, exocytosis is exquisitely regulated to ensure that it happens at only a proper time and place. This characteristic of exocytosis is true for neuronal as well as acrosomal exocytosis. The core neuronal SNARE complex, made up of syntaxin, synaptobrevin and SNAP-25 is sufficient for membrane fusion in vitro, in vivo SNARE complex formation is controlled by a number of regulatory molecules to ensure proper timing of exocytosis (DeBello and O'Connor, 1995; Mochida, 2000).

The process of exocytosis varies widely in different systems. The sperm acrosome is often considered as a specialized type of secretory vesicle. It is assembled after germ cell meiosis and released at fertilization. But it is much larger than a typical secretory vesicle. There are multiple fusion points between the outer acrosomal membrane and plasma membrane during the acrosome reaction compared to a single fusion pore for each vesicle during synaptic vesicle exocytosis. In contrast to exocytosis at synapses, there is no vesicle recycling during the acrosome reaction. The acrosome, unlike secretory vesicles, is not reformed after exocytosis (De Blas et al., 2005). Acrosomal exocytosis also proceeds much more slowly than does neuronal exocytosis (Lopez-Gonzalez et al., 2001; Rettig and Neher, 2002).

There are a number of similarities between membrane fusion in sperm acrosomal exocytosis and synaptic vesicle exocytosis, including the requirements of increased intracellular Ca\(^{2+}\) and SNARE complex formation (Jungnickel et al., 2001; Sollner, 2003; Mayorga et al., 2007). Studies from several laboratories have found that sperm express syntaxins, SNAP-25, VAMP/synaptobrevin and regulatory proteins such as NSF, αSNAP, synaptotagmin and complexins (De Blas et al., 2005; Lopez et al., 2007; Zarelli et al., 2009; Zanetti and Mayorga, 2009). Antibodies to syntaxin and synaptobrevin inhibited the ionophore-induced acrosome reaction in bovine sperm (Ramalho-Santos et al., 2000). However, in this experiment, it is
unclear how the antibodies gained access to SNAREs to inhibit the acrosome reaction. As an alternative to adding SNARE antibodies to live sperm, a model system was developed in which proteins were added to streptolysin-O-permeabilized human sperm and the acrosome reaction was induced with Ca\(^{2+}\). Using this model, Botulinum toxins (that cleave SNARE proteins) inhibit the human sperm acrosome reaction (Tomes et al., 2002). Antibodies to synaptobrevin and syntaxins 1A, 1B, 4, and 6, SNAP-25 and SNAP-23 block the human sperm acrosome reaction in this system (Tomes et al., 2002). Together, these data suggest SNARE complex assembly is required for the acrosome reaction.

The objective of the current study was to determine if mouse sperm capacitation promotes SNARE complexes formation to prepare sperm for the acrosome reaction. The stability of the SNARE complex in SDS was used to assess formation of the sperm SNARE complex. The roles of cholesterol removal, bicarbonate-induced SACY activation and protein kinase A were investigated.

5.3. Material and Methods

5.3.1. Reagents and Animals

Wild type CD-1 mice were purchased from Harlan Sprague-Dawley, Inc. and transgenic (Syb-KI) mice were obtained from Jens Rettig in Hamburg, Germany. Antibodies to Syntaxin 2 and Synaptobrevin 2 were purchased from Synaptic System, Germany. Caveolin antibody was from BD Biosciences, Franklin Lakes, NJ and Synaptic Systems, Goettingen, Germany. Phosphotyrosine and GAPDH antibodies were purchased from Millipore, Billerica, MA and Sigma-Aldrich, St. Louis, MO respectively. Protein markers and precast gels were from Bio-Rad, Hercules, CA. HRP-secondary antibody and ECL substrate was purchased from GE
Healthcare, Little Chalfont, UK. The proteinase inhibitor cocktail (Cat# S8830) was purchased from Sigma-Aldrich, Saint Louis MO) and the phosphatase inhibitor cocktail (Cat# 78428) was purchased from Thermo Scientific, Pittsburgh, PA. The inhibitors, H8, SU6656 and okadaic acid were obtained from Calbiochem (now EMD Millipore, Darmstadt, Germany). Pro-Q Diamond and SYPRO Ruby protein gel stains were purchased from Molecular Probes, Inc., Eugene, OR.

5.3.2. Preparation of Mouse Sperm

To obtain sperm, at least 3 mature male mice were euthanized by CO$_2$ asphyxiation in accordance with Institutional Animal Care and Use Committee guidelines and the cauda epididymides were isolated. The University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee approved experimental protocols. Sperm were collected from the epididymis by piercing it with a 23-gauge needle and squeezing the sperm into a 60 mm petri dish containing 3ml dmKRBT. The petri dish was placed into a 37°C incubator for 10 minutes to allow sperm to swim out. Sperm concentration and motility were checked under a microscope using a hematocytometer. Samples having at least 1.5x10$^6$ sperm/ml and 65% motility were considered for further experiments. The sperm sample was divided into appropriate aliquots and incubated either in capacitating (dmKRBT: 120mM NaCl, 2mM KCl, 2mM CaCl$_2$, 10mM NaHCO$_3$, 1.2mM MgSO$_4$, 0.36mM NaH$_2$PO$_4$, 5.6mM glucose, 1.1mM pyruvic acid, 25mM TAPSO, 18.5mM sucrose, 0.6% BSA, 10units/ml penicillin, and 10μg/ml streptomycin (pH 7.3)) or non-capacitating (dmKRBT without sodium bicarbonate and BSA) medium depending on the experiment. Sperm were then processed through a percoll cushion to get viable and debris-free sperm (Furimsky et al., 2005) Briefly 0.5 mL of 45% percoll was layered on 0.5 mL of 90% percoll in an eppendorf tube. The gradient was centrifuged at 650 × g for 30 min at 25°C. This allowed immotile sperm to layer at interface between the two Percoll layers and
motile sperm to sediment as a pellet. The supernatant containing immotile sperm and percoll solutions were removed. The pellet was suspended in dmKRBT and centrifuged at 450 × g for 10 min at 25°C. The supernatant was removed and the final pellet containing sperm was further processed according to a specific protocol depending on the experiment.

5.3.3. Protein Extraction and Quantification

Sperm were concentrated by centrifugation at 10,000 xg for 3 min in an Eppendorf centrifuge (centrifuge 5810R), suspended in RIPA buffer containing 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, as well as 7 ug/ml each of leupeptin and pepstatin; vortexed for 10 sec at maximum setting using a Fisher vortex (Genie 2TM) and placed on ice for 30 min. Finally, the lysate was centrifuged again and the supernatant containing the protein was collected. The protein concentration of the sperm lysate was determined by bicinchoninic acid protein assay using BSA as a standard (Pierce, Rockford, IL).

5.3.4. Immunoprecipitation

To isolate syntaxin and associated proteins, the sperm lysate was pre-cleared using a non-specific antibody (anti-GAPDH). The pellet was discarded and supernatant was processed for immunoprecipitation. SNARE complex and other associated proteins were immunoprecipitated with a syntaxin antibody and isolated using Dynabeads. Briefly 50 uL of suspended Dynabeads were transferred to a tube. The tube was placed on the magnet to separate beads from the solution. The supernatant was discarded and the tube was removed from the magnet. 10 ug of anti-syntaxin antibody diluted in 200 ul of PBS with Tween 20 were added to the tube and incubated for 10 min with gentle rotation at 21°C. After incubation, the tube was placed on the magnet and supernatant was removed. After adding 100 uL of pre-cleared sample, the tube was
gently pipetted to suspend the Dynabeads-Antibody (Dyn-Ab) complex. The tube was incubated with gentle agitation for 30 min at room temperature to allow the syntaxin and associated proteins (Syn) to bind to the Dyn-Ab complex. After 30 min, the tube was placed on the magnet and the supernatant was removed. The Dyn-Ab-Syn complex was washed three times in 200 ul. The supernatant was removed between each wash on the magnet. The final Dyn-Ab-Syn complex was suspended in 100 ul PBS and transferred to a clean tube. The tube was placed on the magnet and the supernatant was removed. 20 ul of elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS) were added by gentle pipetting to resuspend the Dyn-Ab-Syn complex and resulting suspension was incubated with gentle rotation at room temperature for 5 min to dissociate the complex. The tube was then placed on the magnet and the supernatant containing eluates was transferred to a clean tube. Finally the precipitated protein was either frozen or run directly on an SDS-PAGE.

5.3.5. SDS-PAGE Electrophoresis

To detect SNARE proteins, the sperm lysate or membrane fraction samples were treated for 30 min at RT in a standard Laemmli sample buffer, or were denatured by boiling for 5 min in reducing Laemmli buffer (Laemmli, 1970). 20 μg of sperm protein or 50 μL of fraction samples were loaded on precast 4–16% or 4-20% linear gradient gel (Bio-Rad Laboratories, Inc. Hercules, CA ).

5.3.6. Western Blot Analysis

For western blots, proteins on gels were transferred to a nitrocellulose membrane. The membranes were then blocked with 5% non-fat milk or 3% BSA in Tris-buffered saline (pH 7.6) with 0.1% Tween-20 (TBST) for 1 h at RT or overnight at 4° C with gentle agitation. After
blocking, the membranes were washed three times (10 min each) with TBST and then incubated with primary antibody. After incubation for overnight with a 1:1000 dilution of primary antibody, membranes were washed six times (5 min each), and then incubated at ~25°C for 1 h with HRP-conjugated secondary antibodies at a 1:5,000 dilution. All secondary antibody incubations were with 2.5% Milk in TBST. The membrane was rinsed briefly with water and then HRP activity was visualized with ECL chemiluminescence substrate and data collected using an ImageQuant 4010 (GE Healthcare) imaging system.

5.3.7. Incubation of Sperm with Kinase and Phosphatase Inhibitors

To test the effect of the inhibitor, sperm were pre-incubated with membrane permeable inhibitors in non-capacitating medium for 15 min prior to dilution in capacitating medium and the beginning of the capacitating period. Controls were incubated with vehicle. A negative “incubation control” was performed in medium lacking BSA and bicarbonate that does not allow sperm capacitation.

5.3.8. Data Analysis

All experiments were conducted with pooled semen samples obtained from at least 3 mature male mice and each experiment was replicated a minimum of three times unless otherwise stated. Bands intensities were measured using ImageQuant TL software from GE Healthcare, compared/normalized either with total bands intensities of the same lane and results were shown as a percent change. Depending on the experiment, the band intensity was also normalized with detecting the blots with antibodies to either GAPDH or syntaxin after separating boiled proteins by SDS-PAGE. Statistical significance was determined using an analysis of
variance (ANOVA) and specific comparisons were made using SAS software. Significance was concluded if \( p < 0.05 \).

5.4. Results

5.4.1. Capacitation promotes higher molecular weight SNARE complex formation

My first goal was to detect “neuronal” SNARE proteins, syntaxin and synaptobrevin in mouse sperm. I was able to detect syntaxin from CD-1 WT mouse sperm whereas synaptobrevin was detected by western blot using mRFP-synaptobrevin knock-in mouse sperm using an mRFP antibody as shown in Figure 3.1.

The SNARE core complex is very stable. It is not denatured by SDS (unless boiled) and, once formed, is not cleaved by clostridial neurotoxin (Hayashi et al., 1994). I tested if capacitation promotes SNARE complex formation. I assessed SNARE complex formation by using a syntaxin antibody to immunoprecipitate syntaxin and associated proteins. Proteins of capacitated and non-capacitated sperm were boiled at 95°C for 5 min or heated to 37°C for 30 min and subsequently run on SDS-PAGE, followed by immunoblotting with syntaxin antibody to detect syntaxin complexes. In the gel loaded with boiled samples, only one band corresponding to syntaxin was observed in all groups. This single band of proper size showed the specificity of the antibody. In non-boiled samples, in addition to individual syntaxin, other bands of about 75, 100 and 150 KD size were also observed (Fig. 5.1). These higher molecular weight bands detected by a specific syntaxin antibody suggest the presence of multiple syntaxin complexes (and hence SNARE complexes) of varying size as found in neuronal SNARE complexes.
I validated the presence of syntaxin complexes by precipitating the syntaxin with a syntaxin antibody and then immunoblotting with the same antibody. The detected bands appear to be SNARE complexes because they contain syntaxin and are larger than the molecular weight of free syntaxin. The larger syntaxin-containing SNARE complexes have been reported in other cells when the SDS-PAGE samples were not boiled prior to separation (Kubista et al., 2004; Zhao et al., 2007).

Additional experiments to quantitate the specific size SNARE complexes were performed. Sperm incubated under capacitating conditions for 30 and 60 minutes developed more SNARE complexes and had less free syntaxin than those incubated in medium without BSA or bicarbonate, conditions that do not allow capacitation to occur (Fig. 5.2).

**5.4.2. Membrane cholesterol depletion is required for SNARE complex formation during capacitation**

The overall capacitation pathway is believed to be modulated by cholesterol removal from the sperm plasma membrane (Garbers et al., 1982; Okamura et al., 1985; Visconti et al., 2011). In somatic cells, several signaling pathways involving tyrosine kinases, G protein, etc. are activated by cholesterol-binding reagents such as beta-cyclodextrins (Brown and London, 1998; Kabouridis et al., 2000). Removal of cholesterol may have an effect on sperm protein phosphorylation, which may promote SNARE complex formation during capacitation. Therefore I tested whether cholesterol depletion is needed for SNARE complex formation in preparation for the acrosome reaction. To test the effects of cholesterol depletion on SNARE complex formation, I used incubation medium with no BSA i.e. capacitating dmKRBT without BSA. The medium lacking BSA did not induce SNARE complex formation, suggesting that cholesterol depletion is critical in SNARE complex formation (Fig. 5.3).
5.4.3. **SNARE complex formation during capacitation is dependent on the activation of soluble adenylate cyclase**

Capacitation can be mimicked in calcium and bicarbonate-containing medium supplemented with a cholesterol-acceptor (Visconti et al., 1999). The newly ejaculated sperm encounter a change in HCO$_3^-$ concentration when it enters the female reproductive tract. In addition to changing the pH and the resting membrane potential (Em), HCO$_3^-$ also regulates the cAMP pathway in the presence of calcium through the stimulation of soluble adenylate cyclase (SACY) (Garbers et al., 1982; Okamura et al., 1985; Visconti et al., 2011). Although the precise role of SACY is unclear, SACY knockout mice are sterile and their sperm do not develop hyper-activated motility and may have other capacitation defects (Xie et al., 2006).

In these experiments, I tested whether activation of soluble adenylate cyclase by bicarbonate is needed for SNARE complex formation in preparation for the acrosome reaction. To test the effects of soluble adenylate cyclase on SNARE complex formation, I incubated sperm in capacitating medium (dmKRBT) with no bicarbonate (soluble adenylate cyclase activator). The medium lacking bicarbonate did not induce formation of syntaxin-containing complexes, suggesting that activation of soluble adenylate cyclase is necessary for SNARE complex formation (Fig. 5.3).

5.4.4. **Phosphorylation changes in SNAREs mediated by specific kinases are required for SNARE complex formation**

Using a pharmacological approach, I tested if protein kinase A and tyrosine kinase inhibitors added to sperm during capacitation affect the formation of SNAREs. To separate SNARE complexes, I used syntaxin immunoprecipitations, as described above. Previously I did
not observe any effect of SU6656 (a specific tyrosine kinase inhibitor) and okadaic acid (an inhibitor of type 1 and 2A serine/threonine phosphatases activity) on SNAREs movement into rafts but did find that H8 (a protein kinase A inhibitor) prevented SNARE protein movement into rafts during capacitation.

Because most of higher molecular weight SNARE complexes were formed at a 30 min incubation time in capacitating medium, I incubated sperm with a selective and cell permeable protein kinase A inhibitor, H-8 (Visconti et al., 1995) in capacitating medium for 30 min. Positive and negative controls were performed by incubating sperm for 30 min in capacitating dmKRBT and non-capacitating dmKRBT, respectively.

SNARE complex formation was blocked by H8 but not tyrosine kinase inhibitor (SU6656) and okadaic acid (inhibitors of type 1 and 2A serine/threonine phosphatases) as shown in Figure 5.4. These data suggest that PKA pathway is required for the formation of SNARE complexes during capacitation to prepare sperm for the acrosome reaction.

5.5. Discussion

The major findings of this study are that: 1) mouse sperm capacitation promotes the formation of higher molecular weight SNARE complexes during the early phases of capacitation; 2) formation of higher molecular weight SNARE complexes requires the depletion of cholesterol from the sperm plasma membrane by BSA; 3) activation of SACY is needed for SNARE complex formation; and 4) PKA pathway is involved in the process of SNARE complex formation.

Immunoprecipitation and western blot results showed that, although the SNARE complexes are present in non-capacitated sperm, capacitation promotes further formation of specific syntaxin complexes at 30 min of capacitation (Fig. 5.1 and 5.2). After the initial
increase, there was a slight decrease in abundance of SNARE complexes after 30 min of capacitation. I observed a decrease in intensities of syntaxin and associated proteins after an initial rise at 30 min incubation time in capacitating medium. After 60 min there was no difference among the SNARE bands between capacitated and non-capacitated sperm. This decrease in intensities of SNARE bands may suggest that after 60 min, some sperm may start the acrosome reaction spontaneously and SNAREs in the released membranes are lost.

Capacitation promotes formation of some higher molecular weight SNARE complexes during the early phases of capacitation but free syntaxin and other syntaxin complexes are also present both in capacitated and non-capacitated sperm. This increase and decrease in SNARE bands intensities suggests a transient nature of higher molecular weight SNARE complexes. The transient syntaxin complex formed during the initial phases of capacitation may be the trans-SNARE complex (Tsai et al., 2010). By definition, the trans-SNARE complex is only formed when two membranes contact each other. In case of sperm that have not been capacitated, the outer acrosomal and the sperm plasma membranes are separated from each other (Tsai et al., 2010) so trans-SNARE complex formation is not possible. Using a model system of streptolysin-o-permeabilized sperm that bypass capacitation, the acrosome evaginates and plasma and outer acrosomal membranes of human sperm make contact during the acrosome reaction (Zanetti and Mayorga, 2009). This contact of two membranes may help v-SNAREs and t-SNAREs form a trans-SNARE complex. Therefore, in the absence of signals for capacitation, I propose that the only possible SNARE complexes are cis-SNARE complexes. The role of cis-SNARE complexes in sperm or somatic cells is unclear.

Based on published data and my results, I propose a model for SNAREs complex formation during mouse sperm capacitation to prepare sperm for acrosomal exocytosis (Fig. 5.5).
BSA present in the capacitating medium promotes SNARE movement into lipid rafts, perhaps by affecting raft composition or, through a poorly understood mechanism, activating the PKA pathway and Ca\(^{2+}\) influx through CatSper calcium channels. PKA is also modulated by bicarbonate activation of SACY, which, in turn, activates PKA through cAMP.

The increase in Ca\(^{2+}\) and activation of PKA ultimately activates tyrosine kinases and hence tyrosine phosphorylation/capacitation. Tyrosine phosphorylation/capacitation finally promotes SNAREs re-localization into membrane rafts to form trans-SNARE complex to prepare sperm for the acrosome reaction. SNAREs regulatory proteins such as complexin 1 stabilizes that complex by binding to SNAREs. An increase in cytosolic Ca\(^{2+}\) is detected by synaptotagmin, which drives SNARE-mediated membrane fusion at hundreds of points between the outer acrosome/plasma membrane, releasing acrosomal contents.
5.6. Figures

A

Figure 5.1. Detection of SNARE complexes in capacitated and non-capacitated sperm. 
A. Proteins of capacitated and non-capacitated sperm were boiled at 95°C for 5 min (left panel) or heated to 37°C for 30 min (right panel) and subsequently run on 12% SDS-PAGE gels, followed by immunoblotting with syntaxin antibody to detect syntaxin complexes. In the gel loaded with boiled samples, only one band corresponding to syntaxin was observed in all groups while in samples that were not boiled, in addition to free syntaxin other bands of about 75, 100 and 150 KD were also observed. Samples labeled “C” were capacitated and those labeled “NC” were incubated in medium without bicarbonate and BSA. Incubation times in minutes are given after either “C” or “NC”.

B. Quantitation of the observed SNARE complexes bands in capacitated and non-capacitated group with different incubation time as indicated above. Less free syntaxin was observed in capacitated sperm at 30 and 60 min and the abundance of the 75, 100 and 150 KD bands was greater in these groups. Data are averages of 3 experiments. Each value represents a normalized value of band intensity to total syntaxin with each treatment group.
**Figure 5.2. Proportion of syntaxin in higher molecular weight complexes in capacitated and non-capacitated sperm.**

A. Proteins of capacitated and non-capacitated sperm were immunoprecipitated with syntaxin antibody and subsequently heated to 37°C or boiled at 95°C and separated on 12% SDS-PAGE gel, followed by immunoblotting with syntaxin antibody to detect SNARE complexes. Bands of molecular weights of 34, 75, 100, 150 and 230 KD were observed.

B. Quantitation of the observed SNARE complex bands in capacitated and non-capacitated samples with different incubation time as indicated above. Less free syntaxin was observed in capacitated sperm at 30 and 60 min. The SNARE complexes showed dynamic behavior in capacitating medium at different incubation time, with more bands at 30 min incubation time. A strong band of 150KD was observed in all groups of capacitated and non-capacitated sperm. C0, sperm incubated in capacitation medium for 0 min; C30, sperm incubated in capacitation medium for 30 min; C60, sperm incubated in capacitation medium for 60 min; C90, sperm incubated in capacitation medium for 90 min; NC0, sperm incubated in non-capacitation medium for 0 min; NC30, sperm incubated in non-capacitation medium for 30 min; NC60, sperm incubated in non-capacitation medium for 60 min; NC90, sperm incubated in non-capacitation medium for 90 min. Part B includes averages from 3 experiments. (Reference for normalization procedure; Tsai et al., 2007).
Figure 5.3. **SNARE complexes formation during capacitation is dependent on BSA and bicarbonate.**

**A.** A representative blot of proteins from sperm incubated for 0, 30, 60 and 90 min in capacitating medium, capacitating medium without BSA, capacitating medium without HCO$_3^-$ or medium lacking both BSA and HCO$_3^-$. Samples were boiled at 95°C for 5 min (lower panel) or heated to 37°C for 30 min (upper panel) and separated on 12% SDS-PAGE gels, followed by immunoblotting with syntaxin antibody to detect SNARE complexes. In the gel loaded with boiled samples, only one band corresponding to syntaxin was observed in all groups while in samples that were not boiled, in addition to syntaxin other bands of about 75, 100, 150 and 230 KD size were also observed.

**B.** Quantitation of the observed SNARE complexes bands in capacitated and non-capacitated samples with different incubation time as indicated above. Data are averages of 3 experiments. Each value represents a normalized value of band intensity to free syntaxin in boiled sample with same volume in each treatment group.
Figure 5.4. **SNARE complex formation during capacitation is dependent on the activity of PKA.** **A.** A representative blot of proteins from sperm incubated for 30 min in capacitating medium, non-capacitating medium or capacitating medium with a specific PKA inhibitor (H8) medium. Samples heated to 37°C for 30 min and subsequently run on 12% SDS-PAGE gels, followed by immunoblotting with syntaxin antibody to detect SNARE complexes. **B.** Quantitation of the observed SNARE complexes bands in sperm that were capacitated, non-capacitated and incubated with SU6656, H8 and OA. Data are averages of 3 experiments. Each value represents a normalized value of band intensity to free syntaxin (when boiled) with each treatment group. The control was incubated in non-capacitating medium for zero min.
Figure 5.5. **Proposed Model for SNARe complex formation.** During capacitation, HCO$_3^-$ influx activates SACY and BSA depletes membrane cholesterol. Removal of cholesterol and activation of SACY activate PKA and transient release of internal stored Ca$^{2+}$, which then triggers a sustained influx of extracellular Ca$^{2+}$. The PKA ultimately phosphorylates SNAReS promoting the SNARe complex formation and capacitation.
5.7. References


CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

These studies have advanced our understanding of the mechanism allowing the sperm membrane to undergo the acrosome reaction. A failure of this vital step (during capacitation) is a frequent cause of male infertility. Chapter 1 investigated how capacitation regulates the movement of SNARE proteins into membrane rafts. Chapter 2 provided insight into how capacitation may alter phosphorylation status of SNAREs and other regulatory proteins in preparation for the acrosome reaction whereas chapter 3 determined formation of higher molecular weight SNARE complexes during capacitation. These studies also linked activation/inhibition of kinases and phosphatases during capacitation to SNARE phosphorylation and preparation for acrosomal exocytosis.

My results demonstrate that: 1) syntaxin and synaptobrevin are present in mouse sperm; 2) total phosphorylation of sperm proteins decreases during capacitation; 3) total phosphorylation in syntaxin complexes increases during capacitation; 4) tyrosine phosphorylation changes dynamically in syntaxin complexes during capacitation; and 5) capacitation promotes formation of some higher molecular weight SNARE complexes but free syntaxin and other syntaxin complexes are also present both in capacitated and non-capacitated sperm. SNARE complexes may be cis, on the same membrane, or trans, spanning two different membranes. Higher molecular weight complexes are formed during the early phases of capacitation and their abundance decreases in the later phases of capacitation. This suggests a transient nature of SNARE complexes. The transient syntaxin complex formed during the initial phases of capacitation may be the trans-SNARE complex (Tsai et al., 2010). A trans-SNARE complex can only formed when two membranes are very near to each other. Prior to capacitation, the sperm outer acrosomal membrane and plasma
membrane are separated from each other (Tsai et al., 2010) so trans-SNARE complex formation is not possible and the complexes present may be the cis-SNARE complexes whose precise role is unknown in membranes. However, prior to or at the early stages of the acrosome reaction, the acrosome outer membrane evaginates and plasma and outer acrosomal membranes make contact (Zanetti and Mayorga, 2009). This proximity of these two membranes may allow v-SNAREs and t-SNAREs to form a trans-SNARE complex. Therefore, in the absence of signals for capacitation, I proposed that the only possible SNAREs are the cis-SNAREs as found in both capacitated and in non-capacitated sperm. Subsequently, capacitation would allow formation of trans-SNARE complexes and the initiation of the acrosome reaction.

Rafts are heterogeneous microdomains (Razani et al., 2002), enriched in sterol and sphingolipids that act as platforms for trafficking or docking molecules (Munro, 2003; Pike, 2004; Pike, 2006). Studies have shown that as sperm capacitate, proteins including some SNARE proteins shift into or out of membrane rafts. This movement could allow SNAREpin complex formation to promote membrane fusion at the site of rafts. Using a detergent-free raft extraction method, I found that during capacitation, syntaxin and synaptobrevin move from non-raft fractions into rafts and this movement was dependent on BSA and partially on HCO3− in the capacitating medium. When sperm were incubated with a specific PKA inhibitor, H8, the SNARE re-localization was completely blocked. These results suggest that capacitation re-localizes SNAREs into rafts in a BSA and PKA dependent manner, allowing trans-SNARE complex formation, which may ultimately, promote membrane fusion to occur at sites of rafts.

There is considerable evidence in somatic cells for the hypothesis that phosphorylation of SNAREs and SNARE regulatory proteins promotes the formation of SNARE complex and regulates exocytosis (Tian et al., 2003; Boczan et al., 2004). Therefore, I expected that adding
kinase activators and inhibitors and phosphatase inhibitors would alter the formation of the SNARE complex. When sperm were incubated with a specific PKA inhibitor, H8, the SNARE re-localization and complex formation was completely blocked. These results suggest that capacitation re-localizes SNAREs into rafts in a PKA dependent manner, allowing trans-SNARE complex formation, which ultimately promotes membrane fusion at those sites.

Prior to this work, there was a deficiency in the understanding of how SNAREs were regulated in sperm during capacitation to promote the acrosome reaction. It was unclear if capacitation had any impact on SNARE function. Furthermore, the function of the SNARE proteins during capacitation was uncertain.

Based on my result and other studies, I have developed a model of SNARE function and regulation of acrosomal exocytosis. I propose that capacitation (cholesterol removal and bicarbonate-induced SACY activation) alters sperm protein phosphorylation promoting SNARE complex formation and movement into membrane rafts in preparation for the acrosome reaction. Some regulatory proteins like complexin I and others stabilize that complex by binding SNAREs. An increase in cytosolic Ca\(^{2+}\) is detected by synaptotagmin, which drives SNARE-mediated membrane fusion at hundreds of points between the outer acrosome and plasma membrane, releasing the acrosomal contents. I tested part 2 and 3 of this model (Fig. 6.1) and have suggestions for testing other parts of this model (see Future Studies). The suggested studies will be helpful to understand fully the mechanism of mouse sperm capacitation and the acrosome reaction in addition to validating my results using conventional and innovative techniques.
6.2. Future Studies

I propose the following questions as the continuation of this project:

1. Do sperm begin a spontaneous acrosome reaction in capacitating dmKRBT medium?
2. What is the role of other SNAREs and regulatory proteins during mouse sperm capacitation in preparation for the acrosome reaction?
3. What other activator(s) of PKA signaling pathway (which is independent of SACY) is/are involved in the capacitation of mouse sperm?
4. Are any other signaling pathways (in addition to PKA) involved during mouse sperm capacitation?
5. What types of rafts are present in plasma and acromosal membranes of mouse sperm?
6. What is the precise composition of rafts present in mouse sperm?
7. Which components of the fusion machinery does the inner acrosomal membrane (which becomes the limiting membrane of sperm after the acrosome reaction) has that would ultimately fuse to the egg?
8. Are SNARE proteins necessary for all fusion events of fertilization?

The current research mainly used a biochemical approach but it is important to validate these results using other sophisticated techniques such as modern cell biology and genetic techniques. For example localization and re-localization of SNAREs can be assessed using an innovative microscopy technique (Total Internal Reflection Fluorescence Microscopy; TIRFM, also called evanescent wave microscopy) which is ideal for high-resolution imaging of cellular processes near the plasma membrane. TIRFM has been used successfully to study exocytosis from single vesicles in somatic cells (Steyer and Almers, 2001; Zenisek et al., 2002; Tsuboi et al., 2004). TIRFM selectively illuminates molecules 100-200 nm from the glass-liquid interface,
allowing observation of fluorescently labeled proteins near sites of exocytosis of living cells with minimal fluorescence from outside the plane of interest. Wide field imaging with a CCD camera is ideal to follow localization of two proteins over a large area.

There are various knock-in mice available having SNARE proteins with a fluorescent tag. For example synaptobrevin-mRFP knock-in mice are ideal to study localization of synaptobrevin to the acrosomal membrane that becomes concentrated at sites of fusion (rafts) prior to acrosomal exocytosis as suggested by my biochemical studies. This approach will also be helpful to identify the localization of synaptobrevin in inner acrosomal membrane which becomes the limiting membrane of the sperm after the acrosome reaction. The mRFP signal on the outer acrosomal membrane will be lost following exocytosis but any synaptobrevin-mRFP on the inner acrosomal membrane will remain. I do not expect the mRFP tag to influence synaptobrevin function because the mRFP-tag is intra-acrosomal, the mice are fertile and FP-tagging has not influenced the localization and function of synaptobrevin and other SNAREs in cells in culture (Tsuboi et al., 2004; Sharma et al., 2004; Allersma et al., 2004; Legler et al., 2005; Suyama et al., 2007). Similarly, rafts can also be studied by microscopy using cholera toxin B, which is a convenient and frequently used marker of GM1 and rafts in cells for microscopy (Yanagisawa et al., 2006). Based on my experience with a line of synaptobrevin-mRFP knock-in mice, the signal must be increased for this approach to be used with TIRFM. Perhaps other lines, yet to be developed, that have FP multimers, would provide an adequate signal for these experiments.

As suggested by my results that abundance of higher molecular weight SNARE complexes decreases after an increase 30 min into capacitation, it is possible that some sperm might have started or completed a spontaneous acrosome reaction in the capacitating medium. To confirm this effect, viable spermatozoa could be assessed by CTC staining pattern: 1) F-
pattern (uncapacitated): fluorescence is detected over the whole region of the spermatozoa head; 2) B-pattern (capacitated): fluorescence is detected in the spermatozoa head except in the post-acrosomal region; and 3) AR-pattern (acrosome-reacted): weak fluorescence is observed over the spermatozoa head and a bright band is detected in the equatorial segment (Ward and Storey, 1984).

In view of my data, I expect TIRFM to reveal that SNARE proteins cluster during capacitation or just prior to exocytosis and that they become enriched in stable raft fractions. A biochemical approach allowed me to observe the shifting of proteins through heterogeneous (Pike, 2004; Pike, 2006) and dynamic membrane rafts. The cell biology approach is important as this procedure will minimize concerns about sperm heterogeneity that arise when performing biochemical experiments on sperm that may be at different stages of capacitation and the acrosome reaction. Furthermore this approach will allow use of live sperm.

Finally, to be certain that ejaculated human sperm behave like mouse epididymal sperm as capacitation and the acrosome reaction are concerned, these biochemical experiments I already did with mouse sperm and other proposed experiments should be performed with human sperm using a technique where a lower number of sperm is needed. I do not expect to perform SNARE’s re-localization experiment using western blot with human sperm where I used sperm from 90 mice in one experiment. I anticipate that results of these proposed studies will advance our knowledge and could be used to develop more accurate laboratory fertility assessments. Findings from these studies will also provide clues about how fertility could be improved or sperm could be more effectively prepared for fertilization prior to intra-uterine insemination or in vitro fertilization.
Figure 6.1. Proposed model showing various steps that may be involved in capacitation to prepare the sperm for the acrosome reaction. Please note that the order of number 2 and 3 is unclear.
6.4. References


## Appendix A

### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis-of-variance</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproductive Technologies</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BRIJ96</td>
<td>Trade name for Polyoxyethylene-10-oleyl ether- a surfactant</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CamKII</td>
<td>Ca$^{2+}$/Calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP or 3',5'-cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CatSper</td>
<td>sperm-specific cation channel</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-Coupled Device</td>
</tr>
<tr>
<td>CD-1</td>
<td>Cluster of Differentiation 1</td>
</tr>
<tr>
<td>CTC</td>
<td>Chlortetracycline</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacyl Glycerol</td>
</tr>
<tr>
<td>dmKRBT</td>
<td>A specialized medium (Burkin et al., 2004)</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>Gi1/Gi2</td>
<td>G proteins of the Gi1 and Gi2 family</td>
</tr>
<tr>
<td>GM1</td>
<td>Ganglioside monosialic acid</td>
</tr>
<tr>
<td>HDF</td>
<td>High Density Fraction</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>IAM</td>
<td>Inner Acrosomal Membrane.</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
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<tr>
<td>ICSI</td>
<td>Intra Cytoplasmic Sperm Injection</td>
</tr>
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<td>IP3</td>
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<td>KD or KDa</td>
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<td>Low Density Fraction</td>
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<tr>
<td>mRFP</td>
<td>monomeric Red Fluorescent Protein</td>
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<td>mRFP</td>
<td>Monomeric Red Fluorescent Protein</td>
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ms  millisecond
NBC  Na⁺/HCO₃⁻ Cotransporter
NSF  N-ethylmaleimide sensitive factor/fusion proteins
OA    Okadaic Acid
PAGE PolyAcrylamide Gel Electrophoresis
pHi  Internal pH
PKA  Protein kinase A
PKC  Protein Kinase C
PLC  Phospholipase C
PTK  protein tyrosine kinase
PVS  Perivitelline space
RIPA Radio Immuno Precipitation Assay
SACY soluble adenylate cyclase
SAS  Statistical Analysis System
SDS  Sodium Dodecyl Sulfate
SDS-PAGE sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)
SNAP-25/23 Synaptosomal-associated protein of 25 or 23 KDa
SNARE Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor
Syb-KI Synaptobrevin Knock-in
TAPSO 3-[N-Tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic Acid
TBS  Tris-buffered saline
TBST Tris-buffered saline tween
TIRFM Total Internal Reflection Fluorescence Microscopy
TRPC2 Transient receptor potential cation channel, subfamily C, member 2
t-SNARE Target SNARE
VAMP-2 Vesicle-associated membrane protein 2
v-SNARE Vesicle SNARE
WHO World Health Organization
ZP  Zona Pellucida/Zona Protein
Appendix B

Statistical Analyses

Table B.1. Results of statistical analysis for syntaxin reorganization into rafts (Fig. 3.2).

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<th>Contrast</th>
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<th>Contrast SS</th>
<th>Mean Square</th>
<th>F Value</th>
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Table B.2. Results of statistical analysis for synaptobrevin reorganization into rafts (Fig. 3.3).

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Table B.3. Results of statistical analysis for overall phosphorylation changes in total sperm protein during capacitation (Fig. 4.2).

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Table B.4. Results of statistical analysis for overall phosphorylation changes in total sperm protein during capacitation (Fig. 4.3).

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Table B.5. Results of statistical analysis for total phosphorylation changes in SNARE proteins during capacitation (Fig. 4.4).

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Table B.6. Results of statistical analysis for tyrosine phosphorylation changes in SNARE proteins during capacitation (Fig. 4.5).

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Table B.7. Results of statistical analysis for tyrosine phosphorylation changes in SNARE proteins during capacitation (Fig. 4.6).

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Table B.8. Results of statistical analysis for SNARE complex formation during capacitation (Fig. 5.1).

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Table B.9. Results of statistical analysis for SNARE complex formation during capacitation (Fig. 5.2).

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Table B.10. Results of statistical analysis for SNARE complex formation during capacitation (Fig. 5.3).

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<td>B0 vs B30</td>
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<td>0.22</td>
<td>0.6415</td>
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<td>0.000830</td>
<td>0.00</td>
<td>0.9960</td>
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<tr>
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<td>10.524872</td>
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<td>0.5753</td>
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Table B.11. Results of statistical analysis for SNARE complex formation during capacitation (Fig. 5.4).

<table>
<thead>
<tr>
<th>Contrast</th>
<th>DF</th>
<th>Contrast SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<td>Contrast AvsN</td>
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<td>94.692996</td>
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