Advances in Anatomy, Embryology and Cell Biology

Mariano G. Buffone *Editor*

Sperm Acrosome Biogenesis and Function During Fertilization

Springer

Advances in Anatomy, Embryology and Cell Biology publishes critical reviews and state-of-the-art surveys on all aspects of anatomy and of developmental, cellular and molecular biology, with a special emphasis on biomedical and translational topics.

The series publishes volumes in two different formats:

- Contributed volumes, each collecting 5 to 15 focused reviews written by leading experts
- Single-authored or multi-authored monographs, providing a comprehensive overview of their topic of research

220

Advances in Anatomy, Embryology and Cell Biology

Mariano G. Buffone
Editor

Sperm Acrosome Biogenesis and Function During Fertilization

Springer

Editor
Mariano G. Buffone
Instituto de Biologia y Medicina Experimental
Buenos Aires, Argentina

ISSN 0301-5556 ISSN 2192-7065 (electronic)
Advances in Anatomy, Embryology and Cell Biology
ISBN 978-3-319-30565-3 ISBN 978-3-319-30567-7 (eBook)
DOI 10.1007/978-3-319-30567-7

Library of Congress Control Number: 2016940367

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Preface

Since its discovery in 1952, acrosomal exocytosis (also called acrosome reaction) has been a fascinating process that captured the attention of many researchers. For many years, acrosomal exocytosis was thought as a carriage of proteases. However, several discoveries along the last 64 years have produced a shift in the operational view of the acrosome, from the earlier "large lysosome" model to more complex model where the acrosome possesses a central role in the process of fertilization.

Although acrosomal exocytosis shares many features with other exocytotic processes, it also has unique properties and its occurrence is limited to the male gamete, resulting in an ideal target for developing new drugs for male contraception. However, up to now, no acrosome-specific proteins whose inhibition would block this process have been discovered as has occurred with other sperm functions (i.e., CatSper channel and hyperactivation, Izumo1 and sperm–egg fusion, Slo3 and membrane hyperpolarization).

Despite that, a few years after its discovery, it was noticed that the occurrence of this exocytosis is essential for fertilization, recent evidence has provided a molecular context to that demonstration: Izumo1, an essential protein for sperm–egg fusion is relocalized to the proper site only after acrosomal exocytosis occurs. In a broader context, nowadays, the central dogma that prevails for many years, where a capacitated sperm undergoes exocytosis after binding to the Zona Pellucida of the egg is under debate since reports from different laboratories have claimed that fertilizing sperm undergo exocytosis prior to interacting with the egg's extracellular matrix. Today, it is uncertain where and when mammalian sperm trigger exocytosis. And as a consequence of "where and when," what is the physiological stimulant (s) of this process? Is there a physiological molecule? Or exocytosis occurs as a result of the continuous destabilization of the acrosomal region during capacitation?

It is our hope that this special volume will serve as a reference for many students and researchers who are interested in this particular type of exocytosis. In this special volume, several leaders of our field have extensively reviewed several topics related to the process of acrosomal exocytosis, covering cellular aspects such as where and when exocytosis occurs in mammalian sperm to molecular

aspects such as the proteins of the fusion machinery that participate in the last steps of the process.

Buenos Aires, Argentina Mariano G. Buffone

Contents

Chapter 1
The Acrosome Reaction: A Historical Perspective

Masaru Okabe

1.1 Discovery of Acrosome Reaction

Development of a new tool or method brings us new insight into science. The invention of the phase contrast microscope (yielding a Nobel Prize for Dutch physicist Frits Zernikev in 1953) led American Jean Clark Dan (who married Japanese scientist Katsuma Dan) to observe sea urchin spermatozoa using one of the first commercialized versions of the microscope (Fig. 1.1) and resulted in the world's first "acrosome reaction" paper (Dan 1952). Around the same time, researchers studying mammalian fertilization found that spermatozoa have no fertilizing ability when ejaculated, but require time in the female reproductive tract before fertilizing eggs (Austin 1951; Chang 1951). This phenomenon, specific to mammalian spermatozoa, was termed "capacitation." Initially, the importance of the acrosome reaction in mammalian fertilization was unclear, but the phenomenon soon became recognized as a prerequisite for fertilization (Austin and Bishop 1958). Capacitation represents various changes taking place in spermatozoa, probably initiated by some signals after ejaculation. However, confirming the essentiality of these changes is difficult, especially when spermatozoa are examined as a mass, as even under optimal conditions, not all spermatozoa survive, capacitate, and undergo acrosome reaction during observation (Yanagimachi 1994).

M. Okabe (✉)
Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita 565-0871, Osaka, Japan
e-mail: okabe@biken.osaka-u.ac.jp

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_1

Fig. 1.1 The historical phase contrast microscope with which Jean Clark Dan observed acrosome reaction for the first time in sea urchin spermatozoa. (The microscope is preserved in Misaki Marine Biological Station, The University of Tokyo, Japan. The photo was provided by Professor Hideo Mohri)

1.2 What Triggers the Acrosome Reaction in Mammals?

In animals such as sea urchin (Neill and Vacquier 2004), starfish (Hoshi et al. 2012), and *Xenopus laevis* (Brun 1974), spermatozoa are induced to undergo acrosome reaction when they contact the jelly layers surrounding eggs. The mechanism of acrosome reaction initiation in these animals is well documented from a molecular standpoint. However, acrosome reaction in mammalian spermatozoa is complicated by the requisite capacitation phenomenon (Fig. 1.2), which consists of time-dependent physiological changes in spermatozoa in the female reproductive tract.

If the zona pellucida has characteristics analogous to the jelly layers of sea urchin eggs, it may have the ability to induce acrosome reaction. Initial electron microscopic observation seemed to support this assumption (Saling et al. 1979). The longer the sperm bound to the surface of the egg zonae pellucidae, the more acrosome-reacted spermatozoa were found. Following this report, zona component ZP3 was identified as a sperm receptor (Bleil and Wassarman 1980; Vazquez et al. 1989) and ZP3 was reported to possess both sperm-binding and acrosome reaction-inducing activity by aggregating sperm β 1,4-galactosyltransferase (GalTase) (Macek et al. 1991). These results promoted the hypothesis of zona-induced acrosome reaction. On the sperm side, SP56 was also reported as a ligand for ZP3 (Bookbinder et al. 1995). However, the reality seemed to be less simple than the zona-induced acrosome reaction theory indicated. Spermatozoa from GalTase or SP56 disrupted mice were shown to penetrate zona pellucida with ease (Asano et al. 1997) (Muro et al. 2012). The essential role of ZP3 in fertilization could not be examined in gene-disruption experiments, because when ZP3 was disrupted, zona pellucida was not formed (Rankin et al. 1996). Yet when ZP3 was replaced by human ZP3, human sperm did not bind to the chimeric zona pellucida, and notwithstanding the absence of mouse ZP3, mouse sperm bound to ovulated eggs in vitro and fertility was restored (Rankin et al. 1998).

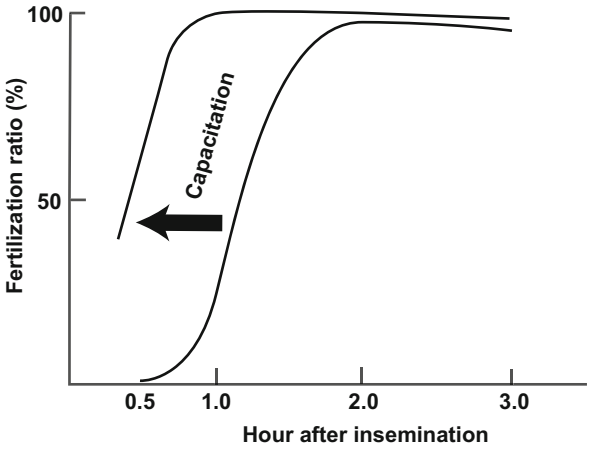

Fig. 1.2 The progression of mouse fertilization in vitro. As indicated, when freshly prepared epididymal spermatozoa were added to eggs, they required time before penetrating the zona pellucida, but when spermatozoa were preincubated in vitro, the required time shortened (Austin 1951; Chang 1951). This difference could be considered requisite time for "capacitation" in mice. From Toyoda Y et al., Studies on the fertilization of mouse eggs in vitro (Toyoda et al. 1971)

1.3 Visualization of Acrosomal Status Using Transgenic Mouse Lines

The green fluorescent protein (GFP) produced by jellyfish opened the door for a vital marker. Soon after the first report in both bacteria and *C. elegans* (Chalfie et al. 1994), we reported that conversion of GFP from its native peptide to the fluorescent chromophore formation could occur in live mice (Ikawa et al. 1995). The initial "green mice" were rather dim, but a mutated enhanced version of GFP (EGFP) was glittery green (Okabe et al. 1997) (Fig. 1.3). Spermatozoa were not fluorescent green in these mice, probably because spermatozoa have a small amount of cytosol, which is where the GFP was designed to localize. However, by using the acrosin promoter (Watanabe et al. 1991), we were able to establish a transgenic mouse line which contained EGFP in sperm acrosome. The sperm showed normal fertilizing ability, and the integrity of their acrosome was easily examined in a noninvasive manner by observing the GFP in an individual "live" spermatozoon under fluorescent microscope. To our surprise, it was shown that spermatozoa did not undergo acrosome reaction only by binding to the zona pellucida (Nakanishi et al. 1999). A similar observation was reported by another group (Baibakov et al. 2007). Observation of acrosome reaction was somewhat inconvenient in the first generation of "green sperm" as the spermatozoa became nonfluorescent after acrosome reaction.

We then produced another transgenic mouse line with EGFP expressed in the acrosome and red fluorescent protein (RFP) arranged to have a mitochondria migration signal (Hasuwa et al. 2010). The dual fluorescent sperm showed normal

Fig. 1.3 The world's first "green mice." Nascent GFP has no fluorescence. A dehydration condensation reaction must take place to form a chromophore in the GFP molecule. It was surprising that this reaction could take place in mouse body (as in jellyfish). The photo shows the second-generation transgenic mouse line in which enhanced GFP (EGFP) was used (Okabe et al. 1997). The EGFP transgenic mouse line under the CAG promoter rendered almost all organs and cells fluorescent green. We have established over 100 lines of "green mice" (Nakanishi et al. 2002). Among them are X-linked GFP lines, which enable us to distinguish male and female embryos before the implantation stage (Isotani et al. 2005)

fertilizing ability in both in vivo and in vitro fertilization and the spermatozoa continued to be visible even after the acrosome reaction. This made it possible to trace sperm behavior in cumulus cells together with their acrosomal status (Fig. 1.4). By using this transgenic mouse line, it was indicated that a majority of spermatozoa underwent acrosome reaction before contacting the zona pellucida in vitro (Hirohashi et al. 2011; Jin et al. 2011). Since spermatozoa with red mitochondria can be observed through the uterine wall, the fate of spermatozoa in vivo is under investigation in various laboratories. Observations of sperm behavior in oviduct and their acrosomal status in vivo will provide us with deeper insight into the mechanism of fertilization.

1.4 Necessity of Acrosome Reaction

In some species like drosophila, the entire spermatozoon is incorporated into egg cytoplasm without acrosome reaction. However, the acrosome itself is still indispensable in drosophila, because acrosomal protein *Snky* is essential for successful fertilization, assisting sperm plasma membrane breakdown in egg (Wilson et al. 2006). Bony fish provide another intriguing example of how fertilization is accomplished, as their spermatozoa do not have an acrosome. How do spermatozoa from these two species penetrate the egg investment without acrosome reaction? In both drosophila and bony fish, the spermatozoa reach the egg by passing through a single hole in the chorion called micropyle. Plants and animals have significantly

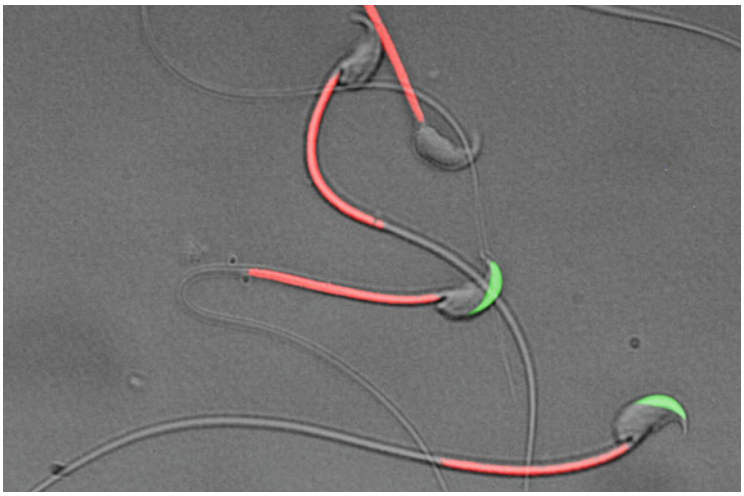

Fig. 1.4 Spermatozoa with green acrosome and red midpiece. RFP (DsRed2) was designed to be expressed ubiquitously under the CAG promoter with a mitochondrial import signal sequence of Atp5g1 (su9) and the EGFP was designed to be expressed under the acrosin promoter with a proacrosin signal sequence (Hasuwa et al. 2010). Note that acrosome-reacted spermatozoa (without EGFP in the acrosomal area) and acrosome-intact sperm (with green fluorescence in the acrosome) are clearly distinguishable from each other. The acrosomal status of fertilizing spermatozoa was investigated using these sperm (Jin et al. 2011; Hirohashi et al. 2011). More importantly, observation of sperm behavior inside the female reproductive tract became possible (Tokuhiro et al. 2012). Reports of more precise observations of behavior of spermatozoa in vivo using fluorescent protein-tagged spermatozoa are expected

different fertilization systems. However, it is interesting to know that plant seeds provide a micropyle for pollen tubes to penetrate. Considering these factors, one can generalize that whereas eggs have no micropyle, spermatozoa have an acrosome and the acrosome reaction is a prerequisite for fertilization. Therefore, one of the essential roles of acrosome reaction might be to invest spermatozoa with the ability to perforate the egg investments.

1.5 "Acrosome Reaction" and "Acrosomal Exocytosis"

Recently, the terms "acrosomal exocytosis" and "acrosome reaction" have been used interchangeably. However, the word "exocytosis" reminds us of Austin's comment from the 1950s that acrosome is "known to contain polysaccharide and probably carries the enzymes and other agents that are responsible for penetration of the cumulus, for attachment to and penetration of the zona, and for eliciting a specific response from the vitellus upon contact with its surface" (Austin and Bishop 1957). However, the same author later speculated that, as acrosome seemed to be lost before entry into the egg, acrosomal exocytosis might not be required for

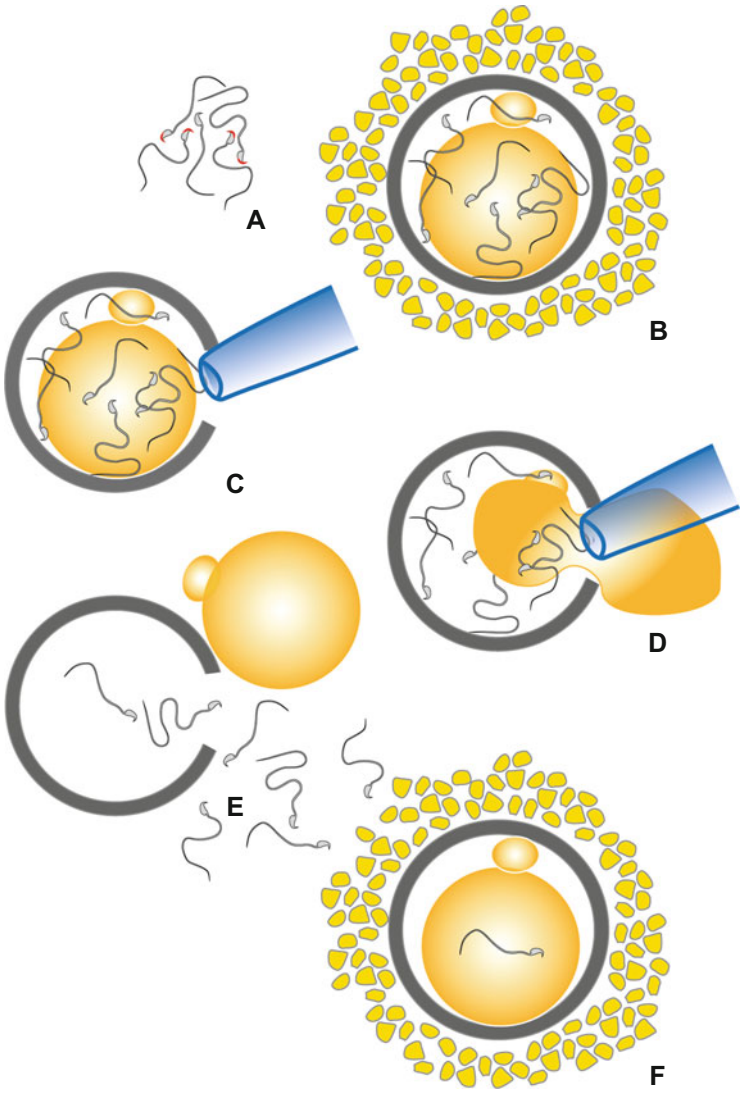

Fig. 1.5 Recovery of spermatozoa from the perivitelline space and subsequent fertilization in vitro. (**a**) Spermatozoa incubated in vitro are a mixture of acrosome-intact and acrosome-reacted spermatozoa. (**b**) IVF was performed in the condition that sperm–egg fusion was inhibited (e.g., IZUMO1$^{-/-}$ spermatozoa vs. wild-type egg, or wild-type spermatozoa vs. CD9$^{-/-}$ eggs). Spermatozoa failing to fuse with eggs accumulated inside the zona pellucida. All spermatozoa in the perivitelline space underwent acrosome reaction. (**c**) A Piezo manipulator was used to create a hole in the zona pellucida using a glass pipet. (**d**) Medium was flushed into the perivitelline space with the same pipet. (**e**) The oocyte and the zona-penetrated spermatozoa were released into the medium. (**f**) Freshly collected oocytes covered by cumulus layers were introduced into the medium and a second penetration of zona pellucida was observed. Wild-type spermatozoa (using Cd9$^{-/-}$ eggs) showed ability to fuse with eggs after a second round of zona penetration and form pronuclei 12 h after insemination. These eggs were shown to develop to term after transplantation into pseudopregnant females (Inoue et al. 2011)

zona penetration (Austin and Bishop 1958). In accordance with this notion, the spermatozoa recovered from the perivitelline space (presumably, post-acrosomal "exocytosis") could penetrate the cumulus layers and zona pellucida of fresh eggs a second time and fertilize eggs (Kuzan et al. 1984; Inoue et al. 2011) (Fig. 1.5). Thus, the acrosome reaction cannot be regarded as a simple "exocytosis."

1.6 Changes in Sperm Membrane During and After Acrosome Reaction

In addition to exocytosing acrosomal content, acrosome-reacted spermatozoa change into a fertilization-competent state during and after exocytosis. The acrosome reaction removes the plasma membrane in the acrosomal cap area and exposes a new membrane including the structure called the perforatorium (Austin and Bishop 1958). Some of the acrosomal contents are now known to remain on spermatozoa even after "exocytosis" (Kim et al. 2001; Nakanishi et al. 2001). These antigens may function during one of the successive transitional stages leading to the complete release of acrosomal components (Kim and Gerton 2003). Acrosome reaction not only enables spermatozoa to perforate the egg investments, it is also essential in rendering spermatozoa competent to fuse with eggs.

Attempts to fuse acrosome-intact spermatozoa with eggs were not successful (Yanagimachi 1994). It should be noted that in normal exocytosis, the vesicle membrane remains on the plasma membrane at the time of excretion. On the other hand, in acrosome reaction, the plasma and outer acrosomal membrane make vesicles and are dispersed in the environment. As a result, the inner acrosomal membrane is exposed and becomes the outermost surface of spermatozoa. Interestingly, sperm–egg fusion does not initiate in the inner acrosomal membrane area, but rather on the membrane of the equatorial segment. How does fertilization competency manifest after the acrosome reaction in this area? At the time of acrosome reaction, the plasma membrane must undergo a drastic change enabling fusion with the underlying outer acrosomal membrane. This change may affect the nature of plasma membrane in the equatorial segment. Additionally or alternatively, the dormant fusion protein might be activated by factors released by acrosomal exocytosis.

1.7 A New Aspect of Membrane Change in Acrosome Reaction

Recently, another mechanism enabling fertilization-competent spermatozoa was postulated. A monoclonal antibody to IZUMO1 was shown to inhibit sperm–egg fusion (Okabe et al. 1988). The disruption of gene Izumo1 did not affect the zona

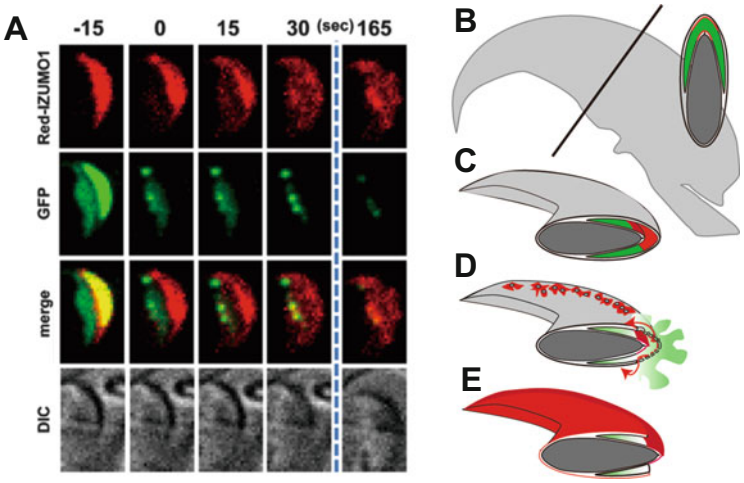

Fig. 1.6 Using OBF13, a sperm–egg fusion inhibitory monoclonal antibody, IZUMO1 was identified by LC/MS/MS and cloned. The spermatozoa lacking IZUMO1 had no ability to fuse with the eggs (Inoue et al. 2005). The localization of IZUMO1 was investigated by producing RFP-Tagged IZUMO1 carrying transgenic spermatozoa. (**a**) indicates the time in relation to the start of acrosome reaction. (**b–e**) indicates the 3-dimensional diagram of sperm membrane. (**b**) Before acrosome reaction, IZUMO1 was localized on inner and outer acrosomal membrane of the acrosomal cap area. (**a**) Within 30 s of the initiation of acrosome reaction, IZUMO1 migrated into the entire head. One explanation for this migration might be that IZUMO1 migrated out to the plasma membrane where the plasma and outer acrosomal membrane were fused. This was illustrated in (**d**) and (**e**) (Satouh et al. 2012)

pellucida penetration ability of spermatozoa at all, but fusing ability with the eggs was completely impaired (Inoue et al. 2005). The fusion-related protein IZUMO1 does not reside on the sperm plasma membrane in the pre-acrosome-reacted state. As an alternative to antibody usage, IZUMO1 was tagged with red fluorescent protein (RFP) and a transgenic mouse line with Red-IZUMO1 was produced. Using the spermatozoa from this transgenic line, the localization of IZUMO1 was investigated in live spermatozoa. As a result, it was found that IZUMO1 is localized on the acrosomal cap area of both outer and inner acrosomal membrane, and at the moment of acrosome reaction, IZUMO1 migrates out from outer (but not inner) acrosomal membrane to plasma membrane. The migration of IZUMO1 to the equatorial segment during/after acrosome reaction was recorded in a movie (Satouh et al. 2012) (Fig. 1.6). The experiment demonstrated that one function of acrosome reaction is to supply protein(s) from outer acrosomal membrane to plasma membrane.

The dynamic movement of IZUMO1 during fusion was also investigated using the spermatozoa from the same transgenic mouse line. IZUMO1 was mainly localized to the equatorial segment before fusion, but it dispersed on egg plasma membrane rapidly when the sperm–egg fusion took place (Fig. 1.7). However, IZUMO1 on the inner acrosomal membrane did not disperse even after fusion, but

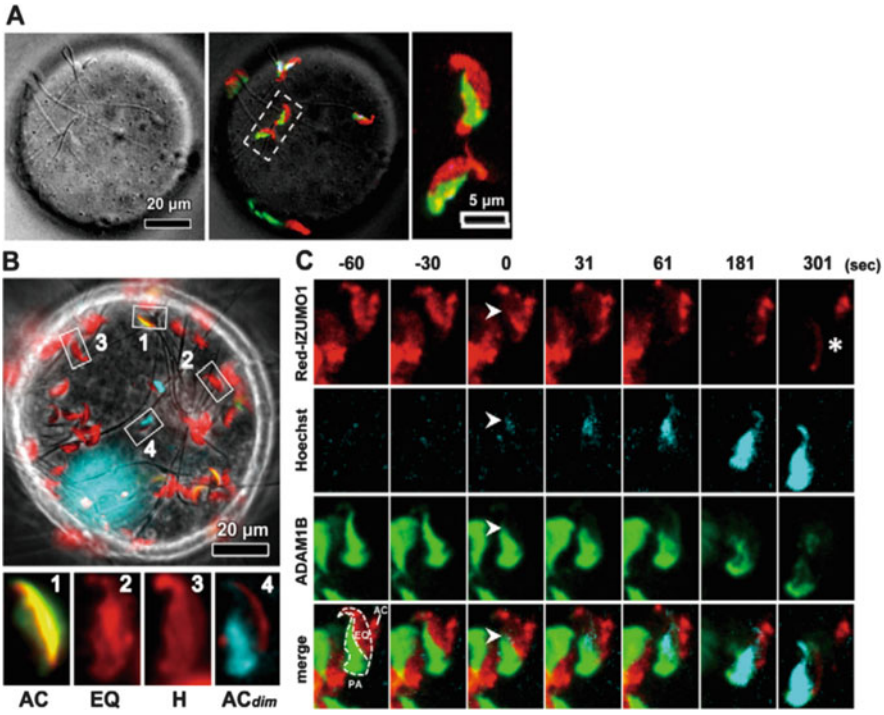

Fig. 1.7 (**a**) Red-IZUMO1 males were mated with females and the eggs were recovered 8 h after copulation. The spermatozoa were in the perivitelline space but failed to fuse with $CD9^{-/-}$ eggs. Spermatozoa were resistant to Hoechst 33258 staining (*cyan*) as they were still vital. In these spermatozoa, ADAM1B was localized in the postequatorial segment of the spermatozoa [stained by Alexa-488-conjugated mAb (*green*)]. A magnified image of spermatozoa showing IZUMO1 on the equatorial segment is seen on the *right*. (**b**) Spermatozoa from Red-IZUMO1 males were mixed with zona-free oocyte. Spermatozoa with variously located IZUMO1 were observed on the egg surface. The acrosomal cap (AC) pattern shows the spermatozoa before acrosome reaction, whereas the acrosome-reacted spermatozoa and spermatozoa imaged before fusion showed Red-IZUMO1 spread out to the entire head ('H'-type distribution), or with a tendency to gather in the equatorial segment ("EQ" type). Fused spermatozoa (Hoechst 33342-positive uptake from the preloaded oolemma) showed the dim acrosomal cap pattern (AC_{dim}) without exception. (**c**) A representative time-lapse view of sperm–egg fusion. The initiation of fusion (time 0) was defined as transfer of Hoechst 33342 dye to the spermatozoa. After the fusion, Red-IZUMO1 dispersed from the same area where fusion started (indicated by *arrowheads*) and mostly finished within 30 s. In contrast, the diffusion of ADAM1B (*green*) from the post-acrosomal area did not take place within the 30-s period, but started about 60 s after the fusion. The dispersal was concomitant with the expansion of the Hoechst 33342 staining area toward the posterior head. The asterisk indicates the emergence of the AC_{dim} pattern (Satouh et al. 2012)

was incorporated into the cytoplasm of the egg as an inner acrosomal membrane structure complex. The two different fates of IZUMO1 on inner and outer acrosomal membrane were recorded in a movie. It has been long understood, but not clearly stated, that sperm–egg fusion is apparently divided into two different phases

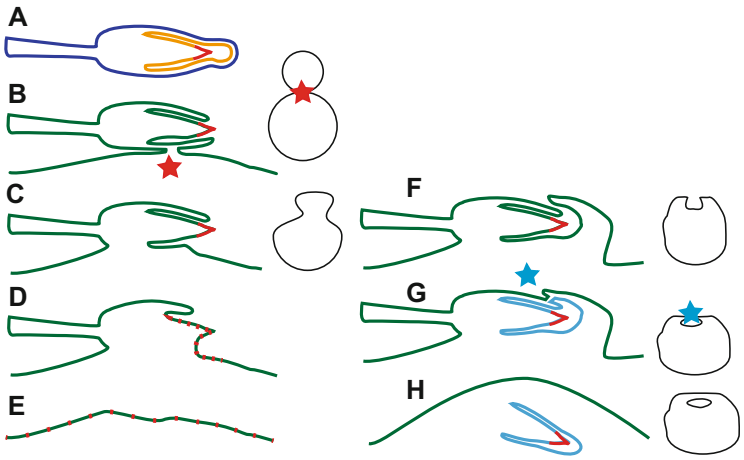

Fig. 1.8 (**a**) Intact spermatozoa have a plasma membrane (*blue*) and an acrosomal membrane (*orange*). (**b**) After the acrosome reaction, these two membranes fuse and form a new sperm membrane. The first fusion event, shown by a *red star*, takes place to join sperm and egg membrane into a single plasma membrane (*green*). A simplified image of the fusion of two independent membranes is illustrated beside Figures (**b**) and (**c**). (**c**–**e**) If fusion is accomplished in this step, IZUMO1 on the acrosomal cap of the inner acrosomal membrane (indicated by *red*) must localize on the newly formed united membrane (*green*). (**f**, **g**) However, the second fusion (indicated by a *blue star*) follows the first, which separates the acrosomal cap and acrosomal sheath areas (*light blue*) from the unified sperm–egg membrane (*green*). (**h**) As a result, IZUMO1 on the inner acrosomal membrane is invaginated in the cytoplasm. A simplified image of the second fusion (dividing fusion of a single membrane) is illustrated beside Figures (**f**–**h**). From live imaging, the disruption of IZUMO1 resulted in the loss of the first fusion. The nature of the second fusion remains totally unknown

as explained in Fig. 1.7. Recently, JUNO on egg was found to interact with IZUMO1 and facilitate sperm–egg fusion (Bianchi et al. 2014). It is interesting to know the micro-localization of JUNO at the time of these two distinctively different fusion processes (Fig. 1.8).

1.8 Emerging Roles of the Acrosome During Spermatogenesis

The degradation of unnecessary or dysfunctional cellular components is achieved through the actions of lysosomes. This catabolic process is called autophagy, which also functions to supply needed nutrients when cells face starvation. It is known that the fertilization-activated autophagy is induced in the developing embryos before implantation (Tsukamoto et al. 2008). Recently, it was reported that autophagy might be involved in spermatogenesis. When one of the key molecules for autophagy was disrupted in germ cells, acrosome formation was impaired and

spermatozoa displayed a phenotype similar to that of human globozoospermia (Wang et al. 2014). From this viewpoint, the acrosome can be regarded as a kind of autolysosome derived from double-membrane autophagosome (Klionsky et al. 2014). If the exocytosed enzymes during acrosome reaction are dispensable as indicated from the experiment shown in Fig. 1.5, the significance of many hydrolyzing enzymes in the acrosome could partly be explained by viewing the acrosome as a catabolic apparatus.

1.9 Future Research

Our experience in producing over 300 gene-disrupted mouse lines for scientists in numerous fields (from 1995 to March 2013: http://www.tgko.biken.osaka-u.ac.jp/tgko/sum/index) revealed that a majority of the gene-disrupted mouse lines showed no phenotype (or only a minor phenotype). This in turn suggested that many genes are dispensable in sustaining the experimental animals kept in animal facilities. The same can be said for various sperm-specific genes. Despite the fact that their gene products are found only in spermatozoa, the disruption of most of these genes did not result in infertile males (Okabe 2015). To our surprise, most of these sperm-specific proteins were indicated to play an important role in various experimental models in vitro. The reasons for this discrepancy could be argued, but as a bottom line, we should heed the importance of incorporating gene-disruption experiments to verify the role of various factors. The recently developed CRISPR/CAS9 system (Wang et al. 2013) should become a powerful means of achieving these goals. This quick and inexpensive revolutionary technique for producing gene-manipulated animals is arguably easier than producing an antibody of a given protein. Moreover, it works in virtually all living creatures from animals to plants (Peng et al. 2014). We are now at the dawn of a deeper and pan-species understanding of the acrosome reaction.

References

Asano M, Furukawa K, Kido M, Matsumoto S, Umesaki Y, Kochibe N, Iwakura Y (1997) Growth retardation and early death of beta-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. EMBO J 16: 1850–1857. doi:10.1093/emboj/16.8.1850

Austin CR (1951) Observations on the penetration of the sperm in the mammalian egg. Aust J Sci Res B 4:581–596

Austin CR, Bishop MWH (1957) Fertilization in mammals. Biol Rev 32:296–348

Austin CR, Bishop MWH (1958) Some features of the acrosome and perforatorium in mammalian spermatozoa. Proc Roy Soc Lond B Biol Sci 149:234

Baibakov B, Gauthier L, Talbot P, Rankin TL, Dean J (2007) Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. Development 134:933–943. doi:10.1242/dev.02752

Bianchi E, Doe B, Goulding D, Wright GJ (2014) Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature 508:483–487. doi:10.1038/nature13203

Bleil JD, Wassarman PM (1980) Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell 20:873–882. doi:10.1016/0012-1606(83)90032-5

Bookbinder LH, Cheng A, Bleil JD (1995) Tissue- and species-specific expression of sp56, a mouse sperm fertilization protein. Science 269:86–89

Brun RB (1974) Studies on fertilization in Xenopus laevis. Biol Reprod 11:513–518

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263:802–805

Chang MC (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:697–698

Dan J (1952) Studies on the acrosome. I. Reaction to egg-water and other stimuli. Biol Bull 103: 54–66

Hasuwa H, Muro Y, Ikawa M, Kato N, Tsujimoto Y, Okabe M (2010) Transgenic mouse sperm that have green acrosome and red mitochondria allow visualization of sperm and their acrosome reaction in vivo. Exp Anim 59:105–107

Hirohashi N, Gerton GL, Buffone MG (2011) Video imaging of the sperm acrosome reaction during in vitro fertilization. Commun Integr Biol 4:471–476. doi:10.4161/cib.4.4.15636

Hoshi M, Moriyama H, Matsumoto M (2012) Structure of acrosome reaction-inducing substance in the jelly coat of starfish eggs: a mini review. Biochem Biophys Res Commun 425:595–598. doi:10.1016/j.bbrc.2012.08.033

Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M (1995) Green fluorescent protein as a marker in transgenic mice. Develop Growth Differ 37:455–459

Inoue N, Ikawa M, Isotani A, Okabe M (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434:234–238. doi:10.1038/nature03362

Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R (2011) Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. Proc Natl Acad Sci USA 108:20008–20011. doi:10.1073/pnas.1116965108

Isotani A, Nakanishi T, Kobayashi S et al (2005) Genomic imprinting of XX spermatogonia and XX oocytes recovered from XX <-> XY chimeric testes. Proc Natl Acad Sci USA 102: 4039–4044. doi:10.1073/pnas.0406769102

Jin M, Fujiwara E, Kakiuchi Y et al (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci USA 108:4892–4896. doi:10.1073/pnas.1018202108

Kim KS, Gerton GL (2003) Differential release of soluble and matrix components: evidence for intermediate states of secretion during spontaneous acrosomal exocytosis in mouse sperm. Dev Biol 264:141–152

Kim KS, Foster JA, Gerton GL (2001) Differential release of guinea pig sperm acrosomal components during exocytosis. Biol Reprod 64:148–156

Klionsky DJ, Eskelinen EL, Deretic V (2014) Autophagosomes, phagosomes, autolysosomes, phagolysosomes, autophagolysosomes... wait, I'm confused. Autophagy 10:549–551. doi:10.4161/auto.28448

Kuzan FB, Fleming AD, Seidel GE Jr (1984) Successful fertilization in vitro of fresh intact oocytes by perivitelline (acrosome-reacted) spermatozoa of the rabbit. Fertil Steril 41:766–770

Macek MB, Lopez LC, Shur BD (1991) Aggregation of beta-1,4-galactosyltransferase on mouse sperm induces the acrosome reaction. Dev Biol 147:440–444

Muro Y, Buffone MG, Okabe M, Gerton GL (2012) Function of the acrosomal Matrix: zona pellucida 3 receptor (ZP3R/sp56) is not essential for mouse fertilization. Biol Reprod 86(1): 1–6. doi:10.1095/biolreprod.111.095877

Nakanishi T, Ikawa M, Yamada S, Parvinen M, Baba T, Nishimune Y, Okabe M (1999) Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein. Febs Lett 449:277–283. doi:10.1016/s0014-5793(99)00433-0

Nakanishi T, Ikawa M, Yamada S, Toshimori K, Okabe M (2001) Alkalinization of acrosome measured by GFP as a pH indicator and its relation to sperm capacitation. Dev Biol 237: 222–231. doi:10.1006/dbio.2001.0353

Nakanishi T, Kuroiwa A, Yamada S et al (2002) FISH analysis of 142 EGFP transgene integration sites into the mouse genome. Genomics 80:564–574. doi:10.1006/geno.2002.7008

Neill AT, Vacquier VD (2004) Ligands and receptors mediating signal transduction in sea urchin spermatozoa. Reproduction 127:141–149. doi:10.1530/rep.1.00085

Okabe M (2015) Mechanisms of fertilization elucidated by gene-manipulated animals. Asian J Androl 17:646–652. doi:10.4103/1008-682X.153299

Okabe M, Yagasaki M, Oda H, Matzno S, Kohama Y, Mimura T (1988) Effect of a monoclonal anti-mouse sperm antibody (OBF13) on the interaction of mouse sperm with zona-free mouse and hamster eggs. J Reprod Immunol 13:211–219. doi:10.1016/0165-0378(88)90002-2

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) 'Green mice' as a source of ubiquitous green cells. Febs Lett 407:313–319. doi:10.1016/s0014-5793(97)00313-x

Peng Y, Clark KJ, Campbell JM, Panetta MR, Guo Y, Ekker SC (2014) Making designer mutants in model organisms. Development 141:4042–4054. doi:10.1242/dev.102186

Rankin T, Familari M, Lee E et al (1996) Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. Development 122:2903–2910

Rankin TL, Tong ZB, Castle PE, Lee E, Gore-Langton R, Nelson LM, Dean J (1998) Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding. Development 125:2415–2424

Saling PM, Sowinski J, Storey BT (1979) An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae in vitro: sequential relationship to the acrosome reaction. J Exp Zool 209:229–238. doi:10.1002/jez.1402090205

Satouh Y, Inoue N, Ikawa M, Okabe M (2012) Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. J Cell Sci 125:4985–4990. doi:10.1242/jcs.100867

Tokuhiro K, Ikawa M, Benham AM, Okabe M (2012) Protein disulfide isomerase homolog PDILT is required for quality control of sperm membrane protein ADAM3 and male infertility. Proc Natl Acad Sci USA 109:3850–3855. doi:10.1073/pnas.1117963109

Toyoda Y, Yokoyama M, Hoshi T (1971) Studies on the fertilization of mouse eggs in vitro. Jpn J Anim Reprod 16:147–157

Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N (2008) Autophagy is essential for preimplantation development of mouse embryos. Science 321:117–120. doi:10.1126/science.1154822

Vazquez MH, Phillips DM, Wassarman PM (1989) Interaction of mouse sperm with purified sperm receptors covalently linked to silica beads. J Cell Sci 92(Pt 4):713–722

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153:910–918. doi:10.1016/j.cell.2013.04.025

Wang H, Wan H, Li X et al (2014) Atg7 is required for acrosome biogenesis during spermatogenesis in mice. Cell Res 24:852–869. doi:10.1038/cr.2014.70

Watanabe K, Baba T, Kashiwabara S, Okamoto A, Arai Y (1991) Structure and organization of the mouse acrosin gene. J Biochem 109:828–833

Wilson KL, Fitch KR, Bafus BT, Wakimoto BT (2006) Sperm plasma membrane breakdown during Drosophila fertilization requires sneaky, an acrosomal membrane protein. Development 133: 4871–4879. doi:10.1242/dev.02671

Yanagimachi R (1994) Mammalian fertilization. Raven, New York

Chapter 2
The Acrosomal Matrix

James A. Foster and George L. Gerton

2.1 Introduction

The acrosome is a single exocytotic vesicle overlying the nucleus of most animal sperm. While the sperm of some taxa (e.g., teleost fish) do not possess an acrosome, it is generally thought that mammalian sperm acrosomes may perform two principal exocytosis-dependent functions: (1) to serve as a site for the sequestration and release of proteins required for binding to and penetration of the zona pellucida and (2) to enable the sperm to fuse with the oolemma. For the past several decades, research has been largely guided by the concept that mammalian sperm acrosomes must be intact to bind to the zona pellucida prior to undergoing exocytosis, enabling the sperm to penetrate this vestment and fertilize the egg (Wassarman et al. 2004). Although this widely accepted dogma has been challenged on many levels, recent experiments utilizing modern, highly sophisticated imaging techniques have enabled investigators to track the acrosomal status of individual fertilizing spermatozoa. Using genetically engineered mouse strains that express green fluorescent protein in their acrosomes, these experiments have shown that almost all fertilizing mouse sperm have undergone acrosomal exocytosis prior to the time they penetrate the zona pellucida (Jin et al. 2011).

These contemporary experiments raise new questions of how the acrosome forms during spermatogenesis and functions in the fertilization process. Specific

J.A. Foster (✉)
Department of Biology, Randolph-Macon College, Ashland, VA 23005, USA
e-mail: jfoster@rmc.edu

G.L. Gerton
Department of Obstetrics and Gynecology, Center for Research on Reproduction and Women's Health, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6160, USA
e-mail: gerton@upenn.edu

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_2

issues that need to be addressed include (1) the contribution of acrosomal biogenesis to the fertility of individual sperm, (2) the timing of acrosomal exocytosis in vivo, (3) the role of acrosomal compartmentalization (including structurally distinct domains within the vesicle and biochemical segregation of overlapping soluble materials and insoluble acrosomal matrix compartments), and (4) the functions of specific proteins in fertilization. While recent results challenge or even contradict experiments that were based upon previous reductionist paradigms, many of the older studies were carefully designed and yielded a solid body of evidence that should be reevaluated from a more holistic standpoint to see whether a reinterpretation sheds new light on the question of the physiological role of the acrosome in fertilization.

In a previous review, we focused on the structure and function of the sperm acrosomal matrix in fertilization, where we espoused three principal points: (1) the acrosome is not a "bag of enzymes," simply comprised of soluble substances that are rapidly released when the outer acrosomal membrane fuses with the plasma membrane, (2) acrosomal exocytosis is not an "all-or-none" event, where the acrosome is either "intact" or "reacted" but instead incorporates intermediate steps involving the progressive loss of acrosomal matrix material, and (3) the acrosomal matrix is a molecular scaffold assembly that is dismantled by a self-regulated mechanism driven, in part, by proteolysis (Buffone et al. 2008). We will not recapitulate these points in this review, but we will discuss them in light of the many new findings from studies using genetically modified mice.

It is abundantly clear that fertilization is a complicated process that requires models to extend from a cluster of hundreds or thousands of sperm to the single-cell level. Given the heterogeneous nature in the structure and physiology in a population of sperm, it is not surprising that aspects of acrosomal structure, including the matrix, could depend on events during spermatogenesis and could impact function at several steps in the female reproductive tract or the cumulus–oocyte complex. Furthermore, the heterogeneity in the timing and location of acrosomal exocytosis in individual cells present within a population of spermatozoa is not yet fully understood. Thus, it is a challenge to sort out real population cell behaviors (e.g., disruption of cumulus cells) from irrelevant or deleterious cell behaviors (spontaneous exocytosis, cell demise). Perhaps the most important question is, what is the location and timing of these events in the one sperm that successfully fertilizes the egg? We must also recognize that there might be multiple means (pathways) to an end (fertilization). In light of the contemporary imaging studies indicating that most mouse sperm that fertilize mouse oocytes enclosed by cumulus masses have lost the green fluorescent protein marker of intact acrosomes (Jin et al. 2011; Hirohashi et al. 2011), we recognize that work in this area is still very active so it would be premature to attempt to develop a consensus model. Nevertheless, it is useful at this time to consider results utilizing another modern approach: gene knockouts in mice. Toward this goal, recently developed, genetically modified mouse models provide valuable new insights into the molecular mechanisms of mammalian fertilization involving the sperm acrosome.

2.2 Gene Knockout Studies in Mice: What They Do and Do Not Tell Us About Acrosomal Matrix Function

The knockout of a specific reproductive gene in the mouse is usually engineered with an explicit outcome (hypothesis) in mind: to demonstrate the importance of a particular protein by interfering with fertility. However, the resulting phenotypes are often different and/or are more complicated than expected. In many cases, knockouts of individual genes encoding acrosomal matrix proteins have not caused sterility; this outcome can be explained away with the understanding that sperm function and fertilization are critical for survival of a species, so it makes sense to have built-in redundancy. Nevertheless, closer inspection of some of these "fertile" knockouts has revealed important insights into the functions of acrosomal matrix and other fertilization proteins. Also, knockouts of multiple proteins can address questions of complementary proteins in a complex system. As the body of literature involving genetically engineered mice has grown, there have been many surprising new observations that force us to think more broadly and to ask different questions than had previously been considered. For example, we now know not to assume that acrosomal exocytosis will only occur on the ZP, and we also understand proteins thought to be involved in sperm–egg interactions can have other functions such as acrosome formation, transiting the uterus and oviduct, etc. Thus, we feel the literature is rich with data from gene knockouts, and this review is an attempt to bring both widely known and understated results to the reader's attention with hope that new avenues for research will come to light (Table 2.1).

2.3 Acrosome Formation/Globozoospermia

The function of the sperm acrosome is dependent upon its structure. The sperm of each species has a characteristic configuration with defined acrosomal shapes that are determined, in part, by the underlying nuclear morphologies. However, each acrosome shares the common features of an inner acrosomal membrane (IAM) that is laminated to the nuclear foundation, a fusogenic outer acrosomal membrane (OAM) that is closely apposed to the plasma membrane over the acrosome, and an equatorial segment (ES), which forms the posterior acrosomal margin where the IAM and OAM meet. Acrosomal formation begins during late meiosis when small, proacrosomal vesicles form in pachytene spermatocytes (Bloom et al. 1975; Holstein and Roosen-Runge 1981; Anakwe and Gerton 1990). After the meiotic divisions, the resulting haploid spermatids inherit the proacrosomal vesicles and continue acrosome biogenesis in earnest. Proacrosomal vesicles eventually coalesce into a single acrosomal vesicle that then attaches to and begins to spread over the future anterior segment of the nucleus. The points of contact between the developing acrosome and spermatid nucleus define the prospective IAM. The acrosome and the nucleus continue developing throughout spermiogenesis until

Table 2.1 Mouse gene knockout models of acrosomal matrix and associated proteins

Mouse gene name	Common protein names	Proposed function	Observed KO phenotype	References
Acrosome formation/globozoospermia				
Agfg1	Hrb	Acrosomal vesicle fusion	Infertile males; globozoospermia; proacrosomal vesicles do not fuse; acrosomal development stops at step 2	Kang-Decker et al. (2001) and Kierszenbaum et al. (2004)
Csnk2a2	Casein kinase II α′ catalytic subunit	Head morphology, acrosome morphology	Infertile males; globozoospermia	Xu et al. (1999)
Dpy19l2		Attachment of acrosomal vesicle to nucleus/acroplaxome	Infertile males; acrosomal vesicle fails to attach to nucleus, acrosome eliminated	Pierre et al. (2012)
Galnt3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	Coalescence of proacrosomal vesicles into acrosome	Infertile males	Miyazaki et al. (2012)
Gmcl1	Germ cell-less (Drosophila)	Acrosome formation, chromatin condensation	Subfertile males; abnormal acrosome/head morphology	Kimura et al. (2003)
Gopc	Golgi-associated PDZ- and coiled-coil motif-containing protein	Head morphology and mitochondrion assembly	Infertile males; globozoospermia and mitochondrial defects	Yao et al. (2002)
Ica1l	Islet cell autoantigen 1-like protein	Head morphology	Subfertile males; globozoospermia	He et al. (2015)
Pafah1b1	Platelet-activating factor acetylhydrolase, regulatory subunit	Acrosome/head formation	Infertile males; Abnormal acrosome morphology	Nayernia et al. (2003)
Pick1	Protein interacting with C kinase 1	Vesicle trafficking from Golgi to the acrosome	Infertile males; globozoospermia	Xiao et al. (2009)
Pla2g3	Phospholipase A2, group III	Acrosome and axoneme formation	Subfertile males; multiple abnormal head & tail morphologies	Sato et al. (2010)
Sec23ip	Sec23 interacting protein	Acrosomal vesicle fusion/formation	Subfertile males; globozoospermia, acrosomal vesicle does not form	Arimitsu et al. (2011)

Spaca1	Sperm acrosome associated 1 protein	Acrosome attachment and lateral spreading over nucleus	Infertile males; globozoospermia, acrosome fails to form	Fujihara et al. (2012)
Tbc1d20	Tbc-1	Acrosomal vesicle formation	KO but no fertility info reported; gene mutation causes disorganized acrosomal granules, arrest at step 3	Sotomayor and Handel (1986) and Liegel et al. (2013)
Zona interacting proteins				
Acr	Proacrosin	Acrosomal matrix disassembly	Fertile males; slow acrosomal matrix dispersal	Baba et al. (1994) and Adham et al. (1997)
Zan	Zonadhesin	Species-specific sperm–egg recognition	Fertile but species-specific gamete recognition affected	Tardif et al. (2010)
Zp3r	Sp56, AM67	Zona binding?	Fertile males	Muro et al. (2012)
Zpbp1	Zona pellucida-binding protein 1	Acrosome formation, compaction	Infertile males; globozoospermia, acrosomal dysplasia	Lin et al. (2007)
Zpbp2	Zona pellucida binding protein 2	Acrosome formation	Subfertile males; abnormal head/acrosome morphology	Lin et al. (2007)
Oocyte cumulus complex and zona pellucida transit				
Acr/Prss21		ZP/cumulus transit	Fertile males with reduced litter size; IVF failure	Kawano et al. (2010)
Acrbp	SP32	Regulate proacrosin activity	No knockout published	Kanemori et al. (2013)
Hyal5	Hyaluronidase 5	Digest hyaluronic acid	Fertile males	Kimura et al. (2009)
Nptx2	AM50, Narp/NP2	Acrosomal matrix structure	Fertile males; no analysis of sperm	Bjartmar et al. (2006)
Pcsk4	Proprotein convertase subtilisin 4/Kexin type 4	Processing/activation of sperm/acrosomal proteins, capacitation, acrosomal exocytosis, ZP binding	Infertile males; abnormal acrosomal morphology	Mbikay et al. (1997), Gyamera-Acheampong et al. (2006) and Tardif et al. (2012)
Prss21	TESP5, testisin	ZP/cumulus transit	Fertile males; in vitro assays showed motility, morphology, and IVF problems	Netzel-Arnett et al. (2009)
Serpina5	Protein C Inhibitor	Sertoli cell–germ cell interactions	Infertile males; premature release of spermatogenic cells, abnormal sperm motility morphology, IVF	Uhrin et al. (2000) and Uhrin et al. (2007)

(continued)

Table 2.1 (continued)

Mouse gene name	Common protein names	Proposed function	Observed KO phenotype	References
Spam1	PH-20	Cumulus transit	Fertile males	Baba et al. (2002)
Spam1/ *Acr*		Work cooperatively for cumulus transit	Delayed cumulus transit	Zhou et al. (2012)
Spam1/ *Prss21*		Work cooperatively for cumulus transit	Delayed cumulus transit	Zhou et al. (2012)
Inner acrosomal membrane proteins				
Cd46	Cellular differentiation marker 46	Stabilize acrosomal membranes, no role in complement protection	Fertile males; enhanced fertilization via rapid capacitation and spontaneous acrosomal exocytosis	Inoue et al. (2003)
Spaca4	SAMP14	Persists following acrosomal exocytosis	No knockout published	Shetty et al. (2003)
Equatorial segment proteins				
Eqtn	Equatorin	Fusion of outer acrosomal and plasma membranes anterior to equatorial segment	Males subfertile and have reduced litter size; sperm do not undergo acrosomal exocytosis	Wolkowicz et al. (2003) and Hao et al. (2014)
Spesp1	Sperm equatorial segment protein 1	Stability of equatorial segment membranes	Males subfertile; fusion of outer acrosomal and plasma membranes in equatorial segment	Fujihara et al. (2010)

they coordinately achieve structures similar to those found in ejaculated sperm. Final steps of metamorphosis occur during epididymal transit.

In recent years, the molecular mechanisms supporting the transmogrification of a round spermatid acrosome to the form seen in mature testicular spermatozoa have been elucidated by many studies utilizing mutations or gene knockouts in the mouse. One of the earliest examples of the effect of acrosomal dysplasia is the blind-sterile mouse, whose round spermatids exhibit disorganized proacrosomal granules with no further development beyond step 3 of spermiogenesis (Sotomayor and Handel 1986). This phenotype is caused by mutations in the *Tbc1d20* gene, which encodes a GTPase-activating protein specific for Rab1 and Rab2 small GTPase families (Liegel et al. 2013). Another GTPase-activating protein, AGFG1 (also known as Hrb), specific in this case for ARF proteins, shows a similar phenotype (Kang-Decker et al. 2001; Kierszenbaum et al. 2004). Vesicle trafficking is key to formation of the acrosome since mutations affecting PICK1, the Golgi-associated PDZ- and coiled-coil motif-containing protein (GOPC), the casein kinase II α′ catalytic subunit (CSNK2A2), and islet cell autoantigen 1-like protein (ICA1L) cause sterility or subfertility in males (Xu et al. 1999; Yao et al. 2002; Xiao et al. 2009; He et al. 2015). Mutations affecting SEC23IP, a phospholipase A1-like protein that interacts with Sec23, also cause male subfertility by interfering with acrosome biogenesis and leading to round-headed spermatozoa (Arimitsu et al. 2011). Spermatids of infertile male mice lacking GalNAc transferase 3 (GALNT3) produce proacrosomal vesicles of various sizes, which attach to the nuclear envelope but do not coalesce to form a single acrosomal vesicle, apparently causing oligoasthenoteratozoospermia through a severe reduction of mucin-type O-glycans, impaired acrosome formation, and increased apoptosis (Miyazaki et al. 2012). Acrosomal dysplasia does occur in cases where certain acrosomal matrix components such as zona pellucida-binding protein 1 (ZPBP) or 2 (ZPBP2) are missing (Lin et al. 2007).

Abnormalities in nuclear envelope structure can also interfere with the ability of the acrosome to form properly. Disruption of the mouse homologue (*Gmcl1*) of the *Drosophila melanogaster* germ cell-less gene causes insufficient chromatin condensation and abnormal acrosome structures in mutant sperm (Kimura et al. 2003). When the inner nuclear membrane protein DPY19L2 is absent, the nuclear dense lamina as well as the junction between the acroplaxome and the nuclear envelope are destabilized. As a result, the linking of the acrosome and the manchette to the nucleus is blocked; this disrupts vesicular trafficking, causes sperm nuclear shaping to fail, and leads to the elimination of the unbound acrosomal vesicle (Pierre et al. 2012). In elongating spermatids, abnormalities of anterior head shaping involving CSNK2A2 or the sperm acrosome-associated 1 protein (SPACA1) also lead to globozoospermia (Xu et al. 1999; Fujihara et al. 2012). Spermatids with mutant PAFAH1B1 protein (the regulatory subunit of platelet-activating factor acetylhydrolase 1b) are sterile because the spermatids fail to form acrosomes properly and exhibit nuclei distorted in size and shape (Nayernia et al. 2003).

Final maturation of the sperm acrosome occurs within the epididymis. The classic case is the morphological alteration in the acrosome of guinea pig sperm

where the head changes drastically from a planar, tennis racket-shaped structure to a form reminiscent of a cupped hand or xistera, the handheld basket used in jai alai, as the sperm transit from the testis to the cauda epididymis (Fawcett and Hollenberg 1963). Another example is seen in the post-testicular development of spermatozoa of the tammar wallaby where the immature, uncondensed acrosome has a distinctive "thumbtack" appearance that is remodeled into a streamlined, condensed structure on the sperm head (Setiadi et al. 1997). Phospholipase A2, group III (PLA2G3), which is secreted in the proximal (caput) region of the mouse epididymis, is reported to have a role in epididymal maturation of sperm (Sato et al. 2010); however, the *Pla2g3* gene is also expressed in the testis, so it is difficult to attribute the subfertility of $Pla2g3^{-/-}$males to the lack of the *Pla2g3* protein in the epididymis rather than the testis. The molecular mechanisms for these structural alterations are not fully understood but could be due, in part, to changes in protein disulfide cross-links, oligosaccharide modifications, and/or repackaging of the acrosomal components (Anakwe et al. 1991; Setiadi et al. 1997; Fléchon 2015).

2.4 Zona Interacting Proteins

For many years, the prevailing notion was that sperm bound to the zona pellucida of unfertilized eggs through cell surface receptors or binding proteins present on the exterior of acrosome-intact sperm. Biochemical studies in the mouse led to the identification of ZP3R (originally called "sp56") as the putative ZP3 receptor protein (Bleil and Wassarman 1990; Cheng et al. 1994; Bookbinder et al. 1995). Subsequent experiments established that ZP3R and its guinea pig homologue are actually components of the acrosomal matrix (Foster et al. 1997; Kim et al. 2001). ZP3R monomers are assembled into a multimeric complex in the particulate acrosomal matrix. During the course of spontaneous acrosomal exocytosis and coincident with release of ZP3R from the acrosome under capacitating conditions, the monomer is proteolytically processed from 67,000 M_r to 43,000 M_r (Buffone et al. 2009). The processing of ZP3R is dramatically reduced when sperm are incubated under non-capacitating conditions. The cleavage probably takes place in complement control protein domain (CCP) 6 or the bridge region between CCP6 and CCP7, which is not present in the guinea pig orthologue AM67. This cleavage has functional consequences since the cleaved form of ZP3R does not bind to unfertilized eggs. However, although ZP3R binds the zona pellucida, the elimination of ZP3R in the mouse by homologous recombination did not affect fertility in the nullizygous mice (Muro et al. 2012).

Other candidates proposed as proteins responsible for zona pellucida binding also turned out to be components of the acrosomal matrix. Zona-binding proteins include the homologues known as sp38 [boar (Mori et al. 1993)], IAM38 [bull (Yu et al. 2006)], ZPBP and ZPBP2 [mouse (Lin et al. 2007)]. In the pig, this protein competes with proacrosin for binding to the zona pellucida (Mori et al. 1993). As discussed previously, elimination of the *Zpbp* and/or *Zpbp2* genes

by homologous recombination results in infertility or subfertility in male mice due to multiple effects on acrosome biogenesis and other spermatogenic defects leading to dysfunctional spermatozoa (Lin et al. 2007). The serine zymogen proacrosin is another zona-binding protein (Howes et al. 2001; Furlong et al. 2005), but, as described below, elimination of the *Acr* gene by homologous recombination does not cause infertility in mice (Baba et al. 1994; Adham et al. 1997). Zonadhesin (ZAN) was originally identified biochemically as a binding protein of the porcine zona pellucida (Hardy and Garbers 1995) and then demonstrated to be associated with the acrosomal matrix and membranes (Bi et al. 2003). Homologues in other species were identified and a gene knockout of *Zan* was performed in the mouse, which does not affect fertility but, instead, enables the mouse sperm to bind more effectively to pig, cow, and rabbit ZP without enhancing attachment to the mouse ZP, essentially eliminating the filter for the species-specific interactions between the mutant mouse sperm and oocytes of other animals (Tardif et al. 2010). From the studies performed to date, it is clear that there are several zona pellucida-binding proteins present within the acrosomal matrix of sperm. Some of these are involved in essential steps of acrosome biogenesis such as ZPBP and ZPBP2. However, the elimination of other zona-binding proteins by homologous recombination does not affect fertility per se but may yield a subtler phenotype such as a loss of species-specific sperm–zona binding or the slower dissipation of the acrosomal matrix when exocytosis is initiated, leading to a delayed rate of fertilization. These traits appear to be redundant, yet they likely impart a competitive advantage to sperm from wild-type mice when one or more males mate with the same female.

2.5 Oocyte Cumulus Complex and Zona Pellucida Transit: Proteases, Regulatory Proteins, and Binding Proteins

Mammalian oocytes are surrounded by two formidable barriers to spermatozoa: the oocyte cumulus complex (OCC) that consists of multiple layers of cumulus cells with an extracellular matrix rich in hyaluronic acid and the ZP that is comprised of three or four glycoproteins (Litscher and Wassarman 2014). The sperm surface and acrosome contain several proteases that are purported to have a role in at least some limited digestion of the OCC extracellular matrix and ZP, but it remains unclear whether these proteases are essential for sperm to transit these barriers or whether mechanical forces produced by the sperm tail are sufficient (Bedford 1998; Litscher and Wassarman 2014). In addition, the acrosomal matrix contains numerous proteins that can bind to the ZP and presumably enable a spermatozoon to burrow into the ZP by the combined mechanical forces of the tail and limited enzymatic digestion by proteases. Gene knockouts of individual and multiple proteases have provided some important and unforeseen insights into this process. Indeed, the pattern of surprising knockout results was set by perhaps the first knockout in this field—the proacrosin (ACR) gene. For many years, proacrosin was purported to be

the main sperm protease for ZP penetration, but, as discussed previously, gene knockout experiments showed that proacrosin was not essential for zona penetration as mice were fertile and proacrosin-null sperm were able to transit the ZP (Baba et al. 1994). Further study showed that proacrosin-null sperm dispersed acrosomal contents more slowly than wild-type sperm, indicating that the role of acrosin was to disperse the acrosomal matrix (Yamagata et al. 1998). This also inspired research to identify novel acrosomal proteases involved in OCC and ZP transit.

PRSS21 (TESP5, testisin) is a candidate serine protease to complement acrosin; it is a GPI-linked protein on the surface of the sperm head, cytoplasmic droplet, and midpiece of the tail (Honda et al. 2002). PRSS21 knockout mice were fertile in long-term mating trials over several weeks, but in vitro analysis showed that the PRSS21-null sperm had decreased motility, abnormal morphology, and poor performance in fertilization assays (Netzel-Arnett et al. 2009).

Double knockout ACR/PRSS21 male mice were able to produce offspring in vivo but with significantly reduced litter size (Kawano et al. 2010). In vitro, ACR/PRSS21-null sperm failed to fertilize both cumulus-free and cumulus-intact oocytes, which suggests a role for these proteases in both cumulus penetration and ZP transit. Sperm penetration through the OCC was slower for ACR/PRSS21 sperm than wild-type sperm, and OCCs incubated with extracts of these sperm showed slower dispersal times than wild-type sperm extracts. Analysis of in vitro fertilization experiments showed numerous effects: the acrosomal contents of ACR/PRSS21 knockout sperm failed to disperse, the number of sperm bound to the ZP and the percentage of oocytes with fused sperm were very low, and the ratio of sperm bound to the ZP, percentage of ZP penetrated by sperm, number of sperm in the perivitelline space, and percent fertilized oocytes were zero or negligible. Scanning electron microscopy showed that oocytes incubated with ACR/PRSS21-null sperm did not have any sperm tracks, the putative paths of sperm through the ZP caused by limited enzymatic digestion. It is notable that the ACR/PRSS21 sperm appear to remain acrosome intact in these images, or perhaps they are acrosome reacted but retain a significant portion of undispersed acrosomal matrix material. The authors hypothesize that this may be due to the lack of PRSS21 activity rather than dispersal of the acrosomal matrix since ACR is known to activate the PRSS21 protease. They propose that ACR-null mice may have compensatory PRSS21 enzymatic activity for penetrating the ZP while PRSS21-null mice may have compensatory acrosomal matrix dispersal activity. ACR/PRSS21 double knockout mice lack both mechanisms and are subfertile due to the failure to fertilize all ovulated oocytes (Kawano et al. 2010). The authors propose that sperm lacking these two proteases are able to fertilize oocytes due to a compensatory mechanism in the uterine fluid; it is also tempting to speculate that at least some sperm lacking these proteases are able to fertilize oocytes due to simple mechanical forces from sperm motility. The authors suggest a role in cumulus dispersal and penetration, which is interesting since sperm lacking known hyaluronidases SPAM1 (Baba et al. 2002) and HYAL5 (Kimura et al. 2009) were able to fertilize oocytes. Together with double knockout experiments of SPAM1 and ACR or

SPAM1 and PRSS21 (Zhou et al. 2012), these results suggest that ACR and PRSS21 may work in concert with hyaluronidases for cumulus penetration. These results are also consistent with the notion that acrosomal exocytosis occurs before or during interaction with the cumulus (Jin et al. 2011).

A likely substrate of acrosin in the guinea pig acrosomal matrix is AM50, the orthologue of NPTX2 (neuronal pentraxin 2 or Narp) in mice. AM50, like other pentraxins, forms large disulfide-bonded pentameric or decameric complexes and is a major structural component of the acrosomal matrix in guinea pigs (Noland et al. 1994; Reid and Blobel 1994). When acrosin is activated during acrosomal exocytosis in guinea pig sperm or in isolated acrosomal matrices, AM50 is processed at acrosin-specific sites coincident with acrosomal matrix disassembly (Westbrook-Case et al. 1994; Kim et al. 2011). NPTX2 is present in the mouse and knockout of this protein yields fertile males (Bjartmar et al. 2006), although analysis of sperm performance has not been done. Curiously, pentraxin 3 (PTX3) is produced by mouse cumulus cells during cumulus expansion and localizes in the matrix; in vivo, *Ptx3*$^{-/-}$ female mice have been reported to be infertile or subfertile due to severe abnormalities of the cumulus oophorus, although oocytes can be successfully fertilized in vitro (Varani et al. 2002; Salustri et al. 2004).

Protein C Inhibitor (PCI), a widely expressed serine protease inhibitor in seminal plasma, is present in the acrosome, has been shown to inhibit human sperm acrosin activity, and is detected on the surface of damaged or abnormal human sperm where it is assumed to prevent inappropriate acrosin activity (Moore et al. 1993; Hermans et al. 1994; Zheng et al. 1994; Uhrin et al. 2007). PCI has also been linked to fertility in humans as infertile patients had inactive PCI (He et al. 1999). PCI-null female mice are fertile but males are infertile and the primary defect appears to be with the Sertoli cell–spermatogenic cell junctions in the testis since spermatogenic cells were prematurely released into the seminiferous tubule lumen and immature and abnormal germ cells were found in the epididymis (Uhrin et al. 2000; Uhrin et al. 2007). Further analysis of PCI-null epididymal sperm showed 95 % abnormal sperm morphology (many with separated heads and tails and abnormally shaped heads), significantly reduced motility, and failure to bind either cumulus-intact or cumulus-free eggs (Uhrin et al. 2000; Uhrin et al. 2007). Whether the observed lack of cumulus and ZP binding and penetration in absence of PCI is due to problems of unregulated acrosin activity is a question to be investigated, but it is interesting to note that ACR/PRSS21-null mice also had this phenotype. Perhaps unregulated acrosin produces premature spontaneous acrosomal exocytosis and proteolysis of the acrosomal matrix such that sperm–ZP binding sites of the acrosomal matrix and/or ZP are prematurely processed and thus nonfunctional.

The pro-protein convertase PCSK4 is reported to be localized in the developing acrosome during spermatogenesis and, in mature sperm, it is present on the plasma membrane over the acrosome (Gyamera-Acheampong et al. 2006); whether there are two PCSK4 pools (acrosomal and cell surface) or it migrates from the acrosome to the sperm surface is unclear. PCSK4-null mice are infertile (Mbikay et al. 1997; Gyamera-Acheampong et al. 2006) and have abnormal acrosomal shapes (Tardif et al. 2012) that are likely caused by reduced ACRBP processing during

spermiogenesis that leads to improper packaging of proacrosin and perhaps other acrosomal matrix proteins into the acrosome (Kanemori et al. 2013). PCSK4-null mature sperm had accelerated tyrosine phosphorylation during capacitation, improper processing of the egg-binding protein ADAM2, increased spontaneous acrosomal exocytosis, and reduced ZP binding. Thus, PCSK4 has multiple functions in developing and mature sperm and identification of substrate proteins will help elucidate the mechanisms through which this protein functions.

2.6 Inner Acrosomal Membrane Proteins

As acrosomal exocytosis proceeds and acrosomal matrix proteins are released, some components of the IAM remain associated with the sperm and are in position to interact with egg vestments. The term "secondary binding" has been used to describe the interactions of these proteins with the ZP, particularly ZP2, but we envision these interactions to be less well defined and to involve multiple binding and release events. In this model, sperm would adhere to the ZP via multiple proteins that are then released as a result of limited proteolysis to produce repeated binding and release at the molecular level; the continual force of the tail would drive the head progressively further into the ZP. Numerous proteins have the ZP-binding and/or enzymatic properties that are consistent with this model.

The cellular differentiation marker CD46 is ubiquitously expressed on the surface of most mammalian cells and is involved in complement protection in many species, including humans, but not in rats, mice, and guinea pigs. In mice, it appears to be expressed solely in the testis, and, in sperm, CD46 is not on the PM as would be expected, but is located inside the acrosome on the IAM. It was thought that this might be to compensate for loss of sperm surface CD proteins that protect the cell from attack by complement, but sperm from CD46 knockout mice are not more susceptible to complement than control sperm, so the function of CD46 is not likely to be complement protection. Interestingly, CD46 knockout mouse sperm have *enhanced* fertility; the sperm show accelerated spontaneous acrosomal exocytosis and have an elevated litter size in one mouse strain (DBA/2N), and CD46-null sperm fertilized superovulated oocytes at a significantly higher rate than controls (Inoue et al. 2003). Thus, CD46 appears to be important in stabilizing the acrosomal membranes. How it does this from the inner acrosomal membrane is a mystery, but it is possible that, during acrosome formation in spermatogenesis, CD46 may interact with other proteins destined for the periacrosomal membranes. Consequently, CD46 might direct or influence sorting of proteins that stabilize these membranes so that, in the absence of CD46, these proteins are misdirected or do not function properly to stabilize the acrosomal membranes.

Several IAM proteins are known to have a role in sperm–ZP binding but do not have knockout models yet. Yu et al. (2006) also have extracted proteasomal activity from the IAM, but, again, there is no gene knockout data available with regard to its role in fertilization. SPACA4 (SAMP14) is present in the IAM, OAM, and ES of

human sperm; it is GPI-linked and is exposed after acrosomal exocytosis (Shetty et al. 2003). Antibodies to recombinant SPACA4 inhibit binding/fusion of human sperm to zona-free hamster eggs, but a knockout mouse line has not been generated.

2.7 Equatorial Segment Proteins

Equatorin (EQTN) localization is limited to the equatorial segment and the protein is resistant to fairly strong extraction conditions (high salt, 0.1 % Triton X-100, sonication), persists following acrosomal exocytosis on sperm in the perivitelline space, and is even present on sperm inside the egg (Manandhar and Toshimori 2001). Following acrosomal exocytosis, some EQTN is redistributed onto the PM over the ES (and some on the IAM), but the significance of this is not clear. Male EQTN-null mice are subfertile (~50 % of controls) and have reduced litter sizes (5.8 vs. 12.3 in control). EQTN-null sperm undergo normal acrosomal biogenesis (acrosomes are normal by immunofluorescence and by transmission electron microscopy), but do not undergo acrosomal exocytosis above control levels. Co-immunoprecipitation with SNAP25 and syntaxin 1a suggests a role in vesicle fusion during acrosomal exocytosis, but since the protein does not have a canonical transmembrane domain, it must do this via a membrane protein that interacts with the vesicle fusion proteins (Wolkowicz et al. 2003; Hao et al. 2014). Also, since the outer acrosomal and plasma membranes don't fuse in the ES, EQTN is not likely to be involved in fusion in that region. The reason why EQTN affects fusion of the OAM and PM in the more anterior zones is an area for more investigation.

Sperm equatorial segment protein 1 (SPESP1) is another ES-limited protein that is important for fertility in both expected and surprising ways. SPESP1-null and heterozygous males had delays in fertility and smaller litter sizes than wild-type males, and heterozygous and null sperm numbers in the oviduct were significantly reduced (Fujihara et al. 2010). Interestingly, in vitro assays showed that the number of sperm, percent progressively motile sperm, and motility patterns were the same for SPESP1-null, heterozygous, and wild-type mice, but the percent of fertilized oocytes and the percent fusibility with zona-free oocytes were significantly less in SPESP1-null and heterozygotes than wild type. Furthermore, in SPESP1-null sperm following acrosomal exocytosis, the plasma and outer acrosomal membranes were found to undergo fusion throughout the entire equatorial segment; since these membranes are typically nonfusogenic, these results show that SPESP1 is important in maintaining the stability of the ES which has an impact on fusibility with the egg plasma membrane (Fujihara et al. 2010).

2.8 Ten Questions

In light of recent developments in the field of fertilization using contemporary imaging techniques and molecular biology to create targeted mutations in genes considered to be important for fertilization, several issues remain that need to be addressed. These include, but are not limited to, the following ten questions: (1) Does acrosomal exocytosis need to occur in a specific region of the female reproductive tract or peri-oocyte environment for fertilization to take place? Current evidence suggests that most sperm have initiated acrosomal exocytosis prior to penetrating the zona pellucida of cumulus-enclosed oocytes. (2) Is binding of the zona pellucida by acrosomal matrix components exposed on the sperm surface required for fertilization or is the affinity of these proteins assistive but not essential for fertilization to take place? For example, ZP3R binds to the oocyte zona pellucida but is not required for fertilization. On the other hand, in the absence of ZAN, the species-specific filter that blocks heterospecific sperm from other phylogenetic classes from binding to mouse oocytes is lost; it would be interesting to determine if ZAN operates to filter the binding of sperm related at the genus or strain level. (3) Can we develop better tools to assess the integrity of the membranes overlying the acrosomal contents during various stages of capacitation and acrosomal exocytosis? (4) Do the components of the acrosome influence the interactions of sperm with the oviductal epithelium, either independently on a single-cell level or perhaps more generally through influencing the reciprocal connectivity between neighboring sperm and the female reproductive tract? (5) Do "acrosome-reacted" sperm that are experimentally recovered from the perivitelline space and are used successfully to fertilize other eggs still contain functional acrosomal matrix material? These experiments raise questions concerning the functions of the acrosomal contents in fertilization (they may be assistive but not required). (6) Are the different terms used to describe the acrosomal matrix important or are they describing the same properties from varied perspectives? Some reports refer to the acrosomal matrix as a particulate compartment of the acrosomal contents (Buffone et al. 2008). Others make reference to a crystalloid nature observed by transmission electron microscopy (Fléchon 2015). Still others classify the acrosomal matrix as an amyloid (Guyonnet et al. 2014). (7) What role does pH play in the intra-acrosomal dynamics and post-exocytotic dispersion of the contents? For example, the pH of the acrosome is acidic and gradually begins to alkalinize during the course of epididymal maturation; once exocytosis is initiated, the contents are rapidly exposed to the more alkaline pH of the milieu surrounding the sperm. (8) Does the change in pH occurring during these periods activate or inactivate acrosomal enzymes? This is true for some proteases such as the proacrosin zymogen activation, but there may be other enzymes exhibiting similar properties. (9) What are the substrates for acrosomal hydrolases? Initially, it was thought that acrosin acted upon the zona pellucida. However, it is clear that this serine protease is involved in the dispersion of the acrosomal matrix, but the actual substrates have yet to be elucidated. (10) Why are acrosomal proteins differentially compartmentalized?

The component segregation could be important for differential binding and/or release during exocytosis. Alternatively, the different domains might simply influence the shape of the acrosome. Clearly, there is still much to learn concerning the involvement of acrosomal matrix components in directing the structure of the acrosome during spermatogenesis and epididymal maturation as well as the function of the acrosome in the fertilization process.

Acknowledgments This work was supported in part by grants R21-ES024527 and P30-ES013508 from the National Institute of Environmental Health Sciences to GLG, and Chenery and Rashkind Research Grants from Randolph-Macon College to JAF.

References

Adham IM, Nayernia K, Engel W (1997) Spermatozoa lacking acrosin protein show delayed fertilization. Mol Reprod Dev 46:370–376. doi:10.1002/(SICI)1098-2795(199703)46:3<370::AID-MRD16>3.0.CO;2-2

Anakwe OO, Gerton GL (1990) Acrosome biogenesis begins during meiosis: Evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis. Biol Reprod 42:317–328

Anakwe OO, Sharma S, Hoff HB et al (1991) Maturation of guinea pig sperm in the epididymis involves the modification of proacrosin oligosaccharide side chains. Mol Reprod Dev 29:294–301. doi:10.1002/mrd.1080290313

Arimitsu N, Kogure T, Baba T et al (2011) p125/Sec23-interacting protein (Sec23ip) is required for spermiogenesis. FEBS Lett 585:2171–2176. doi:10.1016/j.febslet.2011.05.050

Baba T, Azuma S, Kashiwabara S, Toyoda Y (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. J Biol Chem 269:31845–31849

Baba D, Kashiwabara S, Honda A et al (2002) Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. J Biol Chem 277:30310–30314. doi:10.1074/jbc.M204596200

Bedford JM (1998) Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. Biol Reprod 59:1275–1287. doi:10.1095/biolreprod59.6.1275

Bi M, Hickox JR, Winfrey VP et al (2003) Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome. Biochem J 375:477–488. doi:10.1042/bj20030753

Bjartmar L, Huberman AD, Ullian EM et al (2006) Neuronal pentraxins mediate synaptic refinement in the developing visual system. J Neurosci 26:6269–6281. doi:10.1523/JNEUROSCI.4212-05.2006

Bleil JD, Wassarman PM (1990) Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. Proc Natl Acad Sci 87:5563–5567

Bloom W, Fawcett DW, Maximow AA (1975) A textbook of histology, 10th edn. Saunders, Philadelphia

Bookbinder LH, Cheng A, Bleil JD (1995) Tissue- and species-specific expression of sp56, a mouse sperm fertilization protein. Science 269:86–89

Buffone MG, Foster JA, Gerton GL (2008) The role of the acrosomal matrix in fertilization. Int J Dev Biol 52:511–522. doi:10.1387/ijdb.072532mb

Buffone MG, Kim K-S, Doak BJ et al (2009) Functional consequences of cleavage, dissociation and exocytotic release of ZP3R, a C4BP-related protein, from the mouse sperm acrosomal matrix. J Cell Sci 122:3153–3160. doi:10.1242/jcs.052977

Cheng A, Le T, Palacios M et al (1994) Sperm-egg recognition in the mouse: Characterization of sp56, a sperm protein having specific affinity for ZP3. J Cell Biol 125:867–878

Fawcett DW, Hollenberg RD (1963) Changes in the acrosome of the guinea pig spermatozoa during passage through the epididymis. Z Zellforsch Mikrosk Anat 60:276–292

Fléchon J-E (2015) The acrosome of eutherian mammals. Cell Tissue Res 363:147–157. doi:10.1007/s00441-015-2238-0

Foster JA, Friday BB, Maulit MT et al (1997) AM67, a secretory component of the guinea pig sperm acrosomal matrix, is related to mouse sperm protein sp56 and the complement component 4-binding proteins. J Biol Chem 272:12714–12722

Fujihara Y, Murakami M, Inoue N et al (2010) Sperm equatorial segment protein 1, SPESP1, is required for fully fertile sperm in mouse. J Cell Sci 123:1531–1536. doi:10.1242/jcs.067363

Fujihara Y, Satouh Y, Inoue N et al (2012) SPACA1-deficient male mice are infertile with abnormally shaped sperm heads reminiscent of globozoospermia. Development 139:3583–3589. doi:10.1242/dev.081778

Furlong LI, Harris JD, Vazquez-Levin MH (2005) Binding of recombinant human proacrosin/acrosin to zona pellucida (ZP) glycoproteins. I. Studies with recombinant human ZPA, ZPB, and ZPC. Fertil Steril 83:1780–1790. doi:10.1016/j.fertnstert.2004.12.042

Guyonnet B, Egge N, Cornwall GA (2014) Functional amyloids in the mouse sperm acrosome. Mol Cell Biol 34:2624–2634. doi:10.1128/MCB.00073-14

Gyamera-Acheampong C, Tantibhedhyangkul J, Weerachatyanukul W et al (2006) Sperm from mice genetically deficient for the PCSK4 proteinase exhibit accelerated capacitation, precocious acrosome reaction, reduced binding to egg zona pellucida, and impaired fertilizing ability. Biol Reprod 74:666–673. doi:10.1095/biolreprod.105.046821

Hao J, Chen M, Ji S et al (2014) Equatorin is not essential for acrosome biogenesis but is required for the acrosome reaction. Biochem Biophys Res Commun 444:537–542. doi:10.1016/j.bbrc.2014.01.080

Hardy DM, Garbers DL (1995) A sperm membrane protein that binds in a species-specific manner to the egg extracellular matrix is homologous to von Willebrand factor. J Biol Chem 270:26025–26028. doi:10.1074/jbc.270.44.26025

He S, Lin Y-L, Liu Y-X (1999) Functionally inactive protein C inhibitor in seminal plasma may be associated with infertility. Mol Hum Reprod 5:513–519. doi:10.1093/molehr/5.6.513

He J, Xia M, Tsang WH et al (2015) ICA1L forms BAR-domain complexes with PICK1 and is crucial for acrosome formation in spermiogenesis. J Cell Sci 128:3822–3836. doi:10.1242/jcs.173534

Hermans JM, Jones R, Stone SR (1994) Rapid inhibition of the sperm protease acrosin by protein C inhibitor. Biochemistry (Mosc) 33:5440–5444. doi:10.1021/bi00184a012

Hirohashi N, Gerton GL, Buffone MG (2011) Video imaging of the sperm acrosome reaction during in vitro fertilization. Commun Integr Biol 4:471–476. doi:10.4161/cib.4.4.15636

Holstein AF, Roosen-Runge EC (1981) Atlas of human spermatogenesis. Grosse, Berlin

Honda A, Yamagata K, Sugiura S et al (2002) A mouse serine protease TESP5 is selectively included into lipid rafts of sperm membrane presumably as a glycosylphosphatidylinositol-anchored protein. J Biol Chem 277:16976–16984

Howes E, Pascall JC, Engel W, Jones R (2001) Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization. J Cell Sci 114:4127–4136

Inoue N, Ikawa M, Nakanishi T et al (2003) Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. Mol Cell Biol 23:2614–2622. doi:10.1128/MCB.23.7.2614-2622.2003

Jin M, Fujiwara E, Kakiuchi Y et al (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci 108:4892–4896. doi:10.1073/pnas.1018202108

Kanemori Y, Ryu J-H, Sudo M et al (2013) Two functional forms of ACRBP/sp32 are produced by pre-mRNA alternative splicing in the mouse. Biol Reprod 88:105. doi:10.1095/biolreprod.112.107425

Kang-Decker N, Mantchev GT, Juneja SC et al (2001) Lack of acrosome formation in Hrb-deficient mice. Science 294:1531–1533. doi:10.1126/science.1063665

Kawano N, Kang W, Yamashita M et al (2010) Mice lacking two sperm serine proteases, ACR and PRSS21, are subfertile, but the mutant sperm are infertile in vitro. Biol Reprod 83:359–369. doi:10.1095/biolreprod.109.083089

Kierszenbaum AL, Tres LL, Rivkin E et al (2004) The acroplaxome is the docking site of Golgi-derived myosin Va/Rab27a/b-containing proacrosomal vesicles in wild-type and Hrb mutant mouse spermatids. Biol Reprod 70:1400–1410. doi:10.1095/biolreprod.103.025346

Kim KS, Cha MC, Gerton GL (2001) Mouse sperm protein sp56 is a component of the acrosomal matrix. Biol Reprod 64:36–43

Kim K-S, Foster JA, Kvasnicka KW, Gerton GL (2011) Transitional states of acrosomal exocytosis and proteolytic processing of the acrosomal matrix in guinea pig sperm. Mol Reprod Dev 78:930–941. doi:10.1002/mrd.21387

Kimura T, Ito C, Watanabe S et al (2003) Mouse germ cell-less as an essential component for nuclear integrity. Mol Cell Biol 23:1304–1315. doi:10.1128/MCB.23.4.1304-1315.2003

Kimura M, Kim E, Kang W et al (2009) Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in fertilization. Biol Reprod 81:939–947. doi:10.1095/biolreprod.109.078816

Liegel RP, Handley MT, Ronchetti A et al (2013) Loss-of-function mutations in TBC1D20 cause cataracts and male infertility in blind-sterile mice and Warburg micro syndrome in humans. Am J Hum Genet 93:1001–1014. doi:10.1016/j.ajhg.2013.10.011

Lin Y-N, Roy A, Yan W et al (2007) Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Mol Cell Biol 27:6794–6805. doi:10.1128/MCB.01029-07

Litscher ES, Wassarman PM (2014) Evolution, structure, and synthesis of vertebrate egg-coat proteins. Trends Dev Biol 8:65–76

Manandhar G, Toshimori K (2001) Exposure of sperm head equatorin after acrosome reaction and its fate after fertilization in mice. Biol Reprod 65:1425–1436. doi:10.1095/biolreprod65.5.1425

Mbikay M, Tadros H, Ishida N et al (1997) Impaired fertility in mice deficient for the testicular germ-cell protease PC4. Proc Natl Acad Sci 94:6842–6846

Miyazaki T, Mori M, Yoshida CA et al (2012) Galnt3 deficiency disrupts acrosome formation and leads to oligoasthenoteratozoospermia. Histochem Cell Biol 139:339–354. doi:10.1007/s00418-012-1031-3

Moore A, Penfold LM, Johnson JL et al (1993) Human sperm–egg binding is inhibited by peptides corresponding to core region of an acrosomal serine protease inhibitor. Mol Reprod Dev 34:280–291. doi:10.1002/mrd.1080340308

Mori E, Baba T, Iwamatsu A, Mori T (1993) Purification and characterization of a 38-kDa protein, sp38, with zona pellucida-binding property from porcine epididymal sperm. Biochem Biophys Res Commun 196:196–202. doi:10.1006/bbrc.1993.2234

Muro Y, Buffone MG, Okabe M, Gerton GL (2012) Function of the acrosomal matrix: zona pellucida 3 receptor (ZP3R/sp56) is not essential for mouse fertilization. Biol Reprod 86:1–6. doi:10.1095/biolreprod.111.095877

Nayernia K, Vauti F, Meinhardt A et al (2003) Inactivation of a testis-specific Lis1 transcript in mice prevents spermatid differentiation and causes male infertility. J Biol Chem 278:48377–48385. doi:10.1074/jbc.M309583200

Netzel-Arnett S, Bugge TH, Hess RA et al (2009) The glycosylphosphatidylinositol-anchored serine protease PRSS21 (testisin) imparts murine epididymal sperm cell maturation and fertilizing ability. Biol Reprod 81:921–932. doi:10.1095/biolreprod.109.076273

Noland TD, Friday BB, Maulit MT, Gerton GL (1994) The sperm acrosomal matrix contains a novel member of the pentaxin family of calcium-dependent binding proteins. J Biol Chem 269:32607–32614

Pierre V, Martinez G, Coutton C et al (2012) Absence of Dpy19l2, a new inner nuclear membrane protein, causes globozoospermia in mice by preventing the anchoring of the acrosome to the nucleus. Development 139:2955–2965. doi:10.1242/dev.077982

Reid MS, Blobel CP (1994) Apexin, an acrosomal pentaxin. J Biol Chem 269:32615–32620

Salustri A, Garlanda C, Hirsch E et al (2004) PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in vivo fertilization. Development 131:1577–1586. doi:10.1242/dev.01056

Sato H, Taketomi Y, Isogai Y et al (2010) Group III secreted phospholipase A2 regulates epididymal sperm maturation and fertility in mice. J Clin Invest 120:1400–1414. doi:10.1172/JCI40493

Setiadi D, Lin M, Rodger JC (1997) Posttesticular development of spermatozoa of the tammar wallaby (Macropus eugenii). J Anat 190:275–288. doi:10.1046/j.1469-7580.1997.19020275.x

Shetty J, Wolkowicz MJ, Digilio LC et al (2003) SAMP14, a novel, acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily with a role in sperm-egg Interaction. J Biol Chem 278:30506–30515. doi:10.1074/jbc.M301713200

Sotomayor RE, Handel MA (1986) Failure of acrosome assembly in a male sterile mouse mutant. Biol Reprod 34:171–182. doi:10.1095/biolreprod34.1.171

Tardif S, Wilson MD, Wagner R et al (2010) Zonadhesin is essential for species specificity of sperm adhesion to the egg's zona pellucida. J Biol Chem 285(32):24863–24870. doi:10.1074/jbc.M110.123125

Tardif S, Guyonnet B, Cormier N, Cornwall GA (2012) Alteration in the processing of the ACRBP/sp32 protein and sperm head/acrosome malformations in proprotein convertase 4 (PCSK4) null mice. Mol Hum Reprod 18:298–307. doi:10.1093/molehr/gas009

Uhrin P, Dewerchin M, Hilpert M et al (2000) Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. J Clin Invest 106:1531–1539. doi:10.1172/JCI10768

Uhrin P, Schöfer C, Zaujec J et al (2007) Male fertility and protein C inhibitor/plasminogen activator inhibitor-3 (PCI): localization of PCI in mouse testis and failure of single plasminogen activator knockout to restore spermatogenesis in PCI-deficient mice. Fertil Steril 88:1049–1057. doi:10.1016/j.fertnstert.2006.11.193

Varani S, Elvin JA, Yan C et al (2002) Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. Mol Endocrinol 16:1154–1167. doi:10.1210/me.16.6.1154

Wassarman PM, Jovine L, Litscher ES et al (2004) Egg–sperm interactions at fertilization in mammals. Eur J Obstet Gynecol Reprod Biol 115(Suppl 1):S57–S60. doi:10.1016/j.ejogrb.2004.01.025

Westbrook-Case VA, Winfrey VP, Olson GE (1994) A domain-specific 50-kilodalton structural protein of the acrosomal matrix is processed and released during the acrosome reaction in the guinea pig. Biol Reprod 51:1–13. doi:10.1095/biolreprod51.1.1

Wolkowicz MJ, Shetty J, Westbrook A et al (2003) Equatorial segment protein defines a discrete acrosomal subcompartment persisting throughout acrosomal biogenesis. Biol Reprod 69:735–745. doi:10.1095/biolreprod.103.016675

Xiao N, Kam C, Shen C et al (2009) PICK1 deficiency causes male infertility in mice by disrupting acrosome formation. J Clin Invest 119:802–812. doi:10.1172/JCI36230

Xu X, Toselli PA, Russell LD, Seldin DC (1999) Globozoospermia in mice lacking the casein kinase II α′ catalytic subunit. Nat Genet 23:118–121. doi:10.1038/12729

Yamagata K, Murayama K, Okabe M et al (1998) Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. J Biol Chem 273:10470–10474. doi:10.1074/jbc.273.17.10470

Yao R, Ito C, Natsume Y et al (2002) Lack of acrosome formation in mice lacking a Golgi protein, GOPC. Proc Natl Acad Sci 99:11211–11216. doi:10.1073/pnas.162027899

Yu Y, Xu W, Yi Y-J et al (2006) The extracellular protein coat of the inner acrosomal membrane is involved in zona pellucida binding and penetration during fertilization: characterization of its most prominent polypeptide (IAM38). Dev Biol 290:32–43. doi:10.1016/j.ydbio.2005.11.003

Zheng X, Geiger M, Ecke S et al (1994) Inhibition of acrosin by protein C inhibitor and localization of protein C inhibitor to spermatozoa. Am J Physiol 267:C466–C472

Zhou C, Kang W, Baba T (2012) Functional characterization of double-knockout mouse sperm lacking SPAM1 and ACR or SPAM1 and PRSS21 in fertilization. J Reprod Dev 58:330–337. doi:10.1262/jrd.2011-006

Chapter 3
Role of Ion Channels in the Sperm Acrosome Reaction

Carmen Beltrán, Claudia L. Treviño, Esperanza Mata-Martínez, Julio C. Chávez, Claudia Sánchez-Cárdenas, Mark Baker, and Alberto Darszon

3.1 Introduction

The acrosome, a single membrane-delimited specialized organelle, overlies the nucleus of many sperm species. The sperm acrosome reaction (AR) involves fusion and vesiculation at multiple points of the outer acrosomal membrane and the overlying plasma membrane; it is triggered by physiological inducers from the female gamete, its vicinity, or by exposure to appropriate pharmacological stimuli. This unique, tightly regulated, irreversible, single but complex exocytotic process releases the acrosomal contents which include Ca^{2+} and enzymes, modifies membrane components, and exposes the inner acrosomal membrane to the extracellular medium (Dan 1952; Yanagimachi 1994). Sperm–egg coat penetration, fusion with the eggs' plasma membrane, and finally fertilization require the release and exposure of cell components resulting from this exocytotic process (Buffone et al. 2014; Okabe 2014; Vacquier et al. 2014).

Triggering the AR requires the convergence of distinct transduction pathways such as ion permeability changes resulting in intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$) increases, G protein activation, cAMP elevation, lipid alterations, and protein phosphorylation state changes. Certain ion channel antagonists inhibit the AR, emphasizing the preponderant role played by these transporters (Darszon et al. 2011; Florman et al. 2008; Publicover et al. 2007). The fusion

C. Beltrán • C.L. Treviño • E. Mata-Martínez • J.C. Chávez • C. Sánchez-Cárdenas • A. Darszon (✉)
Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad # 2001. Col. Chamilpa., Cuernavaca Morelos, CP 62210, Mexico
e-mail: darszon@ibt.unam.mx

M. Baker
School of Environmental and Life Science, University of Newcastle, Callaghan 2308, NSW, Australia

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_3

machinery involved in many secretory events participates in the sperm AR (see Chap. 4 and Mayorga et al. 2007; Castillo Bennett et al. 2010). However, in contrast to neurotransmitter release which occurs within milliseconds, acrosomal exocytosis takes many minutes (Buffone et al. 2014; Okabe 2014; Vacquier et al. 2014).

As in most exocytotic processes, external and internal Ca^{2+} play a crucial role in the AR (Dan 1954; Yanagimachi 1994); however, which are the molecular entities involved in Ca^{2+} mobilization in this process is far from clear. Sea urchin and mouse sperm have been preferred models to study the ion channels involved in the AR. In sea urchin sperm, binding of the fucose sulfate polymer (FSP) found in the outer layer of the egg (the egg jelly) (Vilela-Silva et al. 2008) to its receptor (suREJ1) triggers changes in membrane potential (Em), increases in $[Ca^{2+}]_i$, $[Na^+]_i$, pH_i, cAMP, IP_3, and NAADP, efflux of K^+ and H^+, as well as activation of PKA, nitric oxide synthase, and phospholipase D, and changes in protein phosphorylation (Domino et al. 1989; Vacquier 2012; reviewed in Darszon et al. 2011). suREJ proteins, of which the sperm plasma membrane contains at least two (suREJ1&3), are homologs of poly-cystin-1 (PKD1/PC1), the protein mutated in autosomal dominant polycystic kidney disease (Vacquier and Swanson 2011). In humans, this is the most common monogenic disease (Gunaratne et al. 2007).

Binding of FSP to suREJ1 triggers Ca^{2+} influx and the AR. Exposing sea urchin sperm populations loaded with Ca^{2+} fluorescent indicators to ion channel blockers (reviewed in Darszon et al. 2011) has evidenced the participation of at least three Ca^{2+} channels in the AR (González-Martínez et al. 2001). The first $[Ca^{2+}]_i$ increase is fast (seconds), transitory, and sensitive to voltage-dependent Ca^{2+} (Ca_v) channel blockers (Schackmann et al. 1978; Guerrero and Darszon 1989a, b; Rodríguez and Darszon 2003; Darszon et al. 2011). The second $[Ca^{2+}]_i$ increase is sustained, is pH_i dependent (activated by alkalinization), and may include channels stimulated by Ca^{2+} release from internal stores (third Ca^{2+} channel), possibly store-operated Ca^{2+} (SOC) channels (Guerrero and Darszon 1989b; González-Martínez et al. 2001; Hirohashi and Vacquier 2003; Darszon et al. 2011).

On the other hand, ZP3 considered the physiological inducer of the mouse sperm AR for many years, but now in doubt (Jin et al. 2011), triggers a Ca^{2+} rise also involving at least three Ca^{2+} channels (reviewed in Darszon et al. 2011). Ethical reasons hamper working with human ZP3 and thus recombinant ZP proteins (rhZP) have been used for research. In this regard, Brewis et al. (1996) showed that rhZP3 induced a transient $[Ca^{2+}]_i$ increase followed by a sustained Ca^{2+} rise, similar to the response of mouse sperm to ZP3 (O'Toole et al. 2000). Linares-Hernandez et al. (1998) described in human sperm a voltage-dependent Ca^{2+} influx which was enhanced when depolarization was preceded by a hyperpolarization, consistent with the hypothesis that Ca_vs were involved in the AR and that the capacitation-associated hyperpolarization was required to remove channel inactivation. Notably, Ca^{2+} influx was also enhanced by pretreating the cells with NH_4Cl (see below). Similar experiments in non-capacitated mouse sperm hyperpolarized artificially for instance with valinomycin showed it was possible to induce AR depolarizing by adding external K^+, consistent with the possible participation of Ca_vs requiring removal of inactivation (De La Vega-Beltran et al. 2012). To date the role of the hyperpolarization that accompanies capacitation is not fully understood.

Alternatively to rhZP3, progesterone was used as a substitute physiological inducer of the AR in human sperm. Progesterone evokes as well a two-component Ca^{2+} rise: one fast and transient and another one slower and sustained. Due to the kinetics and nature of

this dual response and since Ca^{2+} levels are a balance between influx from the exterior and efflux from intracellular stores, intracellular Ca^{2+} channels were also looked for and reported to be present in human sperm (reviewed in Darszon et al. 2011; Correia et al. 2015). These and other important findings (reviewed in Lishko et al. 2011a; Buffone et al. 2014; Darszon and Hernández-Cruz 2014) led to the proposal that binding of ZP3 to its sperm receptor activated (by an unknown mechanism) a Ca_v. Ca^{2+} influx through this channel in turn would stimulate a Ca^{2+}-sensitive PLC, generating IP3 and diacylglycerol (DAG); the first would open IP3 receptors, emptying Ca^{2+} stores and producing the sustained Ca^{2+} elevation observed. This model has been seriously questioned since electrophysiological studies so far have only revealed the presence of a single Ca^{2+} channel, CatSper (Zeng et al. 2013). This channel is responsible for hyperactivation and its involvement in the AR is ill-defined (see later discussion). Paradoxically, in human sperm progesterone is the most likely physiological inducer of the human AR and a potent CatSper activator (Strunker et al. 2011; Lishko et al. 2011b). Defining the precise role of CatSper has been hampered by the lack of specific/clean inhibitors and the impossibility to heterologously express this channel.

Recent years have witnessed intense research and lively debate regarding the molecular identity of the physiological AR inductor(s), when, where, and how this reaction takes place, and the signaling cascades required for its outcome (Jin et al. 2011; Buffone et al. 2014; Nishigaki et al. 2014; Okabe 2014). This issue deals with many of the questions mentioned above. New findings have engaged the field in reevaluating other cellular ligands able to trigger the AR, since the physiological role of ZP in triggering this reaction in mouse sperm has been questioned (Jin et al. 2011; Tateno et al. 2013). These include progesterone (Thomas and Meizel 1989; Blackmore et al. 1990), γ-aminobutyric acid (GABA) (Wistrom and Meizel 1993), glycine (Kumar and Meizel 2008), EGF (Daniel et al. 2010), ATP (Foresta et al. 1992; Luria et al. 2002), acetylcholine (Bray et al. 2005), and sphingosine-1-phosphate (Suhaiman et al. 2010).

Ion channels are most frequently minor components of membranes. Therefore, demonstrating the presence of a particular channel in mature sperm requires complementary strategies. Evidence obtained from controlled immunological or proteomic detection combined with electrophysiological and/or ion-sensitive fluorescent functional assays and pharmacology is needed. Ultimately, elimination of the specific ion channel from sperm, when possible, establishes the role of the channel in sperm physiology, though compensatory mechanisms pose constraints to this approach. It is important to consider that each tool used to detect ion channels has limitations that must be kept in mind. For lack of space, the reader may find details of these restrictions elsewhere (i.e., Nystoriak et al. 2013; Zeng et al. 2013; Cohen et al. 2014; Darszon and Hernández-Cruz 2014).

Taking into account the above, there is still a lot of work needed to unveil and characterize the ion channels that participate in the AR. This chapter focuses on the ion channels known to or suspected to be involved in the AR and their regulation. The list is long but the evidence tenuous. The chapter will first describe the channel candidate, the evidence for its participation in this process, and what is known about its regulation. In addition, we present for the first time an attempt to compile all sea urchin, mouse, and human sperm ion channel peptides that have been detected by proteomics (Tables 3.1, 3.2, and 3.3).

Table 3.1 Ion channels identified by proteomic studies on *Strongylocentrotus purpuratus* sperm

Ion channel (short name)	GI Number	Predicted MW (kDa)	Sperm sample	Proteomic technique	# Peptides (different isolations)	References
Calcium-activated chloride channel alpha subunit (CaCC)			ARVS	M/S	1	[2]
Calcium-activated potassium channel alpha subunit (CaKC)	GI:115621189	144 kDa	AffCol_Co^{2+}	LC-MS/MS	2	[1]
			AffCol_WGA	LC-MS/MS	7	[1]
			SMVS	M/S	1	[2]
Calcium channel voltage-gated α1E, R-type (Cav2.3)			LD_DIM	Gs_LC-MS/MS	2	[1]
Voltage-dependent T-type calcium channel subunit alpha-1H isoform X5 (Cav3.3)	GI:780083229	162	[a]	[a]	[a]	[4]
Cation channel sperm-associated protein 1 (CatSper1)	GI:780014296	55 kDa	Tx-114 extracted	Gs_LC-MS/MS	6	[1]
Cation channel sperm-associated protein 2 (CatSper2)	GI:780132062	60 kDa	Tx-114 extracted	Gs_LC-MS/MS	2	[1]
			AffCol_WGA	M/S	1	[2]
Cation channel sperm-associated protein 4 (CatSper4)	GI:780154800	36 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	4	[1]
Cation channel sperm-associated protein subunit beta-like (CatSperβ-like)	GI:780129693	52 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	(1,10)	[1]
			Flaggella PM	Gs_LC-MS/MS	2	[1]
Cation channel sperm-associated protein subunit gamma 1-like (CatSperγ-like)	GI:780129264	82 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	(5,9)	[1]
Hydrogen channel voltage-gated 1 (HV)			AffCol_WGA	M/S	1	[2]
			ARVS	M/S	1	[2]

Hyperpolarization-activated (Ih) channel (SpIh)	GI:47551101	88 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	11	[1]
Hyperpolarization-gated and cyclic nucleotide-regulated channel (HCN)	GI:69146514	74 kDa	AffCol_Co^{2+}	LC-MS/MS	(5,7)	[1]
			AffCol_ConA	LC-MS/MS	3	[1]
			AffCol_WGA	LC-MS/MS	(3,5)	[1]
			Flaggella (pI)	LC-MS/MS	9	[1]
	GI:74136757		AffCol_Co^{2+}	LC-MS/MS	6	[1]
			AffCol_WGA	LC-MS/MS	1	[1]
			Tx-114 extracted	LC-MS/MS	(11,8,20)	[1]
Piezo-type mechanosensitive ion channel component 2 isoform X1 (Piezo Mech_sensit2_x1)	GI:780013330	128 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	1	[1]
Polycystic kidney disease protein 1-like 2 (PKD1-like2)	GI:780006120	318 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	3	[1]
Polycystic kidney disease protein 2 (PKD2)	GI:30842029	103 kDa	AffCol_Co^{2+}	LC-MS/MS	1	[1]
			Flagella_AffCol_ConA	LC-MS/MS	3	[1]
			AffCol_WGA	LC-MS/MS	46,28	[1]
			AffCol_WGA	M/S	1	[2]
	GI:47551289		Tx-114 extracted	Gs_LC-MS/MS	25	[1]

(continued)

Table 3.1 (continued)

Ion channel (short name)	GI Number	Predicted MW (kDa)	Sperm sample	Proteomic technique	# Peptides (different isolations)	References
Tetrameric potassium-selective cyclic nucleotide gated channel (Tetra-KCNG)			SMVS	M/S	1	[2]
	GI:124746345	253 kDa	AffCol_Co^{2+}	LC-MS/MS	17, 26	[1]
			AffCol_ConA	LC-MS/MS	26	[1]
			AffCol_WGA	LC-MS/MS	23,26	[1]
			AffCol_Co^{2+}	LC-MS/MS	20	[1]
			AffCol_WGA	LC-MS/MS	20	[1]
			Flaggella PM	Gs_LC-MS/MS	8	[1]
			Flagella/Tx-114 extracted	Gs_LC-MS/MS	84,142	[1]
Transmembrane channel-like protein 7	GI:780038035	92 kDa	Flagella/Tx-114 extracted	Gs_LC-MS/MS	1	[1]
Transient receptor potential channel A6 (TRPA6)			AffCol_WGA	M/S	1	[2]
122 kDa protein TMEM16 (TMEM16)	GI:67906860	107 kDa	AffCol_Co^{2+}	LC-MS/MS	1,1	[1]
			AffCol_WGA	LC-MS/MS	1,3,8	[1]
	GI:67906862		AffCol_WGA	LC-MS/MS	2	[1]

Transmembrane protein 16 (TMEM16-like)	GI:118601060		AffCol_Co^{2+}	LC-MS/MS	17	[1]
			AffCol_WGA	LC-MS/MS	27	[1]
			Flaggella PM	Gs_LC-MS/MS	31	[1]
			Flagella/Tx-114 extracted	Gs_LC-MS/MS	93	[1]
			Flaggella (pI)	LC-MS/MS	13	[1]
Voltage-dependent anion channel 2 (VDAC2)	GI:115935979	30 kDa	AffCol_Co^{2+}	LC-MS/MS	(10,12,12)	[1]
			AffCol_ConA	LC-MS/MS	8	[1]
			AffCol_WGA	LC-MS/MS	(2,9,26)	[1]
			LD_DIM	LC-MS/MS	(3,25,35)	[3]

AffColl Affinity column, Co^{2+} Cobalt, *ConA* Concabalamine A, *Gs* Gel stained, *LC* Liquid Chromatography, *LD_DIM* Low-Density Detergent-Insoluble Membranes, *MS* Mass Spectrometry, *MS/MS* Tandem MS, *PM* Plasma Membrane, *SMVS* Sperm Membrane Vesicles, *TX-114* non ionic detergent, *WGA* Wheat germ agglutinin

[1] This work. The proteomic analysis was performed as in [3]; [2] Neill, AT. PhD. Dissertation Thesis (Vacquier VD). University of California, San Diego; [3] Loza-Huerta et al., (2013) Biochimica et Biophysica Acta 1830:5305–15. [4] Strünker T. et al., (2006) Nat Cell Biol. 8(10):1149–54

[a]Partial sequence derived from a PCR-fragment amplified from *S. purpuratus* testis cDNA. Accession number for the human Cav3.3 channel sequence is NP_001003406

Table 3.2 Ion channels identified by proteomic studies on mouse spermatozoa

Ion channel (short name)	Entry (GI:Identification)	MW (kDa)	Sperm sample	Peptides number	Protein identification method	References
Aquaporin 7 (Aqp7)	[a]NM_001170		Sperm	1	Gs/Pe-LC-MS/MS	[3]
Cation channel sperm-associated protein 1 (CatSper1)	Q91ZR5	79	CAM	2	Gs/Pe/MS	[1]
			PM	1	LC-MS/MS	[2]
Cation channel sperm-associated protein 2 (CatSper2)	A2ARP9	69	CAM	3	Gs/Pe/MS	[1]
Cation channel, sperm associated 3 (CatSper3)	Q80W99	46	PM	1	LC-MS/MS	[2]
Cation channel sperm-associated protein 4 (CatSper4)	Q8BVN3	51	CAM	5	Gs/Pe/MS	[1]
			PM	3	LC-MS/MS	[2]
Cation channel sperm-associated protein subunit beta (CatSperβ)	A2RTF1	126	PM	9	LC-MS/MS	[2]
Cation channel sperm-associated protein subunit delta (CatSperγ)	C6KI89	132	PM	8	LC-MS/MS	[2]
Cystic fibrosis transmembrane conductance regulator (CFTR)	P26361	168	PM	1	LC-MS/MS	[2]
Chloride channel calcium activated 1 (CLCA1)	Q9D7Z6	100	Sperm	1	Gs/Pe-LC-MS/MS	[3]
Chloride channel calcium activated 2 (CLCA2)	Q8BG22	104	Sperm	11	Gs/Pe-LC-MS/MS	[3]
Chloride intracellular channel protein 1	Q9D7Z6	27	Sperm	2	MS/MS	[4]
Chloride intracellular channel protein 4(CLIC4)	Q9QYB1	29	Sperm	3	MS/MS	[4]
Polycystic kidney disease (polycystin) and REJ (sperm receptor for egg jelly, sea urchin homolog)-like (PKDR-like)	(GI:1338786)	241	CAM	1	Gs/Pe/MS	[1]
Potassium channel, subfamily U, member 1, isoform CRA_a [Mus musculus] (Kv10.1)	G3X9P7	117	CAM	2	Gs/Pe/MS	[1]
Potassium channel calcium activated intermediate/small conductance subfamily N alpha, member 3 (KCNN3)	P58391	77	PM	1	LC-MS/MS	[2]
Potassium channel calcium activated, subfamily M subunit alpha-3 (mSlo3)	O54982	127	CAM	2	Gs/Pe/MS	[1]

Ryanodine Receptor 2, cardiac (RyR2)	E9Q401	565	CAM	1	Gs/Pe/MS	[1]
Sodium channel type 8α_1 (SCNN8A-1)	[a]NM_001077499.2	225	CAM	1	Gs/Pe/MS	[1]
Sodium channel type 8α_2 (SCNN8A-2)	[a]NM_011323.3	225	CAM	1	Gs/Pe/MS	[1]
Sodium channel voltage-gated (Nav)		220	CAM	3	Gs/Pe/MS	[1]
Sodium channel type 11 subunit alpha (Nav1.3)	[a]NM_018732.3	221	PM	1	LC-MS/MS	[2]
Sodium channel type 4αC (Nav1.4)	Q9ER60	209	CAM	1	Gs/Pe/MS	[1]
Sodium channel type 8α (Nav1.6)	Q9WTU3	225	CAM	1	Gs/Pe/MS	[1]
Sodium channel, voltage-gated, type X, alpha (Nav1.8)	Q6QIY3	221	CAM	3	Gs/Pe/MS	[1]
Sodium channel, voltage-gated, type Ivα (SCN4A)	Q9ER60	209	CAM	1	Gs/Pe/MS	[1]
Sodium channel voltage-gated (SCN8A)	F7D6J5	225	CAM	1	Gs/Pe/MS	[1]
Sodium channel, voltage-gated, type VIIIα CRA_a (SCN8A-A)	Q9WTU3	201	CAM	1	Gs/Pe/MS	[1]
Sodium channel, voltage-gated, type VIIIα CRA_b (SCN8A-B)	Q9WTU3-2	225	CAM	1	Gs/Pe/MS	[1]
Sodium channel type 10α 1 (SCN10A-1)	Q6QIY3	221	CAM	3	Gs/Pe/MS	[1]
Voltage-dependent anion channel 1 (VDAC1)	Q60932	32	DRMs	2	PMF, LC-MS/MS	[5]
Voltage-dependent anion channel 2 (VDAC2)	Q60930	32	CAM	9	Gs/Pe/MS	[1]
			Sperm	1	IPG-strip/-LC-MS/MS	[6]
			Sperm	9	MS/MS	[4]
			DRMs	2	PMF, LC-MS/MS	[5]
Voltage-dependent anion- channel 3 _1 (VDAC3-1)	Q6W2J1	31	CAM	9	Gs/Pe/MS	[1]

CAM Cauda Acrosomal Matrix, *DRMs* Detergent-Resistant membranes, *Entry* in UniProtKB or *GI* identification #; *Gs*/*Pe*/*MS* Gel staining/Protein extraction/Mass Spectrometry, *IPG* Immobilized pH Gradient, *IPI* reference Tissue Distribution, *Iso* Isolation, *LC* Liquid Chromatography, *MS* Mass Spectrometry, *PM* Plasma Membranes, *PMF* peptide mass fingerprints

[1] Guyonnet et al. (2012). Molecular & Cellular Proteomics 11: 10.1074/mcp.M11:020339, 758–774; [2] This Work. Proteomic analysis was performed as in [6]; [3] Stein et al. (2006) Proteomics 6, 3533–43; [4] Guo et al. (2010) J Proteome Res. 5;9(3):1246–56; [5] Nixon, B, et al. (2009) Journal of Cellular Physiology (218)1, 122–134; [6] Baker et al., 2010. Proteomics (10) 3, 482–95.

[a]GenBank accession code

Table 3.3 Ion channels identified by proteomic studies on human spermatozoa

Ion channel (short name)	Entry	MW (kDa)	Proteomic technique	# Peptides	References
Amiloride-sensitive sodium channel subunit alpha Isoform 4 (α-ENaC-4)	P37088-4	74	LC-MS	[b]	[1]
Cation channel sperm-associated protein 1 (CatSper1)	Q8NEC5	90	LC-MS/MS	7	[2]
Cation channel sperm-associated protein 2 (CatSper2)	F8W9H2	62	LC-MS/MS	4	[2]
Cation channel, sperm associated 3 (CatSper3)	Q86XQ3	36	LC-MS/MS	2	[3]
Cation channel sperm-associated protein 4 (CatSper4)	Q7RTX7	54	LC-MS/MS	2	[2]
Cation channel sperm-associated protein subunit beta (CatSperβ)	Q9H7T0	127	Aff Col-CAI/LC-MS/MS	1	[4]a
			Aff Col-CAII/LC-MS/MS	3	[4]a
			Gs/Pe-LC-MS/MS	2	[5]
			LC-MS/MS	13, 17	[2], [3]
Cation channel sperm-associated protein subunit delta (CatSperδ)	Q86XM0	91	LC-MS/MS	8	[2]
			2D-LC-MS/MS	2	[6]
Chloride channel CLIC-like protein 1 (CLCC1 like)	Q96S66	27	2D-LC-MS/MS	18	[6]
Chloride intracellular channel protein 1 (CLCC1)	O00299	27	LC-MS/MS	5, 8	[2], [7]
			iTRA-LC-MS	[b]	[1]
			2D-LC-MS/MS	18	[6]
Chloride intracellular channel protein 2 (CLCC2)	Q9UQC9	104	LC-MSMS	2	[4]b
			2D-LC-MS/MS	5	[6]
Chloride intracellular channel protein 3 (CLCC13)	O95833	27	2D-LC-MS/MS	10	[6]

Chloride intracellular channel protein 4 (CLCC4)	Q9Y696	29	LC-MS/MS	4, 4, 3	[2], [3], [9]
			2D-LC-MS/MS	21	[6]
			2DE_MALDI-TOF MS/MS	5	[8]
Chloride intracellular channel protein 5 (CLCC5)	Q9NZA1	47	sub-LC-MS/MS	4	[4]b
Chloride intracellular channel protein 6 (CLCC6)	Q96NY7	73	LC-MS/MS	1	[2]
			sub-LC-MS/MS	2	[4]b
			2D-LC-MS/MS	10	[6]
Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1)	Q14643	314	2D-LC-MS/MS	8	[6]
Inositol 1,4,5-trisphosphate receptor type 2 (ITPR2)	Q14571	308	2D-LC-MS/MS	12	[6]
Inositol 1,4,5-trisphosphate receptor type 3 (ITPR3)	Q14573	304	LC-MS/MS	1	[2]
			2D-LC-MS/MS	31	[6]
Calcium-activated potassium channel subunit alpha-1 (Slo1)	Q12791	138	2D-LC-MS/MS	1	[6]
Inward rectifier potassium channel 16 (Kir5.1)	Q9NPI9	48	2D-LC-MS/MS	1	[6]
Potassium channel subfamily T member 2 (KCNT2)	Q6UVM3	131	sub-LC-MS/MS	3	[4]b
Potassium channel subfamily U member 1 (KCNU1)	A8MYU2	38	LC-MS/MS	4	[2]
Voltage-gated potassium channel subunit beta-2 (KCNAB2)	Q13303	41	2D-LC-MS/MS	2	[6]
Voltage-gated potassium channel subunit beta-2 Isoform 3 (KCNAB2-3)	Q13303-3	41	LC-MS/MS	3	[2]
Piezo-type mechanosensitive ion channel component 1 (PIEZO1)	Q92508	287	2D-LC-MS/MS	29	[6]
Ryanodine receptor 2 (RYR2)	Q92736	569	Gs/Pe-MS/MS	3	[10]

(continued)

Table 3.3 (continued)

Ion channel (short name)	Entry	MW (kDa)	Proteomic technique	# Peptides	References
Ryanodine receptor 3 (RYR2)	Q15413	552	2D SDS-PAGE_MS/MS	NI	[11]
Transient receptor potential cation channel subfamily A member 1 (TRPA1)	O75762	127	sub-LC-MS/MS	3	[4]b
Short transient receptor potential channel 4 (TRPA4)	Q9UBN4	112	sub-LC-MS/MS	2	[4]b
Short transient receptor potential channel 6 (TRPA6)	Q9Y210	106	2D-LC-MS/MS	9	[6]
Transient receptor potential cation channel family, subfamily M, member 1 (TRPM1)	Q7Z4N2	182	LC-MS/MS	NI	[12]
Transient receptor potential cation channel subfamily M (member 4)	Q8TD43	134	2D-LC-MS/MS	13	[6]
Transient receptor potential cation channel subfamily M member 8 (TRPM8)	Q7Z2W7	128	sub-LC-MS/MS	2	[4]b
Transient receptor potential cation channel subfamily V member 2 (TRPV2)	Q9Y5S1	86	sub-LC-MS/MS	2	[4]b
			2D-LC-MS/MS	15	[6]
Transient receptor potential cation channel subfamily V member 4 (TRPV4)	Q9HBA0	98	2D-LC-MS/MS	1	[6]
Transmembrane channel-like protein 5 (TMC5)	Q6UXY8	115	LC-MS/MS	1	[2]
Transmembrane channel-like protein 8 (TMC8)	Q8IU68	82	2DE-MS/MS & 2DE- MSE	NI	[11]
Trimeric intracellular cation channel type B (TMEM38B)	Q9NVV0	33	sub-LC-MS/MS	42	[4]b
			Gs/Pe/LC-MS/MS		[13]
			iTRAQ- LC-MS	[b]	[1]
			LC-MS/MS	5,5	[2], [3]

Two-pore calcium channel protein 1 (TPCN1)	Q9ULQ1	94	2D-LC-MS/MS	9	6
Voltage-dependent anion-selective channel protein 1 (VDAC1)	P21796	31	iTRA- LC-MS	[b]	[1]
			LC-MS/MS	3, 7, 9	[3, 7, 14]
			2D-LC-MS/MS	20	[6]
			2DE_MALDI-TOF MS/MS	4	[8]
Voltage-dependent anion-selective channel protein 2 (VDAC2)	P45880	32	Gs/Pe-MS/MS	1	[10]
			LC-MS/MS	7, 15, 17, 18	[7, 2, 3, 17]
			2DE/MS	[a]9	[15]
			2DE-MS/MS	5	[16]
			2D-LC-MS/MS	18	[6]
			sub-LC-MS/MS	74	[4]b
			2DE_MALDI-MS/MS	29, 29	[14, 18]
Voltage-dependent anion-selective channel protein 2 Isoform 3 (VDAC2-3)	P45880-3	36	iTRA- LC-MS	[b]	[1]
Voltage-dependent anion-selective channel protein 2 isoform 4 (VDAC2-4)	P45880-4	33	2DE/ MALDI-TOF-MS/LC-MS	9	[19]
Voltage-dependent anion channel 2, isoform CRA_a (VDAC2-CRA-A)	A0A024QZN9	34	Aff Col-CAII/LC-MS/MS	3	[4]a
Voltage-dependent anion-selective channel protein 3 (VDAC3)	Q9Y277	31	Gs/Pe-MS/MS	1	[10]
			LC-MS/MS	10, 13, 9	[2], [7], [9]
			sub-LC-MS/MS	32	[4]b
			2D-LC-MS/MS	15	[6]
Voltage-dependent anion-selective channel protein 3 isoform 1 (VDAC3-1)	Q9Y277-1		iTRA- LC-MS	[b]	[1]
Voltage-dependent anion channel 3 isoform b (VDAC3-b)	Q9Y277-b		LC-MS/MS	9	[16]

(continued)

Table 3.3 (continued)

Ion channel (short name)	Entry	MW (kDa)	Proteomic technique	# Peptides	References
Voltage-dependent anion channel 3, isoform CRA_b (VDAC3-CRA_b)	Q9Y277		Aff Col-CAII/LC-MS/MS	1	[4]a
Voltage-dependent calcium channel subunit alpha-2/delta-1 (CA2D1)	P54289	125	Gs/Pe-LC-MS/MS	2	[5]
			LC-MS/MS	[a]1	[2, 19]
			2D-LC-MS/MS	19	[6]
Voltage-dependent calcium channel subunit alpha-2/delta-2 (CA2D2)	Q9NY47	130	LC-MS/MS	3, 10	[2], [7]
Voltage-dependent L-type calcium channel subunit beta-3 (CACB3)	P54284-5	50	2D-LC-MS/MS	3	[6]
Voltage-dependent R-type calcium channel subunit alpha-1E (Cav2.3)	Q15878	262	sub-LC-MS/MS	2	[4]b
Voltage-gated hydrogen channel 1 (HVCN1)	Q96D96	32	LC-MS/MS	4, 4	[2, 14]
			2D-LC-MS/MS	2	[6]
Voltage-gated hydrogen channel 1 Isoform 1 ((HVCN1)-1)	Q96D96-1	32	iTRA- LC-MS	[b]	[1]
Voltage-gated sodium channel subunit alpha (Nav1.2)	Q99250	230	sub-LC-MS/MS	1	[4]b
			Gs/Pe-MS/MS	2	[10]
Voltage-gated sodium channel subunit alpha (Nav1.4)	P35499	210	Gs/Pe-MS/MS	1	[10]
			sub-LC-MS/MS	1	[4]b
Voltage-gated sodium channel subunit alpha (Nav1.5)	Q14524	229	Gs/Pe-MS/MS	1	[10]
			sub-LC-MS/MS	4	[4]b
Volume-regulated anion channel subunit LRRC8A (LRRC8A)	Q8IWT6	94	2D-LC-MS/MS	23	[6]
Volume-regulated anion channel subunit LRRC8C (LRRC8C)	Q8TDW0	93	2D-LC-MS/MS	18	[6]

Volume-regulated anion channel subunit LRRC8E (LRRC8E)	Q6NSJ5	91	2D-LC-MS/MS	9	[6]
Volume-regulated anion channel subunit LRRC8D (LRRC8D)	Q7L1W4	98	2D-LC-MS/MS	2	[6]

Aff Col-CAI (CAII) Affinity Column-Anti-Carbonic Anhydrase I (II), *Entry* in UniProtKB, *Gs*/*Pe* Gel staining/Protein extraction, *iTRAQ* Isobaric tags for relative and absolute quantitation technology, *LC* Liquid chromatography, *MS* Mass Spectrometry, *MS*/*MS* Tandem MS, *MALDI-TOF* matrix-assisted laser desorption ionization time of flight-mass spectrometry, *MSE* data-label-free quantitative proteomics, *NI* No Information, *2D SDS-PAGE* two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis

[1] Liu Y, et al., (2015) Andrology, 3(2): 247–59; [2] Wang G, et al., (2013) J Proteomics, 79:114–22. [3] Zhou T, et al., (2015) Proteomics, 15(9):1564–73; [4]a This work. Proteomic analysis was performed as in [20]; [4]b This work. Proteomic analysis was performed as in [12], [5] Jumeau F, et al. (2015) J Proteome Res. 14(9):3606–20; [6] Schumacher J, et al. (2013) J Proteome Res. 12(12):5370–82; [7] Amaral A, et al. (2013) Mol Cell Proteomics, 12(2): 330–42; [8] Li J, et al. (2011) Mol Cell Proteomics, 10(3):M110.004630; [9] Sun G, et al. (2014) J Proteomics, 109:199–11; [10] Lefievre L, et al. (2007) Proteomics, 7(17):3066–84; [11] Ferreira B, et al. (2013) Fertil Steril, 100(4):959–69; [12] Baker MA, et al., (2007) Proteomics Clin Appl, 1(5):524–32; [13] de Mateo S, et al. (2011) Proteomics, 11: 2714–26; [14] Martínez-Heredia J, et al. (2006) Proteomics, 6(15): 6(15):4356–4369; [15] Ficarro S, et al. (2003) J Biol Chem. 278(13):11579–89; [16] Sharma R, et al. (2013) Reprod Biol Endocrinol, 11:1–18; [17] Siva AB, et al. (2010) Mol Hum Reprod. 16(7):452–62; [18] Bhande S & Naz RK (2007) Mol Reprod Develop. 74:332–40; [19] Intasqui P, et al. (2015) Fertil Steril. 104(2):292–01; [20] Loza-Huerta et al., 2013. Biochim Biophys Acta 1830 (2013) 5305–15

[a]% Masses match

[b]Relative intensities

3.2 Ca^{2+} Channels

3.2.1 CatSper Channels

The first member of the CatSper family was described in 2001 by Ren and coworkers (Quill et al. 2001; Ren et al. 2001) and recorded electrophysiologically by Kirichok and coworkers in 2006 in mouse sperm (Kirichok et al. 2006). It is now known that CatSper is a heteromultimer channel with all subunits required for proper channel formation and function. The channel constitutes four α subunits (CatSper1–4) and three auxiliary subunits (CatSperβ, γ, and δ) (Liu et al. 2007; Wang et al. 2009; Chung et al. 2011; Lishko et al. 2011a). All CatSper subunits are sperm specific and expressed in the principal piece of the flagella (Ren et al. 2001; Lobley et al. 2003; Chung et al. 2014). Catsper is indispensable for hyperactivation and mutations in any of its subunits cause infertility in mice and so far in CatSper2 in humans (Ren et al. 2001; Carlson et al. 2003; Qi et al. 2007; Ho et al. 2009).

It is known that progesterone, secreted by the ovaries and cumulus cells enveloping the oocyte, stimulates human CatSper at nM concentrations inducing [Ca2+]i increases. At higher μM this hormone can induce the AR in sperm from several mammals including human and mouse. Surprisingly, early this year Lishko's group reported that progesterone stimulates CatSper indirectly by activating a lipid hydrolase ABHD2 that depletes the endocannabinoid 2-arachidonoylglycerol from the human sperm flgellar plasma membrane where it inhibits CatSper at nM concentrations. Also notable is their finding that ABHD2 is found in the mouse acrosomal area and not in the flagella, and inhibition of this enzyme decreases AR (Miller et al. 2016).

The precise role of CatSper in the AR is not clear. In mouse sperm, Ca^{2+} increases can be induced by several stimuli (ZP, BSA, cyclic nucleotide (CN) permeable analogs, a combination of $NaHCO_3$ and 150 mM KCl—pH 7.6, and other agents) (Carlson et al. 2003; Kirichok et al. 2006; Xia and Ren 2009; Xia et al. 2007; Chávez et al. 2014). These Ca^{2+} increases are absent in CatSper null mouse or significantly modified in the case of stimulation by ZP (Xia and Ren 2009). In wild-type mouse sperm such Ca^{2+} responses start in the principal piece and then propagate to midpiece and head within seconds (Xia et al. 2007). The AR is accompanied and requires a $[Ca^{2+}]_i$ elevation in the sperm (Xia and Ren 2009; Xia et al. 2007; Darszon et al. 2011; Buffone et al. 2014). As ZP induces AR in CatSper knockout mouse sperm in vitro experiments in a very similar fashion as in wild-type sperm, the contribution of this channel to the AR is ill-defined (Xia and Ren 2009). Furthermore, since at present time the physiological inducer of the AR is in question, the involvement of CatSper in this process awaits further investigation.

On the other hand, the male reproductive tract and to a lesser extent other tissues express cysteine-rich secretory proteins (CRISPs) that constitute four groups. CRISPs have been shown to regulate ion channels (Battistone et al. 2015). Newly

reported findings show that mouse CRISP1 can inhibit CatSper in sperm from this species and in this manner influence motility regulation (Battistone et al. 2015).

3.2.2 Voltage-Dependent Ca^{2+} Channels

Before the electrophysiological description of CatSper (Ren et al. 2001), it was believed that the main Ca^{2+} channels involved in the sperm AR were the Ca_Vs (Kazazoglou et al. 1985; Florman et al. 1992; Arnoult et al. 1996; Liévano et al. 1996; Darszon et al. 1999).

Early on it was found that the AR in sea urchin sperm was inhibited by Ca_V blockers such as dihydropyridines (Schackmann et al. 1981). Experiments measuring sperm $[Ca^{2+}]_i$ evidenced that these Ca_v antagonists (i.e., nimodipine) inhibited the $[Ca^{2+}]_i$ increases associated with the egg jelly-induced AR [reviewed in (Darszon et al. 2011)]. Furthermore, the rise in $[Ca^{2+}]_i$ triggered by a K^+-induced depolarization in valinomycin-treated sperm was reduced (20–30 %) by nifedipine and nimodipine. Later on transcripts for two Ca_v α subunits similar to those coding for $Ca_v1.2$ and $Ca_v2.3$ were detected in sea urchin testis. In addition, Western blot and immunocytochemistry results with antibodies against Ca_v-Pan and $Ca_v2.3$ from rat were consistent with the presence of Ca_vs in the flagella and acrosome of sea urchin sperm (Granados-Gonzalez et al. 2005). Proteomic efforts in sea urchin sperm have revealed only two Ca_v peptides (see Table 3.1). All the aforementioned results suggest that Ca_v channels might be involved in the AR of sea urchin sperm; however, further experiments are needed to establish or disprove this possibility.

In mouse sperm, it was generally accepted that the capacitation-associated Em hyperpolarization was necessary to remove inactivation from Ca_vs, leaving the channels ready to open upon stimulation with ZP or other factors to trigger the AR (Arnoult et al. 1999; Wennemuth et al. 2000). These observations were supported by biochemical and pharmacological studies, where the localization of low voltage-gated $Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$ was determined in the principal piece, head, and midpiece of mature mouse and human sperm (Trevino et al. 2004; Darszon et al. 2006; Escoffier et al. 2007). In spite of this, Ca_v currents in mature sperm have not been detected and mice null for $Ca_v3.1$ and $Ca_v3.2$ are fertile (Stamboulian et al. 2004; Escoffier et al. 2007). These findings question the role of Ca_vs in the AR, though the limitations of whole cell patch clamp recordings and compensatory mechanism in Ca^{2+} single mutants must be considered (see Concluding Remarks).

The involvement of $Ca_v2.3$ channels in sperm function was previously suggested (Wennemuth et al. 2000; Sakata et al. 2001, 2002). Recently their role in the mouse AR was reevaluated. Sperm from $Ca_v2.3$ null mice display aberrant motility and subfertility. Remarkably, Cohen et al. (2014) also reported that focal enrichment of GM1 lipid regulates $Ca_v2.3$ activity and stimulates Ca^{2+} influx triggering the AR. They also provided plausible explanations as to why Ca_v currents may have

escaped electrophysiological detection. This work exemplifies the need to further examine the participation of Ca_vs in sperm physiology.

3.2.3 TRP/SOC Channels

3.2.3.1 TRPC2

In 1998, Wissenbach et al. (1998) reported the expression of transient receptor potential (TRP) trpc2 mRNA in bovine testis, spleen, and liver but not in brain, heart, adrenal gland, or retina. Trpc2 mRNA expression in bovine testis was limited to spermatocytes suggesting that TRPC2 could contribute to the formation of ion channels in sperm cells. In 2001, Jungnickel et al. (2001) showed that TRPC2 was necessary for Ca^{2+} entry in mouse sperm during the AR. They suggested that this channel was involved in the sustained Ca^{2+} increase in response to ZP3. Inhibiting TRPC2 using an anti-TRPC2 antibody abolished the AR (Jungnickel et al. 2001). However, TRPC2$^{-/-}$ mice are fertile and TRPC2 is a pseudogene in humans; therefore, a compensatory mechanism may rescue fertility since sperm from both species express other TRPC channels (Darszon et al. 2012). In addition, the mechanism by which ZP3 activates TRPC2 is not clearly elucidated. A direct interaction between the channel and the IP3 receptor was suggested (Stamboulian et al. 2005). Later in 2001, Trevino et al. (2001) detected transcripts in mouse testis for the 7 trp genes and immunologically detected TRPC1 and C3 proteins in the flagella and TRPC6 in the post-acrosomal region. As in the case of many of the other channels, the presence and involvement of TRPC2 in sperm physiology and the AR is far from established.

3.2.3.2 TRPM8

The main role of TRPM8 is to function as a cold sensor in neurons (Laing and Dhaka 2015). TRPM8 is also expressed in testis, prostate, spermatogenic cells, and mature sperm (Stein et al. 2004; Zhang and Barritt 2006; De Blas et al. 2009; Martínez-López et al. 2011). This channel was detected by immunoblot and localized to the head and flagella by immunocytochemistry in mouse and human sperm. This labeling pattern was basically absent in sperm from TRPM8$^{-/-}$ mouse (De Blas et al. 2009; Martínez-López et al. 2011). In fact, in testicular sperm Martínez-López and coworkers were able to record TRMP8 currents in response to menthol and icilin, two TRPM8 agonists (Martínez-López et al. 2011). Moreover, TRPM8 antagonists like capsazepine and BCTC inhibited the AR stimulated by ZP and menthol. These data suggest TRPM8 may participate in the AR, although TRPM8$^{-/-}$ male mice are fertile. In human sperm, menthol also strongly induced the AR which was inhibited by capsazepine and BCTC. However, these inhibitors did not decrease AR promoted by either progesterone or ZP3, suggesting that

TRPM8 activation triggers this process by a different signaling pathway (De Blas et al. 2009) or that another channel such as CatSper is involved, since it is also activated by menthol (Brenker et al. 2012) and inhibited by capsazepine and BCTC.

Using electrophysiology, cell assays, and mouse models, the CRISP4-CRISP domain was found to inhibit TRPM8. Functional results suggested that this channel could modulate the progesterone-induced AR (Gibbs et al. 2011). Later research has provided further evidence that CRISP1 may inhibit TRPM8 currents in testicular sperm (Ernesto et al. 2015).

3.2.3.3 TRPP

Polycystin 2 (PC2/PKD2) or TRPP2 is a member of the TRP family of nonselective cation channels. Evidence indicates that PC1/PKD1 and TRPP2 are interacting partners that can form a channel (reviewed in Giamarchi and Delmas 2007). Sea urchin PC2 (suPC2) colocalizes with sperm egg jelly receptor suREJ3 to the plasma membrane over the acrosomal vesicle of sea urchin sperm. suPC2 and suREJ3 are associated in the membrane, suggesting that this complex may function as a cation channel contributing to the Ca^{2+} and Na^{+} influx mediating the AR when sperm contact the egg jelly at fertilization (Kierszenbaum 2004; Neill et al. 2004). PC1-PC2 channels allow Ca^{2+} entry which can produce Ca^{2+} spikes that augment and reshape sensory responses to polymodal stimuli, including Ca^{2+} exposure, voltage repolarization, and acid withdrawal (Hu et al. 2015). New findings show that PKA can directly phosphorylate PC2, which increased the mean open time but not the single channel conductance of the channel (Cantero et al. 2015).

It turns out that *Pkdrej*, which belongs to the PC1 gene family, has been detected only in the mammalian male germ lineage and the protein is expressed in the anterior sperm head. *Pkdrej* null male mice are fertile; however, in mixed population artificial insemination assays and under competing conditions with wild-type males, they are less successful in fertilization. Furthermore, sperm from *Pkdrej* null mice undergo the AR more slowly and take longer to reach the egg–cumulus complex in the oviduct. These findings hint that *Pkdrej* may modulate capacitation and thus the timing of fertilization in vivo (Sutton et al. 2008).

3.2.3.4 Orai and Stim

Sperm possess Ca^{2+} stores, some unusual like the acrosome and the redundant nuclear envelope, and mitochondria. There is evidence of Ca^{2+} store mobilization consistent with the occurrence of store-operated Ca^{2+} entry in sperm (Blackmore 1999; O'Toole et al. 2000; González-Martínez et al. 2001; Costello et al. 2009; Correia et al. 2015).

It is now clear that Orai channels and Stim proteins are key participants for SOC activity, either by themselves or in combination with TRP channels. The presence of Stim and Orai proteins was documented in human (Costello et al. 2009) and

mouse sperm (Darszon et al. 2012) by Western blot and immunocytochemistry. However, few studies have addressed the role of these proteins during the AR. Recently Morris et al. (2015), using cell penetrating peptides attached to a protein domain that stimulates Stim/Orai activity, were able to induce SOC activity at the neck of human sperm, downstream of CatSper stimulation by progesterone. The authors indicate that SOCs contribute to progesterone-induced $[Ca^{2+}]_i$, though the role of this Ca^{2+} mobilization during the AR requires further investigation.

3.2.4 Intracellular Ca^{2+} Channels

Part of SOC activity requires the participation of intracellular Ca^{2+} channels. Accordingly, in 1995 Walensky and Snyder (1995) reported the expression of IP3R, G proteins (Gα/11), and PLCβ1 in the anterior acrosomal membrane. Interestingly, acrosome-reacted sperm lost their acrosomal IP3R immunostaining. The loss of IP3R in the sperm head is consistent with the release of the outer acrosomal membrane and associated IP3R from the sperm head into the medium during acrosomal exocytosis (Walensky and Snyder 1995). The heterotrimeric G proteins G_q and G_{11} activate the β isoforms of the phosphatidylinositol-specific PLC family and most effectively stimulate the β1 isotype. Therefore, the interaction sperm–ZP could trigger the phosphoinositide pathway during the AR (Tomes et al. 1996). The presence of the IP3R (types 1, 2, and 3) using monoclonal and polyclonal antibodies against these receptors was also documented in human sperm probing membrane proteins from reacted and non-reacted sperm (Kuroda et al. 1999). Thapsigargin and cyclopiazonic acid, two agents that release Ca^{2+} from intracellular stores, stimulated the AR in a dose-dependent manner but only when Ca^{2+} was present in the external medium (Meizel and Turner 1993). In contrast, using the model of permeabilized sperm to evaluate the role of SNARE proteins during the AR, it was shown that Rab3A triggers acrosomal exocytosis in the virtual absence of Ca^{2+} in the cytosolic compartment though exocytosis depends on the presence of Ca^{2+} in intracellular stores (De Blas et al. 2002).

3.3 TPC Channels

Two pore channels (TPC1 and TPC2) are NAADP-gated intracellular Ca^{2+}/Na^{+} channels that reside in acidic organelles (Zhu et al. 2010; Xu and Ren 2015) and contribute to Ca^{2+} signaling in most cells. TPC2 transcripts are present in reproductive tissues and TPC1 proteins were documented in sperm from mouse, rat, and human by Western blot (Arndt et al. 2014). Interestingly, in human sperm NAADP induced Ca^{2+} mobilization (Sánchez-Tusie et al. 2014) and the AR (Arndt et al. 2014) in the absence of extracellular Ca^{2+}, a condition also observed by De Blas et al. (2002) but implicating the IP3R instead of TPC. Since Arndt also

observed colocalization of IP3R and TPC1 in lipid rafts, they proposed that acrosomal exocytosis may well fit the "NAADP trigger hypothesis" (Calcraft et al. 2009), in which initial NAADP Ca^{2+} release triggers further Ca^{2+} release through IP3 or Ryanodine receptors located in adjacent Ca^{2+} stores amplifying the signal (Correia et al. 2015).

3.4 Ca^{2+}-Dependent Cl^- Channels (CaCCS)

Early single channel patch clamp recordings in mouse sperm revealed a niflumic acid-sensitive anion-selective channel in mouse sperm (Espinosa et al. 1998). This work also showed that niflumic acid inhibited the AR triggered by ZP, progesterone, and GABA and documented the presence of two larger conductance cationic channels in sperm from this species. Thereafter, it was reported that other anion channel blockers such as 1,9-dideoxyforskolin (DDF) potently inhibited the AR (Espinosa et al. 1999).

Ca^{2+}-dependent whole cell Cl^- currents were recorded by Orta et al. (2012) patch clamping human sperm in the head. The authors proposed that TMEM16A may contribute to these currents and also that sperm CaCCs may participate in the rhZP3-induced AR. These conclusions were based on the fact that niflumic acid (NFA) and 4,4-diisothiocyano-2,2-stilbene disulfonic acid (DIDS), two CaCCs blockers, inhibited the rhZP3-induced AR in a dose-dependent manner. Furthermore, $TMEM16A_{inh}$, the most specific TMEM16A blocker, inhibited nearly 80 % of the AR. By blocking CaCCs, NFA, DIDS, and $TMEM16A_{inh}$ could alter cell volume regulation, which might influence the distance between the outer acrosomal membrane and the plasma membrane that has been found to be critical for acrosome exocytosis (Rossato et al. 1996; Zanetti and Mayorga 2009).

3.5 SLO3 Channels

The SLO family of K^+ channels is composed of four members. SLO1 is modulated by Ca^{2+} (Magleby 2003), SLO2.1 and SLO2.2 by Na^+ and Cl^- (Yuan et al. 2003; Paulais et al. 2006), and SLO3 by pH and voltage (Schreiber et al. 1998). SLO3 is only present in sperm and localized probably to its flagella (Schreiber et al. 1998; Navarro et al. 2007; Miller et al. 2015). In fact, the lack of SLO3 in male mice causes infertility (Santi et al. 2010; Zeng et al. 2011) though they exhibit normal testis size, sperm number (Santi et al. 2010), and capacitation-associated protein phosphorylation (Escoffier et al. 2015). However, sperm null for SLO3 do not hyperactivate, their capacitation-associated hyperpolarization is absent, and their ability to acrosome react significantly diminished (Santi et al. 2010; Zeng et al. 2011; Chávez et al. 2013, 2014). Electrophysiological recordings of SLO3 either heterologously expressed (Xenopus oocytes/CHO cells) or in testicular/

epididymal sperm have allowed its characterization in terms of pH dependence, modulation by the LRRC52 subunit, and the species-specific sensitivity to inhibitors (Martínez-López et al. 2009; Santi et al. 2010; Tang et al. 2010; Zeng et al. 2011; López-González et al. 2014; Sánchez-Carranza et al. 2015; Wrighton et al. 2015; Zeng et al. 2015). The contribution of SLO3 to the AR is not clear; one proposal is that its aperture during capacitation, due to a higher concentration of HCO_3^- and alkaline pH in the female tract, increases the driving force for Ca^{2+} entry, probably through CatSper or other channels, triggering the subsequent events that lead to the AR (Lishko et al. 2011a; Miller et al. 2015). Another hypothesis is that once in the female tract, SLO3 activation hyperpolarizes Em, which in turn stimulates a voltage-dependent Na^+/H^+ exchanger, leading to a further pH_i rise, activating CatSper, depolarizing sperm, and setting the conditions for AR (Chávez et al. 2014).

3.6 Proton Channels

Lishko and coworkers reported in 2010 the presence of H^+ currents (H_V1 channels) in human sperm (Lishko and Kirichok 2010; Lishko et al. 2010). As anticipated, the sperm H_V1 is activated by depolarization, an outward transmembrane pH gradient, and the endocannabinoid anandamide and selectively inhibited by μM Zn^{2+}. They proposed that at the time of ejaculation, the high seminal plasma Zn^{2+} (~2 mM) inhibits sperm H_V1 (Lishko and Kirichok 2010). Once in the female reproductive tract, Zn^{2+} may be chelated by the oviductal fluid, resulting in gradual activation of H_V1 channels (Lu et al. 2008). As the female reproductive tract has a low anandamide concentration, its physiological relevance is controversial (Schuel and Burkman 2005). However, cumulus cells synthesize anandamide (El-Talatini et al. 2009) exposing sperm to this endocannabinoid in the oocyte surroundings and therefore activating H_V1 and increasing pH_i which could contribute to trigger the AR. In contrast, mouse sperm exhibit very small H_V1 currents; their role if any is currently unknown.

3.7 GABA Receptors

GABA receptors have been reported in sperm from several species. In rats, the presence of $GABA_A$ and $GABA_B$ receptors was documented (He et al. 2001; Hu et al. 2002). In mouse, $GABA_A$ subunits, including α1, α5, β1-3, and γ3, were reported in spermatogenic cells as well as in mature sperm (Kanbara et al. 2010, 2011). This group also recorded whole cell Cl^- currents after GABA application in spermatogenic cells (Kanbara et al. 2011). Moreover, a GABA transporter called GAT1 was found in mouse sperm (Ma et al. 2000). Overexpression of this transporter affects testis morphology and fertility in general, reducing reproductive

capacity ~70 % (Hu et al. 2004). Roldan et al. reported that progesterone stimulated the AR, which was inhibited by GABA antagonists in mouse sperm (Roldan et al. 1994). Since it is now known that progesterone does not activate mouse sperm CatSper, reexamining the possible participation of GABA receptors in the AR seems worthy. On the other hand, it was also reported that GABA and progesterone elevated DAG levels, indicating PLC activation. This DAG production could be inhibited with bicuculline, a $GABA_A$ receptor antagonist (Sieghart 1992). The $GABA_A$ subunits α1, α5, β1, and β3 have been identified by immunocytochemistry in the equatorial segment of the human sperm head (Wistrom and Meizel 1993), and Shi et al. (1997) reported AR induction by GABA in this sperm species. A synergistic AR induction with GABA and progesterone was observed. The mechanism and physiological relevance of these GABA responses are still unclear. Interestingly, GABA has also been implicated in the promotion of hyperactivated motility (Fujinoki et al. 2015).

3.8 Glycine Receptors

The Glycine receptor (GlyR) is highly expressed in the nervous system where the amino acid glycine mediates inhibitory neurotransmission. In situ hybridization demonstrated that GlyR β subunit mRNAs are localized in spermatogenic cells (Sato et al. 2002) and immunofluorescence detected GlyR at the tip of the acrosomal region in mature mouse sperm (Sato et al. 2000). In fact, GlyR expression appeared to increase after capacitation and was not detectable after the AR. Moreover, glycine increased the AR in a dose-dependent manner in mouse sperm, which was inhibited by strychnine (a GlyR antagonist) and with a monoclonal GlyR antibody (Sato et al. 2000). Mutations on mouse GlyR α or β subunits produce sperm that are deficient in their ability to undergo the AR stimulated by glycine or by solubilized ZP (Sato et al. 2000). These mutations do not affect the AR stimulated by Ca^{2+} ionophore A23187. These data suggest the sperm GlyR participates in the ZP-initiated mouse AR (Sato et al. 2000; Meizel and Son 2005).

Glycine also induces the AR in human sperm and pharmacological evidence has implicated the GlyR in the ZP- or glycine-induced AR in this species (Melendrez and Meizel 1995). Bray et al. (2002a) observed that strychnine significantly inhibited the AR induced by recombinant human ZP3. The presence of the α_1 subunit of the GlyR was confirmed by Western blot (Bray et al. 2002a). Considering that sperm intracellular [Cl^-] was estimated to be 40 mM in capacitated human sperm, opening of Cl^- channels such as the $GABA_A$ receptor or the GlyR should result in Cl^- efflux, leading to depolarization of the plasma membrane (Garcia and Meizel 1999). Therefore, these neurotransmitter receptors could cause the initial depolarization required to activate Ca_V channels involved in the initial Ca^{2+} rise upon ZP stimulation (Meizel 2004).

3.9 Acetylcholine Receptors

Nicotinic acetylcholine receptors (AChRs) are expressed in the central and peripheral nervous systems and muscle and are involved in fast synaptic transmission. These receptors are composed of various combinations of five different protein subunits (α, β, γ, δ, and ε) (Son and Meizel 2003). Bray and coworkers found α7nAChR transcripts in mouse testis by RT-PCR and protein expression in mouse sperm extracts by immunoblot. In addition, they also found that α7nAChR is present in the midpiece of mature mouse sperm (Bray et al. 2005). Interestingly, α7nAChR ligands like PNU282987 and acetylcholine stimulate the AR in mouse sperm at micromolar concentrations. Moreover, nM concentrations of some antagonists of nAChR such as α-bungarotoxin, α-conotoxin, and methyllycaconitine inhibited the AR initiated by acetylcholine or ZP (Son and Meizel 2003; Jaldety et al. 2012). Furthermore, the synergistic effect between ZP, ACh, and PNU282987 on the AR further supported a possible cooperation between α7nAChR and ZP, suggesting that the two pathways are not redundant (Jaldety et al. 2012). The effect of these nAChR antagonists did not block the AR initiated by Ca^{2+} ionophore A23187, suggesting that the role of α7nAChR is upstream of Ca^{2+} influx (Son and Meizel 2003). Mice null for α7nAChR have impaired fertility and important defects in motility, although the number, morphology, viability, and spontaneous AR are similar to wild-type sperm (Bray et al. 2005). Human sperm express several subunits of the nAChR (α3, α5, α7, α9, and β4); they localize to head, post-acrosomal region, flagella, and connecting piece (Kumar and Meizel 2005). Micromolar concentrations of acetylcholine can initiate the AR in human sperm. This response is inhibited by antagonists (α-bungarotoxin, α-conotoxin, and methyllycaconitine) that block the α7nAChR (Bray et al. 2002b).

3.10 Purinergic Receptors

The Adenosine A1 Receptor (A1R) has been immunolocalized at the post-acrosomal and acrosomal region of rat sperm (Minelli et al. 2000). In human sperm, A1R was found to be coupled to a pertussis toxin (PTX)-sensitive G protein that signals via PLC to produce IP3 promoting $[Ca^{2+}]_i$ increase (Allegrucci et al. 2001). Minelli et al. (2004) showed that A1R knockout mice have reduced progeny. The same group reported afterwards that an A1R agonist increases AR and in vitro fertilization and promotes Ca^{2+} influx into sperm (Minelli et al. 2008).

On the other hand, Navarro et al. recorded a nonselective cation channel in mice sperm upon addition of ATP whose activation promoted $[Ca^{2+}]_i$ increase. They proposed that ATP acts through the P2X2 receptor, which was detected in sperm using RT-PCR and Western blot. These findings were confirmed using sperm from mice lacking the P2X2 receptor which lost the ATP-gated current. P2X2 null mice preserve normal AR and hyperactivation, but fertility declines with frequent mating

(Navarro et al. 2011), suggesting that P2X2 receptor could regulate fertility. Recent observations indicate that ATP induces AR in capacitated rat sperm which is significantly reduced by two ATP antagonists and interestingly also by two P2X7-specific antagonists. These findings suggest the involvement of these particular purinergic receptors in fertilization. The presence of P2X7 was confirmed using Western blot and immunofluorescence (head and flagellum). Quantification of ATP in the rat oviduct showed similar ATP concentrations to those used to induce AR; these values fluctuate during estrous cycle being higher at ovulation (Torres-Fuentes et al. 2015).

Extracellular ATP is a potent activator of the AR in human sperm (Foresta et al. 1992). At that time, the proposal was that ATP would open a plasma membrane channel selective for monovalent cations (Na^+, choline, and methylglucamine) and impermeable to Ca^{2+}. Accordingly, ATP-triggered AR was dependent on extracellular Na^+ and independent of extracellular Ca^{2+} (Foresta et al. 1992). Recently, P2X (1, 2, and 3) receptor subtypes have been immunolocalized on the sperm human head (Banks et al. 2010).

3.11 Na^+ Channels

In mouse, human, and horse sperm capacitation is accompanied by cell membrane hyperpolarization (Sánchez-Carranza et al. 2015). An epithelial Na^+ channel type has been a candidate to contribute to this Em change, particularly in mouse sperm in which a channel of this type was detected by Western blot and immunocytochemistry (Hernández-González 2005). However, further experiments are needed to verify the presence of this channel in mammalian sperm and define its specific role during AR.

The presence of voltage-gated Na^+ channels (Na_V) has been documented in human sperm by mRNA expression (Na_V 1.1-1.9), proteomics (see Table 3.3), and immunofluorescence (all except Na_V 1.1 and 1.3) (Pinto et al. 2009). Though the role of these channels in sperm motility was investigated, their exact function remains poorly understood. Patrat et al. (2000) reported that AR induced with progesterone was significantly higher when sperm were incubated in Na^+-containing medium as compared to Na^+-depleted medium. Further research is needed to establish the role of these channels during the AR.

3.12 Voltage-Dependent Anion Channels (VDAC)

Two-dimensional gel electrophoresis and mass spectrometry identified VDAC as one of the proteins that undergo tyrosine phosphorylation during mouse sperm capacitation (Arcelay et al. 2008). More than one VDAC isoform was immunodetected, consistent with previous reports in bovine sperm (Hinsch

et al. 2004). As anticipated, VDAC was immunolocalized to the midpiece where mitochondria are found, though surprisingly it was also detected in the mouse sperm anterior head from where it was lost upon AR (Arcelay et al. 2008).

Using immunofluorescence and Western blot, VDAC1, VDAC2, and VDAC3 were detected in human sperm flagella and head (Liu et al. 2011; Kwon et al. 2013). It was proposed that VDAC2 may act as an integral membrane protein that participates in the maintenance of acrosomal morphology by regulating ion transport, as reported for bovine sperm (Triphan et al. 2008). Several VDAC peptides have been reported in sperm proteomic studies (Table 3.1 (sea urchin), 3.2 (mouse), and 3.3 (human)). The function of this protein in mitochondria is well known; however, its function in the acrosome is not well understood (see Thinnes 2015).

3.13 Ion Channel Proteomics

It is worth pointing out, though not surprising, that from the sea urchin, mouse, and human sperm proteins identified by proteomics, the number of ion channels is quite scarce (see Tables 3.1, 3.2, and 3.3). Despite the use of a variety of proteomic strategies and sample isolation methods, several ion channels known to be functionally present in sperm from these species have not yet been identified (i.e., SOCs and Ca_Vs). This is mainly due to their very low abundance in general. Furthermore, as with some membrane proteins, ion channels can be hard to solubilize and enrich; they aggregate and are difficult to digest (Baker 2011). On the other hand, there is proteomic evidence for the presence of different channels for which no function has been described.

3.14 Concluding Remarks

This review has described functional evidence that points to the involvement of channels other than CatSper and SLO3 in the AR. In spite of this, cytoplasmic droplet whole cell patch clamp recordings from corpus epididymal sperm from the double CatSper and SLO3 null mouse indicate that at pH_i 8 these two channels are the main contributors of cationic currents (Zeng et al. 2013). Under these conditions, the presence of a few Ca_Vs or other Ca^{2+}-permeable channels such as TRPs or SOCs in the sperm head plasma membrane cannot be discarded. It is worth pointing that SOCs display a 10 femtosiemens single channel conductance that would be difficult to discern within the pipette leak conductance (Dynes et al. 2015). This is even more difficult at physiologically relevant pH_i of ~7.2–7.4 of capacitated sperm (Visconti 2012), where CatSper and SLO3 are incompletely activated; thus, other currents could have a more important influence on sperm physiology (Zeng et al. 2013; Darszon and Hernández-Cruz 2014). Furthermore, since these cells have a high input impedance, a single channel might significantly contribute to their

resting potential. Additionally, as discussed elsewhere (i.e., Darszon and Hernández-Cruz 2014), whole cell patch clamp is ideal for small round cells, where good space clamp can be achieved, but cells displaying long processes such as the sperm flagella present space clamp problems.

The above considerations emphasize the importance of carrying out single channel recordings on the sperm head to examine the ion channels present. Only few attempts have been reported in this direction, as described earlier in sea urchin (Guerrero et al. 1987), mouse (Espinosa and Darszon 1995), and human sperm (Gu et al. 2004). These studies have detected cationic and anionic channels which must be further characterized to determine their possible role in the AR. Novel approaches involving total internal reflectance fluorescence (TIRF) microscopy are beginning to shed light regarding single channel organization in Ca_Vs (Nystoriak et al. 2013) and SOC channels (Dynes et al. 2015). These combined strategies are needed to determine which ion channels are indeed involved in the physiologically relevant AR. Certainly, the search for the physiological conditions that lead to the AR is being reevaluated as this knowledge is instrumental to identifying and understanding the channels and transporters that participate in this key reaction.

Other important matters to consider in future experiments are (1) the significant species-specific differences in ion channel properties that have emerged in recent years. Striking examples are the progesterone sensitivity of human CatSper as compared to that of mouse, and in the case of SLO3 the differential potency pH_i and Ca^{2+} have on this channel in human and mouse sperm (Lishko et al. 2011b). Since CatSper is a polymodal channel (Brenker et al. 2012) with a promiscuous pharmacology, species differences may confuse ligand and inhibitor effects and difficult channel identity assignment. Furthermore, as CatSper has been shown to influence sperm flagellar protein organization, its absence in null mice could alter other channels ensemble and function.

Acknowledgements We thank Shirley Ainsworth, Roberto Rodríguez, Arturo Ocádiz, Juan Manuel Hurtado, David Santiago, Paulina Torres-Rodríguez, and Jose Luis de la Vega-Beltrán for their excellent library, computer service, and technical assistance. This work was supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development grants (NIH RO1 HD38082 to Pablo Visconti); the Alexander von Humboldt Foundation (to CT); CONACyT-Mexico Fronteras de la Ciencia 71 to AD, CB, and CT; and DGAPA/UNAM (IN204112 to CB, IN203116 to CT and IN205516 to AD).

References

Allegrucci C, Liguori L, Minelli A (2001) Stimulation by N 6-cyclopentyladenosine of A1 adenosine receptors, coupled to G alpha i2 protein subunit, has a capacitative effect on human spermatozoa. Biol Reprod 64:1653–1659

Arcelay E, Salicioni AM, Wertheimer E, Visconti PE (2008) Identification of proteins undergoing tyrosine phosphorylation during mouse sperm capacitation. Int J Dev Biol 52:463–472

Arndt L, Castonguay J, Arlt E et al (2014) NAADP and the two-pore channel protein 1 participate in the acrosome reaction in mammalian spermatozoa. Mol Biol Cell 25:948–964

Arnoult C, Cardullo RA, Lemos JR, Florman HM (1996) Activation of mouse sperm T-type Ca2+ channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci U S A 93:13004–13009

Arnoult C, Kazam IG, Visconti PE et al (1999) Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc Natl Acad Sci U S A 96:6757–6762

Baker MA (2011) The "omics" revolution and our understanding of sperm cell biology. Asian J Androl 13(1):6–10

Banks FCL, Calvert RC, Burnstock G (2010) Changing P2X receptor localization on maturing sperm in the epididymides of mice, hamsters, rats, and humans: a preliminary study. Fertil Steril 93:1415–1420

Battistone M, Brukman N, Carvajal G et al (2015) From the epididymis to the egg: participation of CRISP proteins in mammalian fertilization. Asian J Androl 17(5):711–715

Blackmore PF (1999) Extragenomic actions of progesterone in human sperm and progesterone metabolites in human platelets. Steroids 64:149–156

Blackmore PF, Beebe SJ, Danforth DR, Alexander N (1990) Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. J Biol Chem 265:1376–1380

Bray C, Son JH, Kumar P et al (2002a) A role for the human sperm glycine receptor/Cl(-) channel in the acrosome reaction initiated by recombinant ZP3. Biol Reprod 66:91–97

Bray C, Son JH, Meizel S (2002b) A nicotinic acetylcholine receptor is involved in the acrosome reaction of human sperm initiated by recombinant human ZP3. Biol Reprod 67:782–788

Bray C, Son JH, Meizel S (2005) Acetylcholine causes an increase of intracellular calcium in human sperm. Mol Hum Reprod 11:881–889

Brenker C, Goodwin N, Weyand I et al (2012) The CatSper channel: a polymodal chemosensor in human sperm. EMBO J 31:1654–1665

Brewis IA, Clayton R, Barratt CL et al (1996) Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. Mol Hum Reprod 2: 583–589

Buffone MG, Hirohashi N, Gerton GL (2014) Unresolved questions concerning mammalian sperm acrosomal exocytosis. Biol Reprod 90(112):1–8

Calcraft PJ, Ruas M, Pan Z et al (2009) NAADP mobilizes calcium from acidic organelles through two-pore channels. Nature 459:596–600

Carlson AE, Westenbroek RE, Quill T et al (2003) CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm. Proc Natl Acad Sci U S A 100:14864–14868

Castillo Bennett J, Roggero CM, Mancifesta FE, Mayorga LS (2010) Calcineurin-mediated dephosphorylation of synaptotagmin VI is necessary for acrosomal exocytosis. J Biol Chem 285:26269–26278

Chávez JC, de la Vega-Beltrán JL, Escoffier J et al (2013) Ion permeabilities in mouse sperm reveal an external trigger for SLO3-dependent hyperpolarization. PLoS One 8:1–13

Chávez JC, Ferreira JJ, Butler A et al (2014) SLO3 K+ channels control calcium entry through CATSPER channels in sperm. J Biol Chem 289:32266–32275

Chung JJ, Navarro B, Krapivinsky G et al (2011) A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. Nat Commun 2(153):1–12

Chung J-J, Shim S-H, Everley RA et al (2014) Structurally distinct Ca2+ signaling domains of sperm flagella orchestrate tyrosine phosphorylation and motility. Cell 157:808–822

Cohen R, Buttke DE, Asano A et al (2014) Lipid modulation of calcium flux through CaV2.3 regulates acrosome exocytosis and fertilization. Dev Cell 28:310–321

Correia J, Michelangeli F, Publicover S (2015) Regulation and roles of Ca^{2+} stores in human sperm. Reproduction 150:R65–R76

Costello S, Michelangeli F, Nash K et al (2009) Ca2+-stores in sperm: their identities and functions. Reproduction 138:425–437

Dan JC (1952) Studies on the acrosome. I. Reaction to egg-water and other stimuli. Biol Bull 103: 54–66

Dan JC (1954) Studies on the acrosome. III. Effect of calcium deficiency. Biol Bull 107:335–349
Daniel L, Etkovitz N, Weiss SR et al (2010) Regulation of the sperm EGF receptor by ouabain leads to initiation of the acrosome reaction. Dev Biol 344:650–657
Darszon A, Hernández-Cruz A (2014) T-type Ca2+ channels in spermatogenic cells and sperm. Pflugers Arch 466:819–831
Darszon A, Labarca P, Nishigaki T, Espinosa F (1999) Ion channels in sperm physiology. Physiol Rev 79:481–510
Darszon A, Lopez-Martinez P, Acevedo JJ et al (2006) T-type Ca2+ channels in sperm function. Cell Calcium 40:241–252
Darszon A, Nishigaki T, Beltrán C, Treviño CL (2011) Calcium channels in the development, maturation, and function of spermatozoa. Physiol Rev 91:1305–1355
Darszon A, Sánchez-Cárdenas C, Orta G et al (2012) Are TRP channels involved in sperm development and function? Cell Tissue Res 349:749–764
De Blas G, Michaut M, Trevino CL et al (2002) The intraacrosomal calcium pool plays a direct role in acrosomal exocytosis. J Biol Chem 277:49326–49331
De Blas GA, Darszon A, Ocampo AY et al (2009) TRPM8, a versatile channel in human sperm. PLoS One 4, e6095
De La Vega-Beltran JL, Sanchez-Cardenas C, Krapf D et al (2012) Mouse sperm membrane potential hyperpolarization is necessary and sufficient to prepare sperm for the acrosome reaction. J Biol Chem 287:44384–44393
del Cantero MR, Velázquez IF, Streets AJ et al (2015) The cAMP signaling pathway and direct protein kinase a phosphorylation regulate polycystin-2 (TRPP2) channel function. J Biol Chem 290:23888–23896
Domino SE, Bocckino SB, Garbers DL (1989) Activation of phospholipase D by the fucose-sulfate glycoconjugate that induces an acrosome reaction in spermatozoa. J Biol Chem 264: 9412–9419
Dynes JL, Amcheslavsky A, Cahalan MD (2015) Genetically targeted single-channel optical recording reveals multiple Orai1 gating states and oscillations in calcium influx. Proc Natl Acad Sci U S A 13(2):440–445
El-Talatini MR, Taylor AH, Elson JC et al (2009) Localisation and function of the endocannabinoid system in the human ovary. PLoS One 4, e4579
Ernesto JI, Weigel Munoz M, Battistone MA et al (2015) CRISP1 as a novel CatSper regulator that modulates sperm motility and orientation during fertilization. J Cell Biol 210:1213–1224
Escoffier J, Boisseau S, Serres C et al (2007) Expression, localization and functions in acrosome reaction and sperm motility of Ca(V)3.1 and Ca(V)3.2 channels in sperm cells: an evaluation from Ca(V)3.1 and Ca(V)3.2 deficient mice. J Cell Physiol 212:753–763
Escoffier J, Navarrete F, Haddad D et al (2015) Flow cytometry analysis reveals that only a subpopulation of mouse sperm undergoes hyperpolarization during capacitation. Biol Prod 92:1–11
Espinosa F, Darszon A (1995) Mouse sperm membrane potential: changes induced by Ca2+. FEBS Lett 372:119–125
Espinosa F, de la Vega-Beltran JL, Lopez-Gonzalez I et al (1998) Mouse sperm patch-clamp recordings reveal single Cl- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction. FEBS Lett 426:47–51
Espinosa F, Lopez-Gonzalez I, Serrano CJ et al (1999) Anion channel blockers differentially affect T-type Ca(2+) currents of mouse spermatogenic cells, alpha1E currents expressed in Xenopus oocytes and the sperm acrosome reaction. Dev Genet 25:103–114
Florman HM, Corron ME, Kim TD, Babcock DF (1992) Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. Dev Biol 152:304–314
Florman HM, Jungnickel MK, Sutton KA (2008) Regulating the acrosome reaction. Int J Dev Biol 52(5–6):503–510

Foresta C, Rossato M, Di Virgilio F (1992) Extracellular ATP is a trigger for the acrosome reaction in human spermatozoa. J Biol Chem 267:19443–19447

Fujinoki M, Takei GL, Kon H (2015) Non-genomic regulation and disruption of spermatozoal in vitro hyperactivation by oviductal hormones. J Physiol Sci. Nov 5. [Epub ahead of print]

Garcia MA, Meizel S (1999) Determination of the steady-state intracellular chloride concentration in capacitated human spermatozoa. J Androl 20:88–93

Giamarchi A, Delmas P (2007) Activation mechanisms and functional roles of TRPP2 cation channels. In: Liedtke WB, Heller S (eds) TRP ion channel function sensory transduction and cellular signaling cascades, Chapter 14. CRC Press/Taylor & Francis, Boca Raton, FL

Gibbs GM, Orta G, Reddy T et al (2011) Cysteine-rich secretory protein 4 is an inhibitor of transient receptor potential M8 with a role in establishing sperm function. Proc Natl Acad Sci U S A 108:7034–7039

Gonzalez-Martinez MT, Galindo BE, de De La Torre L et al (2001) A sustained increase in intracellular Ca(2+) is required for the acrosome reaction in sea urchin sperm. Dev Biol 236: 220–229

Granados-Gonzalez G, Mendoza-Lujambio I, Rodriguez E et al (2005) Identification of voltage-dependent Ca2+ channels in sea urchin sperm. FEBS Lett 579:6667–6672

Gu Y, Kirkman-Brown JC, Korchev Y et al (2004) Multi-state, 4-aminopyridine-sensitive ion channels in human spermatozoa. Dev Biol 274:308–317

Guerrero A, Darszon A (1989a) Egg jelly triggers a calcium influx which inactivates and is inhibited by calmodulin antagonists in the sea urchin sperm. Biochim Biophys Acta 980: 109–116

Guerrero A, Darszon A (1989b) Evidence for the activation of two different Ca2+ channels during the egg jelly-induced acrosome reaction of sea urchin sperm. J Biol Chem 264:19593–19599

Guerrero A, Sanchez JA, Darszon A (1987) Single-channel activity in sea urchin sperm revealed by the patch-clamp technique. FEBS Lett 220:295–298

Gunaratne HJ, Moy GW, Kinukawa M et al (2007) The 10 sea urchin receptor for egg jelly proteins (SpREJ) are members of the polycystic kidney disease-1 (PKD1) family. BMC Genomics 8(235):1–19

He XB, Hu JH, Wu Q et al (2001) Identification of GABA(B) receptor in rat testis and sperm. Biochem Biophys Res Commun 283:243–247

Hernandez-Gonzalez EO (2005) Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyperpolarization in mouse sperm. J Biol Chem 281: 5623–5633

Hinsch KD, De Pinto V, Aires VA et al (2004) Voltage-dependent anion-selective channels VDAC2 and VDAC3 are abundant proteins in bovine outer dense fibers, a cytoskeletal component of the sperm flagellum. J Biol Chem 279:15281–15288

Hirohashi N, Vacquier VD (2003) Store-operated calcium channels trigger exocytosis of the sea urchin sperm acrosomal vesicle. Biochem Biophys Res Commun 304:285–292

Ho K, Wolff CA, Suarez SS (2009) CatSper-null mutant spermatozoa are unable to ascend beyond the oviductal reservoir. Reprod Fertil Dev 21:345–350

Hu JH, He XB, Wu Q et al (2002) Subunit composition and function of GABAA receptors of rat spermatozoa. Neurochem Res 27:195–199

Hu JH, Zhang JF, Ma YH et al (2004) Impaired reproduction in transgenic mice overexpressing Gamma-aminobutyric acid transporter I (GAT1). Cell Res 14:54–59

Hu M, Liu Y, Wu J, Liu X (2015) Influx-operated Ca2+ entry via PKD2-L1 and PKD1-L3 channels facilitates sensory responses to polymodal transient stimuli. Cell Rep 13:1–14

Jaldety Y, Glick Y, Orr-Urtreger A et al (2012) Sperm epidermal growth factor receptor (EGFR) mediates α7 acetylcholine receptor (AChR) activation to promote fertilization. J Biol Chem 287:22328–22340

Jin M, Fujiwara E, Kakiuchi Y et al (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci U S A 108:4892–4896

Jungnickel MK, Marrero H, Birnbaumer L et al (2001) Trp2 regulates entry of Ca2+ into mouse sperm triggered by egg ZP3. Nat Cell Biol 3:499–502
Kanbara K, Okamoto K, Nomura S et al (2010) The cellular expression of GABA(A) receptor alpha1 subunit during spermatogenesis in the mouse testis. Histol Histopathol 25:1229–1238
Kanbara K, Mori Y, Kubota T et al (2011) Expression of the GABAA receptor/chloride channel in murine spermatogenic cells. Histol Histopathol 26:95–106
Kazazoglou T, Schackmann RW, Fosset M, Shapiro BM (1985) Calcium channel antagonists inhibit the acrosome reaction and bind to plasma membranes of sea urchin sperm. Proc Natl Acad Sci U S A 82:1460–1464
Kierszenbaum AL (2004) Polycystins: what polycystic kidney disease tells us about sperm. Mol Reprod Dev 67:385–388
Kirichok Y, Navarro B, Clapham DE (2006) Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. Nature 439:737–740
Kumar P, Meizel S (2005) Nicotinic acetylcholine receptor subunits and associated proteins in human sperm. J Biol Chem 280:25928–25935
Kumar P, Meizel S (2008) Identification and spatial distribution of glycine receptor subunits in human sperm. Reproduction 136:387–390
Kuroda Y, Kaneko S, Yoshimura Y et al (1999) Influence of progesterone and GABAA receptor on calcium mobilization during human sperm acrosome reaction. Arch Androl 42:185–191
Kwon W-S, Park Y-J, Mohamed E-SA, Pang M-G (2013) Voltage-dependent anion channels are a key factor of male fertility. Fertil Steril 99:354–361
Laing RJ, Dhaka A (2015) ThermoTRPs and pain. Neuroscientist 1–17
Liévano A, Santi CM, Serrano CJ et al (1996) T-type Ca2+ channels and alpha1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. FEBS Lett 388:150–154
Linares-Hernandez L, Guzman-Grenfell AM, Hicks-Gomez JJ, Gonzalez-Martinez MT (1998) Voltage-dependent calcium influx in human sperm assessed by simultaneous optical detection of intracellular calcium and membrane potential. Biochim Biophys Acta 1372:1–12
Lishko PV, Kirichok Y (2010) The role of Hv1 and CatSper channels in sperm activation. J Physiol 588:4667–4672
Lishko PV, Botchkina IL, Fedorenko A, Kirichok Y (2010) Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. Cell 140:327–337
Lishko P, Kirichok Y, Ren D et al (2011a) The control of male fertility by spermatozoan ion channels. Annu Rev Physiol 2012 74:453–475, Published online 2011 Oct 13
Lishko PV, Botchkina IL, Kirichok Y (2011b) Progesterone activates the principal Ca2+ channel of human sperm. Nature 471:387–391
Liu J, Xia J, Cho KH et al (2007) CatSperbeta, a novel transmembrane protein in the CatSper channel complex. J Biol Chem 282:18945–18952
Liu B, Wang P, Wang Z, Zhang W (2011) The use of anti-VDAC2 antibody for the combined assessment of human sperm acrosome integrity and ionophore A23187-induced acrosome reaction. PLoS One 6, e16985
Lobley A, Pierron V, Reynolds L et al (2003) Identification of human and mouse CatSper3 and CatSper4 genes: characterisation of a common interaction domain and evidence for expression in testis. Reprod Biol Endocrinol 1(53):1–15
Lopez-Gonzalez I, Torres-Rodriguez P, Sanchez-Carranza O et al (2014) Membrane hyperpolarization during human sperm capacitation. Mol Hum Reprod 20:619–629
Lu J, Stewart AJ, Sadler PJ et al (2008) Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. Biochem Soc Trans 36:1317–1321
Luria A, Rubinstein S, Lax Y, Breitbart H (2002) Extracellular adenosine triphosphate stimulates acrosomal exocytosis in bovine spermatozoa via P2 purinoceptor. Biol Reprod 66:429–437
Ma YH, Hu JH, Zhou XG et al (2000) Gamma-aminobutyric acid transporter (GAT1) overexpression in mouse affects the testicular morphology. Cell Res 10:59–69

Magleby KL (2003) Gating mechanism of BK (Slo1) channels: so near, yet so far. J Gen Physiol 121:81–96
Martínez-López P, Santi CM, Treviño CL et al (2009) Mouse sperm K+ currents stimulated by pH and cAMP possibly coded by Slo3 channels. Biochem Biophys Res Commun 381:204–209
Martínez-López P, Treviño CL, de la Vega-Beltrán JL et al (2011) TRPM8 in mouse sperm detects temperature changes and may influence the acrosome reaction. J Cell Physiol 226:1620–1631
Mayorga LS, Tomes CN, Belmonte SA (2007) Acrosomal exocytosis, a special type of regulated secretion. IUBMB Life 59:286–292
Meizel S (2004) The sperm, a neuron with a tail: "neuronal" receptors in mammalian sperm. Biol Rev Camb Philos Soc 79:713–732
Meizel S, Son JH (2005) Studies of sperm from mutant mice suggesting that two neurotransmitter receptors are important to the zona pellucida-initiated acrosome reaction. Mol Reprod Dev 72:250–258
Meizel S, Turner KO (1993) Initiation of the human sperm acrosome reaction by thapsigargin. J Exp Zool 267:350–355
Melendrez CS, Meizel S (1995) Studies of porcine and human sperm suggesting a role for a sperm glycine receptor/Cl- channel in the zona pellucida-initiated acrosome reaction. Biol Reprod 53: 676–683
Miller MR, Mansell SA, Meyers SA, Lishko PV (2015) Flagellar ion channels of sperm: similarities and differences between species. Cell Calcium 58:105–113
Miller MR, Mannowetz N, Iavarone AT et al (2016) Unconventional endocannabinoid signaling governs sperm activation via sex hormone progesterone. Science. Mar 17. pii:aad6887 [Epub ahead of print]
Minelli A, Allegrucci C, Piomboni P et al (2000) Immunolocalization of A1 adenosine receptors in mammalian spermatozoa. J Histochem Cytochem 48:1163–1171
Minelli A, Liguori L, Bellaza I et al (2004) Involvement of A1 adenosine receptors in the acquisition of fertilizing capacity. J Androl 25:286–292
Minelli A, Bellezza I, Collodel G, Fredholm BB (2008) Promiscuous coupling and involvement of protein kinase C and extracellular signal-regulated kinase 1/2 in the adenosine A1 receptor signalling in mammalian spermatozoa. Biochem Pharmacol 75:931–941
Morris J, Jones S, Howl J et al (2015) Cell-penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced [Ca2+]i signal in human sperm. Mol Hum Reprod 21:563–570
Navarro B, Kirichok Y, Clapham DE (2007) KSper, a pH-sensitive K+ current that controls sperm membrane potential. Proc Natl Acad Sci U S A 104:7688–7692
Navarro B, Miki K, Clapham DE (2011) ATP-activated P2X2 current in mouse spermatozoa. Proc Natl Acad Sci U S A 108(34):14342–14347
Neill AT, Moy GW, Vacquier VD (2004) Polycystin-2 associates with the polycystin-1 homolog, suREJ3, and localizes to the acrosomal region of sea urchin spermatozoa. Mol Reprod Dev 67:472–477
Nishigaki T, José O, González-Cota AL et al (2014) Intracellular pH in sperm physiology. Biochem Biophys Res Commun 450:1149–1158
Nystoriak MA, Nieves-Cintrón M, Navedo MF (2013) Capturing single L-type Ca(2+) channel function with optics. Biochim Biophys Acta 1833:1657–1664
O'Toole CM, Arnoult C, Darszon A et al (2000) Ca(2+) entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol Biol Cell 11: 1571–1584
Okabe M (2014) Mechanism of fertilization: a modern view. Exp Anim 63:357–365
Orta G, Ferreira G, Jose O et al (2012) Human spermatozoa possess a calcium-dependent chloride channel that may participate in the acrosomal reaction. J Physiol 590:2659–2675
Patrat C, Serres C, Jouannet P (2000) Induction of a sodium ion influx by progesterone in human spermatozoa. Biol Reprod 62:1380–1386

Paulais M, Lachheb S, Teulon J (2006) A Na+- and Cl—activated K+ channel in the thick ascending limb of mouse kidney. J Gen Physiol 127:205–215
Pinto FM, Ravina CG, Fernández-Sánchez M et al (2009) Molecular and functional characterization of voltage-gated sodium channels in human sperm. Reprod Biol Endocrinol 7(71):1–9
Publicover S, Harper CV, Barratt C (2007) $[Ca^{2+}]_i$ signalling in sperm—making the most of what you've got. Nat Cell Biol 9(3):235–242
Qi H, Moran MM, Navarro B et al (2007) All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc Natl Acad Sci U S A 104:1219–1223
Quill TA, Ren D, Clapham DE, Garbers DL (2001) A voltage-gated ion channel expressed specifically in spermatozoa. Proc Natl Acad Sci U S A 98:12527–12531
Ren D, Navarro B, Perez G et al (2001) A sperm ion channel required for sperm motility and male fertility. Nature 413:603–609
Rodriguez E, Darszon A (2003) Intracellular sodium changes during the speract response and the acrosome reaction in sea urchin sperm. J Physiol 546:89–100
Roldan ER, Murase T, Shi QX (1994) Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science 266(5190):1578–1581
Rossato M, Di Virgilio F, Foresta C (1996) Involvement of osmo-sensitive calcium influx in human sperm activation. Mol Hum Reprod 2:903–909
Sakata Y, Saegusa H, Zong S et al (2001) Analysis of Ca2+ currents in spermatocytes from mice lacking Cav2.3 (α1E) Ca2+ channel. Biochem Biophys Res Commun 288:1032–1036
Sakata Y, Saegusa H, Zong S et al (2002) Ca(v)2.3 (alpha1E) Ca2+ channel participates in the control of sperm function. FEBS Lett 516:229–233
Sánchez-Carranza O, Torres-Rodríguez P, Darszon A et al (2015) Pharmacology of hSlo3 channels and their contribution in the capacitation-associated hyperpolarization of human sperm. Biochem Biophys Res Commun 466:554–559
Sánchez-Tusie AA, Vasudevan SR, Churchill GC et al (2014) Characterization of NAADP-mediated calcium signaling in human spermatozoa. Biochem Biophys Res Commun 443: 531–536
Santi CM, Martinez-Lopez P, de la Vega-Beltran JL et al (2010) The SLO3 sperm-specific potassium channel plays a vital role in male fertility. FEBS Lett 584:1041–1046
Sato Y, Son JH, Meizel S (2000) The mouse sperm glycine receptor/chloride channel: cellular localization and involvement in the acrosome reaction initiated by glycine. J Androl 21:99–106
Sato Y, Tucker RP, Meizel S (2002) Detection of glycine receptor/Cl- channel beta subunit transcripts in mouse testis. Zygote 10:105–108
Schackmann RW, Eddy EM, Shapiro BM (1978) The acrosome reaction of strongylocentrotus purpuratus sperm. Ion requirements and movements. Dev Biol 65:483–495
Schackmann RW, Christen R, Shapiro BM (1981) Membrane potential depolarization and increased intracellular pH accompany the acrosome reaction of sea urchin sperm. Proc Natl Acad Sci U S A 78:6066–6070
Schreiber M, Wei A, Yuan A et al (1998) Slo3, a novel pH-sensitive K+ channel from mammalian spermatocytes. J Biol Chem 273:3509–3516
Schuel H, Burkman LJ (2005) A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. Biol Reprod 73:1078–1086
Shi QX, Yuan YY, Roldan ER (1997) Gamma-aminobutyric acid (GABA) induces the acrosome reaction in human spermatozoa. Mol Hum Reprod 3:677–683
Sieghart W (1992) GABAA receptors: ligand-gated Cl- ion channels modulated by multiple drug-binding sites. Trends Pharmacol Sci 13:446–450
Son JH, Meizel S (2003) Evidence suggesting that the mouse sperm acrosome reaction initiated by the zona pellucida involves an alpha7 nicotinic acetylcholine receptor. Biol Reprod 68: 1348–1353
Stamboulian S, Kim D, Shin HS et al (2004) Biophysical and pharmacological characterization of spermatogenic T-type calcium current in mice lacking the CaV3.1 (alpha1G) calcium channel:

CaV3.2 (alpha1H) is the main functional calcium channel in wild-type spermatogenic cells. J Cell Physiol 200:116–124
Stamboulian S, Moutin MJ, Treves S et al (2005) Junctate, an inositol 1,4,5-triphosphate receptor associated protein, is present in rodent sperm and binds TRPC2 and TRPC5 but not TRPC1 channels. Dev Biol 286:326–337
Stein RJ, Santos S, Nagatomi J et al (2004) Cool (TRPM8) and hot (TRPV1) receptors in the bladder and male genital tract. J Urol 172:1175–1178
Strunker T, Goodwin N, Brenker C et al (2011) The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature 471:382–386
Suhaiman L, De Blas GA, Obeid LM et al (2010) Sphingosine 1-phosphate and sphingosine kinase are involved in a novel signaling pathway leading to acrosomal exocytosis. J Biol Chem 285: 16302–16314
Sutton KA, Jungnickel MK, Florman HM (2008) A polycystin-1 controls postcopulatory reproductive selection in mice. Proc Natl Acad Sci U S A 105:8661–8666
Tang Q-Y, Zhang Z, Xia X-M, Lingle CJ (2010) Block of mouse Slo1 and Slo3 K+ channels by CTX, IbTX, TEA, 4-AP and quinidine. Channels (Austin) 4:22–41
Tateno H, Krapf D, Hino T et al (2013) Ca2+ ionophore A23187 can make mouse spermatozoa capable of fertilizing in vitro without activation of cAMP-dependent phosphorylation pathways. Proc Natl Acad Sci U S A 110:18543–18548
Thinnes FP (2015) Biochimica et biophysica acta phosphorylation, nitrosation and plasminogen K3 modulation make VDAC-1 lucid as part of the extrinsic apoptotic pathway—resulting thesis: native VDAC-1 indispensable for finalisation of its 3D structure. BBA Biomembr 1848: 1410–1416
Thomas P, Meizel S (1989) Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx. Biochem J 264: 539–546
Tomes CN, McMaster CR, Saling PM (1996) Activation of mouse sperm phosphatidylinositol-4,5 bisphosphate-phospholipase C by zona pellucida is modulated by tyrosine phosphorylation. Mol Reprod Dev 43:196–204
Torres-Fuentes JL, Rios M, Moreno RD (2015) Involvement of a P2X7 receptor in the acrosome reaction induced by ATP in rat spermatozoa. J Cell Physiol 230(12):3068–3075
Trevino CL, Serrano CJ, Beltran C et al (2001) Identification of mouse trp homologs and lipid rafts from spermatogenic cells and sperm. FEBS Lett 509:119–125
Trevino CL, Felix R, Castellano LE et al (2004) Expression and differential cell distribution of low-threshold Ca(2+) channels in mammalian male germ cells and sperm. FEBS Lett 563: 87–92
Triphan X, Menzel VA, Petrunkina AM et al (2008) Localisation and function of voltage-dependent anion channels (VDAC) in bovine spermatozoa. Pflugers Arch 455:677–686
Vacquier VD (2012) The quest for the sea urchin egg receptor for sperm. Biochem Biophys Res Commun 425:583–587
Vacquier VD, Swanson WJ (2011) Selection in the rapid evolution of gamete recognition proteins in marine invertebrates. Cold Spring Harb Perspect Biol 3:1–17
Vacquier VD, Loza-Huerta A, Garcia-Rincon J et al (2014) Soluble adenylyl cyclase of sea urchin spermatozoa. Biochim Biophys Acta 1842:2621–2628
Vilela-Silva AC, Hirohashi N, Mourao PA (2008) The structure of sulfated polysaccharides ensures a carbohydrate-based mechanism for species recognition during sea urchin fertilization. Int J Dev Biol 52:551–559
Visconti PE (2012) Sperm bioenergetics in a nutshell. Biol Reprod 87:72
Walensky LD, Snyder SH (1995) Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. J Cell Biol 130:857–869
Wang H, Liu J, Cho KH, Ren D (2009) A novel, single, transmembrane protein CATSPERG is associated with CATSPER1 channel protein. Biol Reprod 81:539–544

Wennemuth G, Westenbroek RE, Xu T et al (2000) CaV2.2 and CaV2.3 (N- and R-type) Ca2+ channels in depolarization-evoked entry of Ca2+ into mouse sperm. J Biol Chem 275: 21210–21217

Wissenbach U, Schroth G, Philipp S, Flockerzi V (1998) Structure and mRNA expression of a bovine trp homologue related to mammalian trp2 transcripts. FEBS Lett 429:61–66

Wistrom CA, Meizel S (1993) Evidence suggesting involvement of a unique human sperm steroid receptor/Cl- channel complex in the progesterone-initiated acrosome reaction. Dev Biol 159: 679–690

Wrighton DC, Muench SP, Lippiat JD (2015) Mechanism of inhibition of mouse Slo3 (K Ca 5. 1) potassium channels by quinine, quinidine and barium. Br J Pharmacol 3:4355–4363

Xia J, Ren D (2009) Egg coat proteins activate calcium entry into mouse sperm via CATSPER channels. Biol Reprod 80:1092–1098

Xia J, Reigada D, Mitchell CH, Ren D (2007) CATSPER channel-mediated Ca2+ entry into mouse sperm triggers a tail-to-head propagation. Biol Reprod 77:551–559

Xu H, Ren D (2015) Lysosomal physiology. Annu Rev Physiol 77:57–80

Yanagimachi R (1994) Mammalian Fertilization. In: Knobile E, Neill JD (eds) The physiology of reproduction, Second Edition. Raven, New York, pp 189–317

Yuan A, Santi CM, Wei A et al (2003) The sodium-activated potassium channel is encoded by a member of the Slo gene family. Neuron 37:765–773

Zanetti N, Mayorga LS (2009) Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. Biol Reprod 81:396–405

Zeng XH, Yang C, Kim ST et al (2011) Deletion of the Slo3 gene abolishes alkalization-activated K+ current in mouse spermatozoa. Proc Natl Acad Sci U S A 108:5879–5884

Zeng X-H, Navarro B, Xia X-M et al (2013) Simultaneous knockout of Slo3 and CatSper1 abolishes all alkalization- and voltage-activated current in mouse spermatozoa. J Gen Physiol 142:305–313

Zeng X, Yang C, Xia X et al (2015) SLO3 auxiliary subunit LRRC52 controls gating of sperm KSPER currents and is critical for normal fertility. Proc Natl Acad Sci U S A 112(8): 2599–2604

Zhang L, Barritt GJ (2006) TRPM8 in prostate cancer cells: a potential diagnostic and prognostic marker with a secretory function? Endocr Relat Cancer 13:27–38

Zhu MX, Ma J, Parrington J et al (2010) Calcium signaling via two-pore channels: local or global, that is the question. Am J Physiol Cell Physiol 298:C430–C441

Chapter 4
The Molecules of Sperm Exocytosis

Silvia A. Belmonte, Luis S. Mayorga, and Claudia N. Tomes

4.1 Introduction

During fertilization in eutherian mammals, the spermatozoon must penetrate the cumulus oophorus and the zona pellucida to reach and fuse with the oolemma. Only hyperactivated sperm that have completed the AR can successfully accomplish this task. Therefore, the AR is an essential secretory process that must occur at the appropriate time and location for a productive sperm–egg interaction. The AR relies on the same highly conserved molecules that drive intracellular membrane fusion and exocytosis in all other cells [see (Tomes 2015) for a recent review]. In this chapter, we will describe how sperm prepare for and accomplish bilayer mixing and will provide a perspective on how does the AR compare to regulated exocytosis undergone by other cells.

Different cell types carry secretory granules, such as neuronal, neuroendocrine, endocrine, and exocrine cells. Regulated exocytosis varies among these cells in terms of cargo, kinetics, probability, and modality of release. Secretory organelles are heterogeneous in size, ranging from 50 nm in synaptic vesicles in neurons to several 100 nm in lytic granules in hematopoietic cells. The secreted material is also very different; synaptic vesicles transport primarily small molecular weight neurotransmitters whereas granules characterized by an electron-dense core contain larger neuropeptides, hormones, heparin, enzymes, mucin, surfactants, and other complex materials [see (Shitara and Weigert 2015; Masedunskas et al. 2012; Porat-Shliom et al. 2013) for recent reviews]. The acrosome is a very large electron-dense granule covering about 50 % of the nucleus in human sperm (Florman and

S.A. Belmonte • L.S. Mayorga • C.N. Tomes (✉)
Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología, IHEM-CONICET, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, 5500 Mendoza, Mendoza, Argentina
e-mail: ctomes@fcm.uncu.edu.ar

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_4

Ducibella 2005). Its secretion does not quite fit into any of the known modes of release (full collapse, kiss and run, or compound exocytosis). Rather, the acrosomal and sperm's plasma membranes mix through a unique membrane fusion mode.

Exocytosis of secretory vesicles causes the release of granules' contents into the extracellular medium and the incorporation of the granules' membranes into the plasma membrane (full collapse). In some instances, the vesicle membrane is not incorporated into the plasma membrane after the opening of the fusion pore; instead, the fusion pore reseals after the release of a quantum of the secretory content (kiss and run) (Rizzoli and Jahn 2007). Finally, in some cell types such as mast cells, secretory granules fuse among them and with the plasma membrane (compound exocytosis). In some exocytic events what is important is not so much the release of cargo to the extracellular domain but the incorporation of membrane proteins transported by trafficking vesicles into the plasma membrane. For example, fusion of vesicles carrying the glucose transporter GLUT4 with the plasma membrane is the mechanism by which insulin facilitates glucose incorporation into muscle cells and adipocytes (Govers 2014). During sperm exocytosis, both the release of the acrosomal contents and changes in the plasma membrane are important. Soluble hydrolytic enzymes interact with an insoluble glycoprotein matrix inside the acrosome; they are both released at fertilization (Kim et al. 2001). In some species, acrosomal hydrolytic enzymes are released on the zona pellucida [see for instance (Sumigama et al. 2015)]. It was initially proposed that their function was to carve a path across it for fertilizing sperm although this notion has been challenged later (Bedford 2004). Moreover, we have learnt recently that previously reacted sperm can sort the zona barrier and fertilize eggs in mice (Hirohashi et al. 2011; Jin et al. 2011; Inoue et al. 2011). This does not mean that released acrosomal contents have no function in facilitating sperm–egg encounters. It has been suggested, for instance, that the AR exposes proteins that stabilize the contact between acrosome-reacted sperm and the zona pellucida. Perhaps as important for sperm physiology as acrosomal content release is the peculiar way in which acrosomal exocytosis modifies the topology and composition of the cell membrane. This is accomplished through a remarkable process in which multiple fusion pores open connecting the outer acrosomal membrane and the overlying plasma membrane. Joining of pores generates hybrid plasma–outer acrosomal membrane vesicles, which are shed, together with parts of the cytosol and the acrosomal contents. Thus, unlike in full collapse exocytosis, only a portion of the acrosomal membrane is incorporated into the plasma membrane following membrane fusion. As a consequence of the AR, the inner acrosomal membrane joins the remaining plasma membrane and becomes the leading cell membrane. After this dramatic reorganization, the topology and profile of the apical part of the sperm head change to expose membrane proteins that are essential for sperm–egg interaction and fusion (Okabe 2013). It has been suggested that this remodeling would favor the penetration of the zona assisted by physical thrust caused by vigorous flagellar beat (Bedford 1998). The concept that only reacted sperm are able to fuse with the oolemma has not yet been challenged; therefore, the AR continues to be a central

event during mammalian fertilization. These and other topological aspects of the AR will be discussed later in the chapter.

4.2 Delivery of Impermeant Probes into Sperm

During the last couple of decades, significant advances were made in our understanding of secretion biology. In contrast, the molecular basis of the AR remained comparatively poorly studied until not too long ago. This knowledge deficit was due in part to the fact that sperm are unable to synthesize proteins, complicating the use of standard molecular biological approaches—such as gene overexpression or silencing—widely used to study regulated secretion in other cells. The possibility of studying the role of macromolecules, i.e., the fusion proteins Rabs, SNAREs, Muncs, charged lipids, etc., is severely limited because such molecules are unable to cross the cell membrane. To overcome this limitation, we have applied in sperm a widespread methodology in the study of exocytosis in other cells, that of controlled plasma membrane permeabilization with streptolysin O (SLO) (Yunes et al. 2000; Hutt et al. 2005; Hernandez-Gonzalez et al. 2000; Kitamura et al. 2003; Ackermann et al. 2008; Hu et al. 2010) and perfringolysin O (Pocognoni et al. 2013). The permeable sperm preparations we have established are readily manipulated and reflective of the in vivo organization of the cell and allow us to measure a functional, calcium-regulated AR that is morphologically indistinguishable from that in non-permeabilized cells. Moreover, the protocol allows to isolate pathways that would take place after the sustained influx of calcium from the extracellular milieu in non-permeabilized cells from those that happen before the opening of calcium channels in the plasma membrane. Experiments with this assay and several auxiliary techniques have generated a large body of evidence that gave origin to our current working models (simplified) for acrosomal exocytosis [cartoons in Figs. 4.1 and 4.2, and 3 in Tomes (2015)].

As stated above, permeabilized sperm are useful to scrutinize events that take place after the opening of store-operated calcium channels in the plasma membrane of non-permeabilized cells but not to investigate earlier events or processes involving the regulation of gradients across the plasma membrane. Or to analyze the AR elicited by progesterone, perhaps the most studied trigger. Therefore, we became interested in developing tools to characterize the molecules of exocytosis in non-permeabilized cells. Needless to say, pharmacological approaches have been extensively applied to study the AR in intact cells for many years. But pharmacology has serious limitations when it comes to investigating membrane fusion mechanisms because there are no reliable tools available. More recently, experiments conducted in sperm from genetically modified animals have also advanced our understanding of the fertilization process (Okabe 2014). As powerful as it is, this technology also has its shortcomings. In what specifically pertains to the study of the AR, genetic models may perturb the genesis of the acrosome or simply allow time for compensatory mechanisms within the endogenous fusion machinery. Thus,

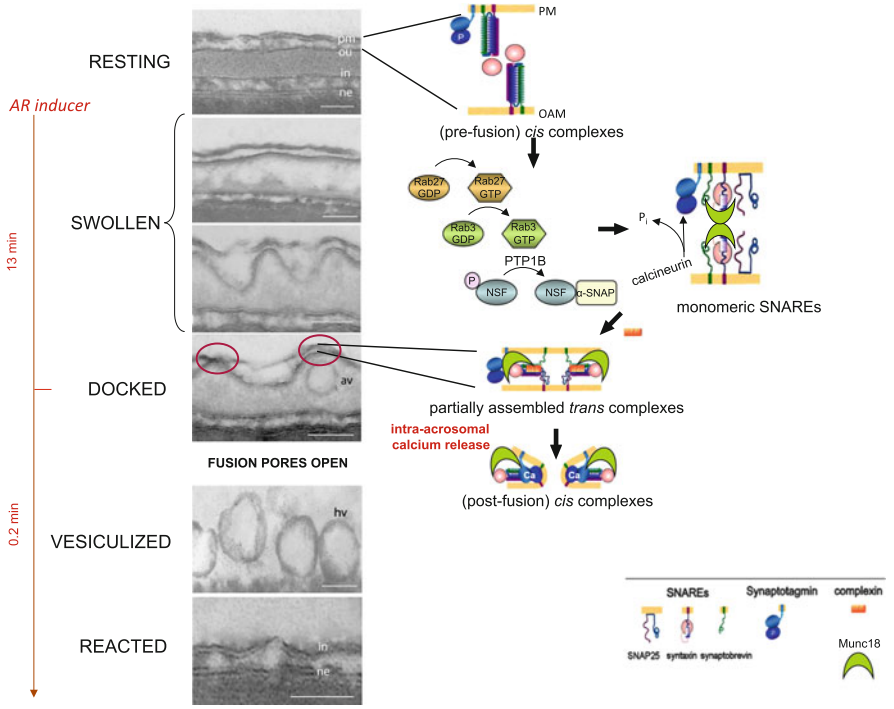

Fig. 4.1 Molecular correlates for the morphological stages of the AR. **RESTING**, the acrosome is flat. SNAREs, synaptotagmin, and NSF (not shown) are inactive, the former engaged in *cis* complexes on both outer acrosomal (OAM) and plasma membranes (PM) and the latter phosphorylated on threonine and tyrosine, respectively. **SWOLLEN**, exposure to AR inducers elicits the swelling of the acrosome. Rab27 exchanges GDP for GTP; subsequently, Rab27-GTP promotes the activation of Rab3. Next, calcineurin dephosphorylates synaptotagmin and PTP1B dephosphorylates NSF. Once dephosphorylated, NSF together with α-SNAP disassembles *cis* SNARE complexes. Munc18 binds monomeric syntaxin, keeping it temporarily in a closed configuration. **DOCKED**, the swollen acrosome docks to the plasma membrane at multiple points (*red ovals*). Munc18 and complexin help SNAREs to initiate their assembly in *trans* complexes. Munc18 reaccommodates on the SNARE complex. It takes about 13 min for 50 % of reacting sperm to reach docking from the moment sperm are stimulated. **FUSION PORES OPEN** (not captured by electron microscopy yet), calcium is released from the acrosome through IP_3-sensitive channels; this calcium binds synaptotagmin. Afterwards, a series of sub-reactions that include displacement of complexin and full zippering of the SNARE complex promote the opening of fusion pores. SNAREs are now on a single (fused) membrane and therefore in a *cis* configuration. **VESICULIZED**, portions of plasma and outer acrosomal membranes (those between the red ovals in the **DOCKED** picture) detach from the sperm head as hybrid vesicles (hv). Post-fusion *cis* SNARE complexes remain trapped inside the vesicles. Rab3 must hydrolyze GTP to reach this state (not shown). 50 % of the docked acrosomes become vesiculated in only 0.2 min. **REACTED**, hybrid vesicles, acrosomal contents, and cytosol are shed. The inner acrosomal membrane (in) is now the leading cell membrane. A time *arrow* is shown on the *left*. *pm*/*PM* plasma membrane, *ou*/*OAM* outer acrosomal membrane, *in* inner acrosomal membrane, *ne* nuclear envelope, *av* intra-acrosomal vesicle, *hv* hybrid vesicle. Electron micrographs are taken from (Zanetti and Mayorga 2009). The model was modified from (Roggero et al. 2007) and the drawings from (Sollner 2003)

short incubations to deliver recombinant proteins into sperm represent a technical advantage because the results obtained are basically a straightforward readout for the role of the introduced proteins in exocytosis. Both microinjection and patch clamp are used in somatic cells to deliver proteins; neither technology has been reported to work in sperm.

The discovery of peptides able to cross cellular membranes launched a novel field in molecular delivery based on these noninvasive vectors, most commonly called "cell-penetrating peptides" or "protein transduction domains." These peptides efficiently transport biologically active molecules inside living cells, and thus there has been a recent explosion in the literature regarding the use of these peptides in medicine (Bechara and Sagan 2013). Typically, these peptides are rich in basic residues as is the one identified within the sequence of the TAT protein from AIDS virus (Joliot and Prochiantz 2004). Our laboratories have pioneered the application of this technique by delivering first a permeant version of recombinant Rab3A and more recently of VPS4A to live human sperm. By means of this strategy, we have been able to establish the relationship between these proteins and the exocytosis mechanism initiated by progesterone (Lopez et al. 2007) and A23187 (Pocognoni et al. 2015). Dr Publicover and coworkers have published a study screening the incorporation of several cell-penetrating peptides into sperm (Jones et al. 2013) and more recently another one analyzing calcium-related responses in human sperm challenged with progesterone (Morris et al. 2015). In these four papers, CPPs were attached to their cargo proteins either after or during peptide synthesis. To make this application more versatile, we have recently designed a plasmid for bacterial expression that contains DNA sequences encoding a purification/detection tag (His_6) and the TAT peptide upstream a polylinker for subcloning the gene of interest. By means of this technology, we have been able to deliver a permeable version of a cAMP sequestering reagent [cAMP sponge (Lefkimmiatis et al. 2009)] into live sperm. This tool allowed us to unveil complex cAMP-related signaling pathways that operate in the AR induced by progesterone (Lucchesi et al. 2016). We are currently conducting experiments with membrane-permeant versions of Rab27A and the light chain of tetanus toxin and have available permeable versions of α-SNAP, α-synuclein, and the catalytic domain of PTP1B.

4.3 Somatic Cell vs Sperm Exocytosis: General Features

Most cells contain a large number of secretory vesicles. They may be fully or partially releasable in response to extracellular signals. The sequential stages within the exocytotic pathway include physical movement of vesicles to the subplasmalemmal region, tethering and then docking at release sites on the plasma membrane, opening of fusion pores, release of granule contents, and, depending on the cell type, retrieval of the granule membrane by endocytosis (Burgoyne and Morgan 2003). The AR is also a multistage process, in this case driven by unidirectional cascades that consist of the activation of multiple signaling pathways, and

the opening of ion—mainly calcium—channels. In human sperm, the acrosome changes its morphology upon activation. The granule swells and the outer acrosomal membrane forms inward invaginations; the protruding edges of these invaginations contact the plasma membrane (Zanetti and Mayorga 2009; Sosa et al. 2015). The opening and expansion of fusion pores at the contact points result in the formation of hybrid vesicles, which, together with the acrosomal contents, are released to complete exocytosis (transmission electron micrographs in Fig. 4.1).

A brief comparison between the AR and other exocytoses is offered next: (i) In some cells, the same substances are secreted again and again, requiring multiple rounds of fusion such that, in addition to generating new granules, both membranes and fusion machinery recycle several times. Sperm contain one preformed granule, without biogenesis of new ones. Sperm exocytosis is thus a singular event; post-exocytosis membrane recycling and/or endocytosis do not exist in these cells. As such, all components of the fusion machinery can only exhibit pre-fusion roles. (ii) Most exocytotic cells house numerous (sometimes thousands) secretory vesicles, often divided into functionally different pools. In contrast, each sperm contains a single secretory granule. (iii) In many exocytotic cells, a pool of granules is situated very close to or in direct contact with the plasma membrane even before the application of a stimulus; these granules are defined as docked. Granules in this pool are believed to release their contents faster than others upon calcium influx. In contrast, all acrosomes (in all sperm cells) are undocked before the application of an exocytosis stimulus. In other words, docking does not preexist but is secondary to the challenge of the cells [(Zanetti and Mayorga 2009; Rodriguez et al. 2011, 2012) but see (Tsai et al. 2010)].

4.3.1 Kinetics of the Acrosome Reaction

Most of what is known in exocytosis comes from the abundant bibliography on secretion in neuroendocrine cells. Small vesicles and their fast fusion with the plasma membrane characterize exocytosis in these cells. Much less is known about secretion of the large granules harbored in exocrine cells, such as pneumocytes and acinar and salivary cells. There is a striking correlation between granule size and secretion kinetics (Porat-Shliom et al. 2013). The AR is not an exception to this rule: the large acrosomal granule exhibits slow (minutes) kinetics of release. The end point of most studies of AR is the staining of cells at different times after stimulation to calculate the percentage of reacted sperm. This experimental setting measures three time components: (i) that required for stimulated sperm to activate the molecular machinery of membrane fusion, (ii) the actual opening of fusion pores, and (iii) the expansion of fusion pores to release the acrosomal contents. It also includes the probability of each cell to respond to the stimulus received. Our rationale for adding a probability parameter to the interpretation of kinetics data comes from the consistent observation that not all sperm react at the same time (Harper et al. 2008; Sanchez-Cardenas et al. 2014; Sosa et al. 2015; Zoppino et al. 2012). If the speed of release were solely

a consequence of slow events required to accomplish exocytosis, then all sperm would react synchronously after a lag period of time. This is not the case and that is particularly evident in human sperm. Only a small percentage of these cells respond to physiological and pharmacological inducers within the first few minutes. Over time, the number of cells recruited to the reacted state increases following roughly a first-order kinetics. A lag period is not observed, which indicates that some sperm undergo secretion faster than others. The reason for this asynchrony is not understood, although it is licit to speculate that it may be related to different capacitation stages. In other words, some sperm may be fully capacitated and are activated as soon as they are stimulated, whereas others require some time to achieve full capacitation and respond to the stimulus. This concept has some caveats. The asynchrony now should be during capacitation, which in itself is not a well-defined process. Moreover, the asynchrony is observed in sperm capacitated for different periods of time (Sosa and Mayorga, unpublished observations). We suggest that capacitation and the AR are asynchronic because some stochastic events are involved in both processes.

We have recently conducted a study aimed at defining the rate-limiting step for acrosomal exocytosis. The kinetics of swelling is similar to that of acrosomal loss. Hence, we propose that swelling is the rate-limiting step for secretion (Sosa et al. 2015). Moreover, not all acrosomes swell at the same time, nor there is a clear lag period for this process, which indicates that swelling is in itself a stochastic event in stimulated sperm.

4.4 Molecular Correlates of Exocytotic Stages

We have reviewed the molecular mechanisms that drive human sperm exocytosis with focus on the role and point of action of members of the fusion machinery before (Mayorga et al. 2007; Tomes 2007, 2015). The review from earlier this year summarizes what was known about the role of each fusion factor in exocytosis at the time it was written and provides numerous references to reviews and primary literature. Thus, we take the liberty to refer interested readers to these publications for detailed information instead of revisiting these topics here. In the next section, we will focus on correlating molecular mechanisms with exocytotic stages defined morphologically, kinetically, biochemically, and/or functionally.

4.4.1 Resting Human Sperm

In unstimulated sperm, the acrosome has an electron-dense content and a flat outer acrosomal membrane evenly (about 18 nm) separated from the plasma membrane (Fig. 4.1). The fact that the membranes do not interact suggests that the fusion machinery is inactive. SNAREs constitute the minimal membrane fusion machinery (Rizo and Sudhof 2012; Jahn and Fasshauer 2012). Syntaxin1, SNAP-25, and

synaptobrevin2 are the synaptic isoforms of the SNARE superfamily. Based on the identity of highly conserved residues, syntaxins and SNAPs are classified as Q-SNAREs (glutamine-containing SNAREs), whereas synaptobrevins are R-SNAREs (arginine-containing SNAREs) (Fasshauer et al. 1998). The Q- and R-SNAREs join into tight four-helix bundles during all intracellular fusion processes; R-SNAREs contribute one and Q-SNAREs three helices to these complexes. Isoforms of all SNARE families involved in regulated exocytosis in certain cells serve as targets of clostridial neurotoxins. Tetanus toxin (TeTx) and seven botulinum neurotoxins (BoNT/A, B, C1, D, E, F, and G) are highly specific zinc-dependent proteases that cleave members of the SNARE superfamily only when not packed in tight heterotrimeric complexes (Hayashi et al. 1994). Whatever the steady-state configuration of SNAREs in neuroendocrine cells might be, exocytosis is blocked by neurotoxins, suggesting that SNAREs go through toxin-sensitive stages (Xu et al. 1998; Chen et al. 1999). In contrast, both R- and Q-SNAREs are stably protected from toxin cleavage in resting sperm on both plasma and outer acrosomal membranes (De Blas et al. 2005). This is why we assign a *cis* configuration to the SNARE complex in unstimulated sperm (top cartoon in Fig. 4.1).

During membrane fusion, Rab proteins direct the recognition and physical attachments of the compartments that are going to fuse (Barr 2013; Stenmark 2009). Rab3 and Rab27 constitute the two main Rab subfamilies directly implicated in regulated exocytosis. These "secretory Rabs" localize to vesicles and secretory granules in a variety of secretory cell types (Burgoyne and Morgan 2003; Tolmachova et al. 2004; Fukuda 2008; Graham et al. 2008; Pavlos et al. 2010). They control the recruitment and attachment of secretory vesicles to the plasma membrane through interaction with effectors (Tsuboi and Fukuda 2005). Sperm Rabs 27 and 3 do not cycle constantly between their inactive, GDP-bound and active, GTP-bound forms as described for Rab families that drive intracellular membrane traffic; instead, their activation state and localization are coupled to exocytotic stimuli (Bustos et al. 2012). Thus, a high percentage of resting sperm contains these Rabs in their GDP-bound, inactive conformation.

Synaptotagmins detect the concentration of calcium by binding it and thereafter signaling to other targets via conformational changes. Synaptotagmins also bind proteins and membrane phospholipids (Pang and Sudhof 2010; Jahn and Fasshauer 2012; Chapman 2008; Park et al. 2015; Seven et al. 2013). Synaptotagmin and the ATPase NSF are inactive in resting sperm. Their activities are repressed by threonine (synaptotagmin) (Roggero et al. 2005) and tyrosine (NSF) (Zarelli et al. 2009) phosphorylation.

4.4.2 *Stimulated Sperm: Morphological Aspects of the Early Stages*

Because of its size and shape, the acrosome cannot dislodge from its position to reach the plasma membrane when sperm are challenged with exocytosis inducers.

The solution nature found to this problem is that at an early stage during the AR, the acrosomal contents swell, pushing the outer acrosomal membrane toward the plasma membrane (Fig. 4.1, left). Swelling is caused by physiological and pharmacological AR triggers, such as progesterone (Lucchesi et al. 2016; Sosa et al. 2016) and the calcium ionophore A23187 (Zanetti and Mayorga 2009; Sosa et al. 2015). Little is known about the molecular mechanisms that drive swelling other than it is blocked by KH7, an inhibitor of the soluble adenylyl cyclase, and by the cAMP sponge (Lucchesi et al. 2016). Likewise, cAMP analogs induce swelling by themselves (Sosa et al. 2016). In contrast to cAMP blockers, AR inhibitors such as recombinant complexin II, Rab-GDIα, neurotoxins, and reagents that interfere with intracellular calcium mobilization or phospholipase C(PLC) activity (Zanetti and Mayorga 2009) or with the proteinaceous fusion machinery described later (Bello et al. 2012; Rodriguez et al. 2011, 2012) do not impair swelling, which indicates that their targets are situated downstream of this event in the exocytotic cascade.

Swelling is accompanied by inward deformations of the outer acrosomal membrane, which limits its contacts with the plasma membrane to the edges of the invaginations, and by the appearance of intra-acrosomal vesicles. The latter exhibit a topology that resembles the luminal vesicles observed in the endocytic pathway in endosomes called multivesicular bodies (Scott et al. 2014). The mechanism of deformation of the endosomal membrane in these bodies has been characterized by other authors and involves ESCRT complexes (McCullough et al. 2013). A role for ESCRT has been proposed in mouse acrosome biogenesis (Berruti et al. 2010). We tested whether these complexes are involved in acrosomal exocytosis. Perturbing the function of TSG101, CHAMP4 (belonging to ESCRT complexes I and III, respectively) or VPS4 (the ATPase required for disassembling ESCRT complexes) blocks secretion in permeabilized human spermatozoa. The same effect was observed using membrane-permeant VPS4A in intact sperm (Pocognoni et al. 2015). We propose that ESCRT complexes are recruited to the bent areas of the outer acrosomal membrane invaginations, from where they promote the organization of microdomains that are necessary for interaction with complementary sites on the plasma membrane.

4.4.3 Stimulated Sperm: The Fusion Machinery in Preparation for Docking

Rab27 and Rab3A are activated (exchange GDP for GTP) in response to exocytosis inducers concomitantly with acrosomal swelling during the exocytotic cascade (Branham et al. 2009; Lopez et al. 2012; Bustos et al. 2012, 2014; Ruete et al. 2014) (cartoon in Fig. 4.1).

Disentangling fusion-incompetent *cis* SNARE complexes to regenerate monomeric SNAREs requires metabolic energy provided by NSF via the hydrolysis of

ATP. It is believed that the activity of NSF is constitutive in most cells to ensure that *cis* complexes are disassembled under normal steady-state conditions. In sperm, however, the picture is different, perhaps owing to their need to coordinate the AR carefully with the exact moment when they encounter the egg. Upon initiation of the AR and following Rab3's activation, protein tyrosine phosphatase 1B (PTP1B) dephosphorylates NSF, de-repressing its activity. Dephospho-NSF binds α-SNAP, which stimulates the ATPase activity of NSF to disassemble *cis* SNARE complexes (Zarelli et al. 2009). Broadly during the same window of time, calcineurin dephosphorylates synaptotagmin VI (Castillo et al. 2010) (Fig. 4.1).

In addition to SNAREs, members of the Munc13 and 18 protein families participate in membrane fusion [for recent reviews, see (Rizo and Sudhof 2012; Burgoyne et al. 2009; Carr and Rizo 2010; Sudhof and Rothman 2009; Lang and Jahn 2008; Jahn and Fasshauer 2012)]. Syntaxin1 contains a SNARE motif (purple helix in Fig. 4.1) and an N-terminal, three-helix bundle called the Habc domain (pink balloon in Fig. 4.1). The Habc domain and the SNARE motif bind intramolecularly, forming a "closed conformation" that is self-inhibited and binds Munc18-1. Syntaxin opening by Munc13 gates the assembly of a fusion complex constituted by three SNAREs plus Munc18 (Rizo and Sudhof 2012; Rizo and Xu 2015). Munc13 (not shown in Fig. 4.1 for simplicity) is necessary for the AR elicited by calcium in permeabilized human sperm (Bello et al. 2012). Munc18-1 binds syntaxin going transiently through a monomeric, closed configuration (Rodriguez et al. 2012) (Fig. 4.1).

4.4.4 Stimulated Sperm: Docking

Roughly 13 min after the initiation of the AR (Sosa et al. 2015), 50 % of the sperm exhibit swollen acrosomes with numerous tight appositions (distances between 0 nm and 8 nm) between the outer acrosomal and plasma membranes (Zanetti and Mayorga 2009). These distances are comparable to those reported in the literature for morphologically docked secretory vesicles (Verhage and Sorensen 2008; Schweizer et al. 1998) and are a hallmark of docked acrosomes. Differently from our findings in the human, docked acrosomes have been detected in capacitated boar sperm not stimulated to undergo exocytosis (Tsai et al. 2010). The tight appositions between the acrosome and the plasma membrane during the docking stage of the AR are stabilized by partially assembled *trans* SNARE complexes, envisioned as molecular bridges between the two membranes that are going to fuse. Assembly of these complexes is facilitated by Munc18-1 (see before) and complexin (Roggero et al. 2007) (Fig. 4.1). Intact SNAREs are required for the docking of the acrosome to the plasma membrane because cleavage of SNAREs with botulinum or tetanus toxins prevents morphological (Zanetti and Mayorga 2009) and functional (De Blas et al. 2005) docking. Likewise, sequestration of free syntaxin with recombinant Munc18-1 (Rodriguez et al. 2012) or an excess of α-SNAP (Rodriguez et al. 2011) halts the AR exocytotic cascade before it reaches

this stage. So does sequestration of endogenous Munc18-1 (Rodriguez et al. 2012), Rab3, and its effector RIM (Bello et al. 2012) with specific antibodies.

4.4.5 *Beyond Docking: Calcium Mobilization, Opening of Fusion Pores, Vesiculization, and Shedding of the Acrosomal Portion of the Cell*

After docking, the remaining exocytotic steps until the acrosome is lost completely happen comparatively fast [12 s, (Sosa et al. 2015)]. One of the first post-docking events during the AR cascade is the efflux of calcium from the acrosomal reservoir through inositol 1,4,5-trisphosphate (IP_3)-sensitive channels. That the acrosome behaves as an internal store of releasable calcium is covered elsewhere in this volume. Calcium mobilization from the acrosomal store is readily observed with the fluorescent, membrane-permeant calcium sensor Fluo3-AM in SLO-permeabilized human sperm. The AR inducers active Rab3A (De Blas et al. 2002), diacylglycerol (Lopez et al. 2012), external calcium (Ruete et al. 2014), and 8-pCPT-2-O-Me-cAMP (Lucchesi et al. 2016) decrease the concentration of calcium inside the acrosome assessed by single-cell fluorescence microscopy. So does adenophostin A, an IP_3 receptor agonist (Ruete et al. 2014) that rescues all AR blockers that interfere with intra-acrosomal calcium mobilization. The local increase in calcium achieved by this pathway activates synaptotagmin, which displaces complexin from *trans* SNARE complexes and allows their full zippering (Roggero et al. 2007) (Fig. 4.1). This does not rule out a potentially direct role for synaptotagmin in acrosome-to-plasma membrane fusion as has been suggested in other models (Seven et al. 2013; Honigmann et al. 2013; Park et al. 2015). Halting exocytosis with intra-acrosomal calcium chelators or exogenous complexin II captures SNAREs in a partially assembled *trans* configuration and the acrosome morphologically docked to the plasma membrane (De Blas et al. 2005; Roggero et al. 2007; Zanetti and Mayorga 2009; Bello et al. 2012; Rodriguez et al. 2011, 2012) (Fig. 4.1).

Following docking, the outer acrosomal membrane fuses with the adjacent plasma membrane at the multiple points of apposition. In most cells, fusion pores widen, the membrane surrounding the secretory vesicles is incorporated into the plasma membrane, and the granule contents are discharged. In sperm, however, pore widening produces hybrid plasma membrane–outer acrosomal membrane vesicles and tubules that are shed upon completion of exocytosis. We have developed conditions that allow us to stop secretion when fusion pores are opened, but pore expansion is inhibited, preventing hybrid vesicle formation (Quevedo et al. submitted). Visualization of this stage requires probes that permeate into the acrosome through the fusion pores that connect the extracellular medium to the acrosomal lumen. Bathing sperm suspensions with such fluorescent probes allows detection of exocytosis in real time by single-cell microscopy (Harper et al. 2008;

Zoppino et al. 2012). The lectin *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA) stains the acrosomal region of unreacted (fixed and permeabilized) sperm and therefore is extensively used to score the AR. In addition, PSA rapidly permeates into the acrosome of (neither fixed nor permeabilized) cells through open fusion pores. PSA cross-links the remaining acrosomal matrix adhered to hybrid vesicles to the sperm head, thus preventing the complete dispersal of the granule contents. In other words, this protocol freezes the AR at the vesiculization stage, visualized because fluorescence in reacted sperm is permanent rather than transient (Zoppino et al. 2012).

In combination with standard methods, this protocol allowed us to demonstrate that Rab3 must be inactivated (hydrolyze GTP) to accomplish vesiculization of the acrosomal and plasma membranes but not the opening of fusion pores (Bustos et al. 2014). These data agree with those from rat brain synaptosomes, where global Rab3A-GTP hydrolysis correlates with massive exocytosis of synaptic vesicles (Stahl et al. 1994). In brief, our observations indicate that Rab3 must be activated to initiate the AR and must be inactivated to allow fusion pore expansion and acrosome vesiculization.

4.5 Unusual Lipid Composition in Sperm Membranes

Due to the loss of most cell organelles and DNA transcription machinery, spermatozoa lack protein expression and vesicular transport. In consequence, phospholipids, cholesterol, and other components of the plasma membrane cannot be newly synthesized. In spite of these, sperm plasma membrane is not a stable and metabolically inert structure. Lipid remodeling is accomplished during sperm maturation [reviewed in Flesch and Gadella (2000) and Dacheux and Dacheux (2013)], capacitation (Asano et al. 2013; Visconti 2009), and the AR (see below).

Sperm membranes have an unusual lipid composition. In addition to the phospholipids and sphingolipids present in all cell membranes, spermatozoa from many different species are rich in plasmalogens, which may elevate membrane fluidity or play a role in membrane fusion (Aveldaño et al. 1992; Petcoff et al. 2008). Another hallmark of sperm membranes is that glycerophospholipids and sphingomyelin are rich in species containing long-chain and very-long-chain polyunsaturated fatty acids. Important biochemical changes in these lipid species occur during capacitation and the AR in rat sperm, resulting in shifts in membrane biophysical properties such as lipid mobility (Oresti et al. 2010; Zanetti et al. 2010; Oresti et al. 2015). Xenopus, boar, bull, rat, and ram sperm have very high levels of sphingomyelin (~12–20 mol %), which may be associated with decreased membrane fluidity and/or high levels of precursors for ceramide production (Stith 2015). Uneven distribution of ceramides, sphingomyelin, and glycerophospholipids was found between heads and tails (Furland et al. 2007; Oresti et al. 2011). The fact that sperm membranes are highly compartmentalized with regard to lipid species suggests specific roles for them in sperm functions.

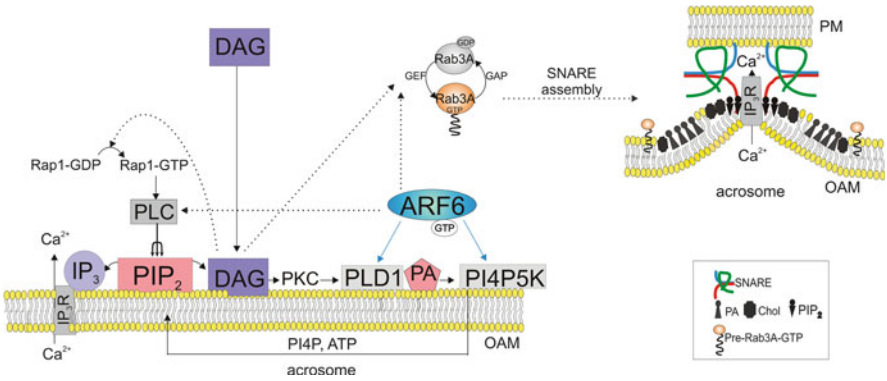

Fig. 4.2 Model highlighting the importance of lipids as signaling molecules for the AR. Rap1 GDP/GTP exchange is promoted by DAG (and/or Epac1, not shown) and triggers PLC epsilon activation, generating the loop shown in the cartoon. DAG also activates PLD1 via PKC. Active PLD1 hydrolyzes phosphatidylcholine, generating choline and PA. The latter activates a phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) which synthesizes PIP_2 and closes a positive feedback loop. PIP_2 hydrolysis also generates IP_3, which elicits the efflux of calcium from the acrosome. Simultaneously, DAG connects with the proteinaceous membrane fusion machinery by activating Rab3A, which leads to the assembly of *trans* SNARE complexes (Fig. 4.1). Exocytic stimuli also promote GDP/GTP exchange in ARF6 that stimulates PLD1, PI4P5K, and PLC activities, driving PIP_2 turnover and contributing to the lipid cascade. The micrographs in Fig. 4.1 show that there is a remarkable bending of the outer acrosomal membrane associated with acrosomal swelling. We postulate a significant role for cone (PA)- and inverted cone (PIP_2)-shaped lipids during the AR. Lipid modifications in the cytosolic hemileaflet of the OAM during the exocytotic stimulus may induce changes in membrane curvature that may foster the growing of deep invaginations of the OAM and the recruitment of SNARE proteins. *Solid arrows* mean that there is one step between the terms connected, and *dashed arrows* mean that the number of steps is either unknown or not depicted for simplicity

4.5.1 Lipid Dynamics During Sperm Exocytosis

Lipids are multitasking molecules in exocytosis: (i) they cluster in platforms defining membrane domains and recruiting proteins from the cytosol; (ii) they can act as first and second messengers in signal transduction and molecular recognition processes; and (iii) they define the shape of membranes that are going to fuse. Membrane fusion requires that flat lipid bilayers deform into shapes with very high curvatures. This makes membrane bending a critical force in determining fusion mechanisms. Here, we will analyze the role of different lipids in acrosomal exocytosis considering these points of view and focusing mainly on our own contributions to the field.

4.5.1.1 Cholesterol

Cholesterol and sphingomyelin cluster into discrete, lipid-enriched microdomains (lipid rafts) in cellular membranes. These domains recruit proteins from the cytosol

that subsequently organize secondary signaling or effector complexes. Due to the physical properties of these microdomains, distinct classes of membrane proteins are incorporated into them whereas others are excluded; SNAREs belong in the first category (van den Bogaart et al. 2013; Chamberlain et al. 2001). Isoprenylated proteins, such as Rabs, are normally excluded from rafts (Pyenta et al. 2001). It has been proposed that the association of SNAREs with lipid rafts concentrates these proteins at defined sites of the plasma membrane. In neuroendocrine cells and neurons, depletion of cholesterol results in a reduced vesicular release [reviewed in Chasserot-Golaz et al. (2010)]. Recently, Zick et al. showed that cholesterol is essential for fusion at physiological SNARE concentrations (Zick et al. 2014). Syntaxin 2 localizes to both raft and non-raft fractions in guinea pig and murine sperm (Travis et al. 2001; Asano et al. 2009). Syntaxin and synaptobrevin association with lipid rafts depends on cholesterol depletion in capacitating sperm, presumably preparing SNAREs for acrosomal exocytosis (Tsai et al. 2007).

Cholesterol efflux has been extensively studied as a hallmark of sperm capacitation. We have shown an additional, direct effect of cholesterol efflux during the AR (Belmonte et al. 2005). Surprisingly, decreasing cholesterol concentrations in sperm membranes affects an early event in the exocytic cascade rather than bilayer mixing. This event is the increased targeting of Rab3A to sperm membranes. We postulate that initially sperm SNAREs concentrate in rafts, whereas Rab3A localizes to the cytosol. When cholesterol levels decrease, Rab3A is able to insert its geranylgeranyl groups into sperm membranes, where it recruits effectors and initiates tethering of the outer acrosomal to the plasma membrane. In short, cholesterol efflux appears to confer fertilization competence, at least in part, by optimizing the conditions for the productive assembly of the fusion machinery required for the AR.

4.5.1.2 Diacylglycerol (DAG)

DAG, a phospholipid-cleavage product generated by PLC activity, triggers the fusion of liposomes, is necessary for the fusion of isolated organelles (Fratti et al. 2004), and mediates vesicle fusion through the activation of a TRP channel (Albert 2011). It is well known that phorbol esters, functional analogs of DAG, potently enhance neurotransmitter release at synapses and facilitate exocytosis in PC12 cells (Xue et al. 2009). Phorbol esters and DAG activate C1-domain-containing proteins, such as PKCs and Munc13 (Rhee et al. 2002).

Addition of DAG or phorbol esters triggers acrosomal exocytosis in spermatozoa from different species. Endogenous synthesis of DAG is differentially regulated by transducing pathways activated by physiological agonists (O'Toole et al. 1996). DAG can activate TRP channels on the plasma membrane, promoting a calcium influx from extracellular medium (Jungnickel et al. 2001). This lipid can also impact on other factors involved in exocytosis. To unveil a signaling pathway bypassing any effect of this lipid on plasma membrane channels, we have used SLO-permeabilized human sperm. In this experimental model, we showed that

DAG promotes the activation of Rab3A, which in turn initiates a cascade of protein–protein interactions that culminates in the assembly of SNARE complexes (Fig. 4.2). In addition, DAG fosters the production of PIP_2 by feeding into a PKC- and PLD1-dependent positive loop (Lopez et al. 2012).

4.5.1.3 Phosphatidylinositol-4,5-bisphosphate (PIP_2)

PIP_2 is abundant in the inner leaflet of the plasma membrane (about 2 mol %). This lipid clusters in small membrane microdomains (Slochower et al. 2014) that engage in strong electrostatic-based interactions with positively charged molecules (McLaughlin and Murray 2005). For instance, Van den Bogaart et al. showed that PIP_2 increases 40-fold the affinity of synaptotagmin-1 for calcium (van den Boggart et al. 2012). Recently, it has been demonstrated that synaptotagmin-1 binds to PIP_2-containing membrane but not to SNAREs at physiological ionic strength (Park et al. 2015). PIP_2 microdomains can induce changes in membrane curvature given that the molecule has the shape of an inverted cone (the hydrophobic part occupies a relatively smaller surface area than the head). Its role in the exocytosis of neurons and neuroendocrine cells has been profusely demonstrated [for a review, see (Martin 2015)].

Agonist–receptor interactions promote calcium influx from the external medium into the cytosol, this ion being essential for the activation of phospholipases. Current thinking is that phospholipases hydrolyze PIP_2 at an early stage during sperm exocytosis, initiating complex signaling cascades [reviewed in Roldan and Shi (2007)]. We have demonstrated that PIP_2 is hydrolyzed also at a later stage during the AR as well as regenerated through the loop shown in Fig. 4.2.

A recent publication from our laboratory highlights that ADP Ribosylation Factor 6 (ARF6) present in human spermatozoa exchanges GDP by GTP in response to exocytic stimuli and subsequently regulates lipid turnover. Once active, ARF6 increases PLC activity, causing PIP_2 hydrolysis generating DAG and IP_3-dependent intra-acrosomal calcium release. Furthermore, active ARF6 increases the exchange of GDP for GTP on Rab3A, a prerequisite for secretion (Pelletan et al. 2015). Our study describes a novel molecular link between ARF6, PLC, and Rab3A and provides insights into the interplay between lipids and proteins during acrosomal exocytosis (summarized in Fig. 4.2)

4.5.1.4 Phosphatidic Acid (PA)

PLDs are responsible for PA production in most cells. PLD1 participates in the fusion of secretory granules with the plasma membrane during regulated exocytosis in neuroendocrine and neural cells [for a review, see (Vitale 2010)]. A PLD activity is required for actin polymerization during bovine spermatozoa capacitation (Cohen et al. 2004). PLD1 is present in human sperm and its activity increases when cells are challenged with DAG. Inhibition of the pathway that activates PLD

is rescued by PA, indicating that this lipid is crucial for acrosomal exocytosis (Lopez et al. 2012). In terms of interactions with the proteinaceous fusion machinery, PA binds the juxtamembrane domain of the SNARE protein syntaxin-1 (Lam et al. 2008) and Epac1 (Consonni et al. 2012), a cAMP-dependent Rap-GEF. Epac and Rap are key factors in the signaling cascade connecting cAMP with the activation of the loop that produces PIP_2, DAG, and IP_3 (Branham et al. 2006, 2009; Ruete et al. 2014) (Fig. 4.2).

In addition to recruiting and regulating the activity of various important proteins, PA tends to form microdomains, through intermolecular hydrogen bonding, which serve as membrane insertion sites. PA contains a small headgroup and is considered cone-shaped lipid that generates a membrane curvature favoring exocytosis [reviewed in Chasserot-Golaz et al. (2010)].

4.6 Conclusions

In this chapter, we attempted to summarize some of the recent findings from our laboratories regarding the AR, while putting them in the context of what is known about exocytosis in somatic cells. The unusual size of the acrosome makes its release special. For the acrosomal and plasma membrane to fuse, the bilayers must contact. This is accomplished through acrosomal content swelling and membrane waving, which pushes one membrane against the other at the protruding regions. The approximation of membranes and the activation of the proteinaceous fusion machinery allow components on one membrane to bind its partners/effectors on the other, thus creating molecular bridges between them. Simultaneously, complex signaling cascades trigger the synthesis of specific lipids that work as second messengers and as modifiers of membrane topology. An active cross talk between proteins and lipids prepares the scenario where calcium mobilization from the acrosome triggers the opening and expansion of fusion pores to release hybrid vesicles together with the acrosomal contents, and to expose membrane proteins on the sperm surface that are essential for sperm–egg interaction and fusion.

References

Ackermann F, Zitranski N, Heydecke D, Wilhelm B, Gudermann T, Boekhoff I (2008) The multi-PDZ domain protein MUPP1 as a lipid raft-associated scaffolding protein controlling the acrosome reaction in mammalian spermatozoa. J Cell Physiol 214:757–768

Albert AP (2011) Gating mechanisms of canonical transient receptor potential channel proteins: role of phosphoinositols and diacylglycerol. Adv Exp Med Biol 704:391–411

Asano A, Selvaraj V, Buttke DE, Nelson JL, Green KM, Evans JE, Travis AJ (2009) Biochemical characterization of membrane fractions in murine sperm: identification of three distinct sub-types of membrane rafts. J Cell Physiol 218:537–548

Asano A, Nelson-Harrington JL, Travis AJ (2013) Phospholipase B is activated in response to sterol removal and stimulates acrosome exocytosis in murine sperm. J Biol Chem 288:28104–28115

Aveldaño MI, Rotstein NP, Vermouth NT (1992) Lipid remodelling during epididymal maturation of rat spermatozoa. Enrichment in plasmenylcholines containing long-chain polyenoic fatty acids of the n-9 series. Biochem J 283:235–241

Barr FA (2013) Review series: Rab GTPases and membrane identity: causal or inconsequential? J Cell Biol 202:191–199

Bechara C, Sagan S (2013) Cell-penetrating peptides: 20 years later, where do we stand? FEBS Lett 587:1693–1702

Bedford JM (1998) Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. Biol Reprod 59:1275–1287

Bedford JM (2004) Enigmas of mammalian gamete form and function. Biol Rev Camb Philos Soc 79:429–460

Bello OD, Zanetti MN, Mayorga LS, Michaut MA (2012) RIM, Munc13, and Rab3A interplay in acrosomal exocytosis. Exp Cell Res 318:478–488

Belmonte SA, Lopez CI, Roggero CM, De Blas GA, Tomes CN, Mayorga LS (2005) Cholesterol content regulates acrosomal exocytosis by enhancing Rab3A plasma membrane association. Dev Biol 285:393–408

Berruti G, Ripolone M, Ceriani M (2010) USP8, a regulator of endosomal sorting, is involved in mouse acrosome biogenesis through interaction with the spermatid ESCRT-0 complex and microtubules. Biol Reprod 82:930–939

Branham MT, Mayorga LS, Tomes CN (2006) Calcium-induced acrosomal exocytosis requires cAMP acting through a protein kinase A-independent, Epac-mediated pathway. J Biol Chem 281:8656–8666

Branham MT, Bustos MA, De Blas GA, Rehmann H, Zarelli VE, Trevino CL, Darszon A, Mayorga LS, Tomes CN (2009) Epac activates the small G proteins Rap1 and Rab3A to achieve exocytosis. J Biol Chem 284(37):24825–24839

Burgoyne RD, Morgan A (2003) Secretory granule exocytosis. Physiol Rev 83:581–632

Burgoyne RD, Barclay JW, Ciufo LF, Graham ME, Handley MT, Morgan A (2009) The functions of Munc18-1 in regulated exocytosis. Ann N Y Acad Sci 1152:76–86

Bustos MA, Lucchesi O, Ruete MC, Mayorga LS, Tomes CN (2012) Rab27 and Rab3 sequentially regulate human sperm dense-core granule exocytosis. Proc Natl Acad Sci U S A 109(30): E2057–E2066

Bustos MA, Roggero CM, De la Iglesia PX, Mayorga LS, Tomes CN (2014) GTP-bound Rab3A exhibits consecutive positive and negative roles during human sperm dense-core granule exocytosis. J Mol Cell Biol 6:286–298

Carr CM, Rizo J (2010) At the junction of SNARE and SM protein function. Curr Opin Cell Biol 22:488–495

Castillo BJ, Roggero CM, Mancifesta FE, Mayorga LS (2010) Calcineurin-mediated dephosphorylation of synaptotagmin VI is necessary for acrosomal exocytosis. J Biol Chem 285:26269–26278

Chamberlain LH, Burgoyne RD, Gould GW (2001) SNARE proteins are highly enriched in lipid rafts in PC12 cells: Implications for the spatial control of exocytosis. Proc Natl Acad Sci U S A 98:5619–5624

Chapman ER (2008) How does synaptotagmin trigger neurotransmitter release? Annu Rev Biochem 77:615–641

Chasserot-Golaz S, Coorssen JR, Meunier FA, Vitale N (2010) Lipid dynamics in exocytosis. Cell Mol Neurobiol 30:1335–1342

Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH (1999) SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion. Cell 97:165–174

Cohen G, Rubinstein S, Gur Y, Breitbart H (2004) Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. Dev Biol 267:230–241

Consonni SV, Gloerich M, Spanjaard E, Bos JL (2012) cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane. Proc Natl Acad Sci U S A 109:3814–3819

Dacheux JL, Dacheux F (2013) New insights into epididymal function in relation to sperm maturation. Reproduction 147:R27–R42

De Blas G, Michaut M, Trevino CL, Tomes CN, Yunes R, Darszon A, Mayorga LS (2002) The intraacrosomal calcium pool plays a direct role in acrosomal exocytosis. J Biol Chem 277:49326–49331

De Blas GA, Roggero CM, Tomes CN, Mayorga LS (2005) Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. PLoS Biol 3, e323

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci U S A 95:15781–15786

Flesch FM, Gadella BM (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 1469:197–235

Florman HM, Ducibella T (2005) Fertilization in mammals. In: Neill JD (ed) Knobil and Neill's physiology of reproduction, 3rd edn. Elsevier-Academic Press, San Diego, CA, pp 55–112

Fratti RA, Jun Y, Merz AJ, Margolis N, Wickner W (2004) Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. J Cell Biol 167:1087–1098

Fukuda M (2008) Regulation of secretory vesicle traffic by Rab small GTPases. Cell Mol Life Sci 65:2801–2813

Furland NE, Oresti GM, Antollini SS, Venturino A, Maldonado EN, Aveldaño MI (2007) Very long-chain polyunsaturated fatty acids are the major acyl groups of sphingomyelins and ceramides in the head of mammalian spermatozoa. J Biol Chem 282:18151–18161

Govers R (2014) Molecular mechanisms of GLUT4 regulation in adipocytes. Diabetes Metab 40:400–410

Graham ME, Handley MT, Barclay JW, Ciufo LF, Barrow SL, Morgan A, Burgoyne RD (2008) A gain-of-function mutant of Munc18-1 stimulates secretory granule recruitment and exocytosis and reveals a direct interaction of Munc18-1 with Rab3. Biochem J 409:407–416

Harper CV, Cummerson JA, White MR, Publicover SJ, Johnson PM (2008) Dynamic resolution of acrosomal exocytosis in human sperm. J Cell Sci 121:2130–2135

Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, Sudhof TC, Niemann H (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. EMBO J 13:5051–5061

Hernandez-Gonzalez EO, Lecona-Valera AN, Escobar-Herrera J, Mujica A (2000) Involvement of an F-actin skeleton on the acrosome reaction in guinea pig spermatozoa. Cell Motil Cytoskeleton 46:43–58

Hirohashi N, Gerton GL, Buffone MG (2011) Video imaging of the sperm acrosome reaction during in vitro fertilization. Commun Integr Biol 4:471–476

Honigmann A, van den Bogaart G, Iraheta E, Risselada HJ, Milovanovic D, Mueller V, Mullar S, Diederichsen U, Fasshauer D, Grubmuller H, Hell SW, Eggeling C, Kuhnel K, Jahn R (2013) Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. Nat Struct Mol Biol 20:679–686

Hu XQ, Ji SY, Li YC, Fan CH, Cai H, Yang JL, Zhang CP, Chen M, Pan ZF, Hu ZY, Gao F, Liu YX (2010) Acrosome formation-associated factor is involved in fertilization. Fertil Steril 93:1482–1492

Hutt DM, Baltz JM, Ngsee JK (2005) Synaptotagmin VI and VIII and Syntaxin 2 are essential for the mouse sperm acrosome reaction. J Biol Chem 280:20197–20203

Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R (2011) Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. Proc Natl Acad Sci U S A 108:20008–20011

Jahn R, Fasshauer D (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490(7419):201–207

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci U S A 108:4892–4896

Joliot A, Prochiantz A (2004) Transduction peptides: from technology to physiology. Nat Cell Biol 6:189–196

Jones S, Lukanowska M, Suhorutsenko J, Oxenham S, Barratt C, Publicover S, Copolovici DM, Langel U, Howl J (2013) Intracellular translocation and differential accumulation of cell-penetrating peptides in bovine spermatozoa: evaluation of efficient delivery vectors that do not compromise human sperm motility. Hum Reprod 28:1874–1889

Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM (2001) Trp2 regulates entry of Ca^{2+} into mouse sperm triggered by egg ZP3. Nat Cell Biol 3:499–502

Kim KS, Foster JA, Gerton GL (2001) Differential release of guinea pig sperm acrosomal components during exocytosis. Biol Reprod 64:148–156

Kitamura K, Tanaka H, Nishimune Y (2003) Haprin, a novel haploid germ cell-specific RING finger protein involved in the acrosome reaction. J Biol Chem 278:44417–44423

Lam AD, Tryoen-Toth P, Tsai B, Vitale N, Stuenkel EL (2008) SNARE-catalyzed fusion events are regulated by Syntaxin1A-lipid interactions. Mol Biol Cell 19:485–497

Lang T, Jahn R (2008) Core proteins of the secretory machinery. Handb Exp Pharmacol 184:107–127

Lefkimmiatis K, Moyer MP, Curci S, Hofer AM (2009) "cAMP sponge": a buffer for cyclic adenosine 3′, 5′-monophosphate. PLoS ONE 4, e7649

Lopez CI, Belmonte SA, De Blas GA, Mayorga LS (2007) Membrane-permeant Rab3A triggers acrosomal exocytosis in living human sperm. FASEB J 21:4121–4130

Lopez CI, Pelletan LE, Suhaiman L, De Blas GA, Vitale N, Mayorga LS, Belmonte SA (2012) Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate. Biochim Biophys Acta 1821:1186–1199

Lucchesi O, Ruete MC, Bustos MA, Quevedo MF, Tomes CN (2016) The signaling module cAMP/Epac/Rap1/PLCε/IP3 mobilizes acrosomal calcium during sperm exocytosis. Biochim Biophys Acta 1863(4):544–561. doi:10.1016/j.bbamcr.2015.12.007

Martin TF (2015) PI(4,5)P_2-binding effector proteins for vesicle exocytosis. Biochim Biophys Acta 1851:785–793

Masedunskas A, Porat-Shliom N, Weigert R (2012) Regulated exocytosis: novel insights from intravital microscopy. Traffic 13:627–634

Mayorga L, Tomes CN, Belmonte SA (2007) Acrosomal exocytosis, a special type of regulated secretion. IUBMB Life 59:286–292

McCullough J, Colf LA, Sundquist WI (2013) Membrane fission reactions of the mammalian ESCRT pathway. Annu Rev Biochem 82:663–692

McLaughlin S, Murray D (2005) Plasma membrane phosphoinositide organization by protein electrostatics. Nature 438:605–611

Morris J, Jones S, Howl J, Lukanowska M, Lefievre L, Publicover S (2015) Cell-penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced $[Ca^{2+}]_i$ signal in human sperm. Mol Hum Reprod 21:563–570

O'Toole CM, Roldan ER, Hampton P, Fraser LR (1996) A role for diacylglycerol in human sperm acrosomal exocytosis. Mol Hum Reprod 2:317–326

Okabe M (2013) The cell biology of mammalian fertilization. Development 140:4471–4479

Okabe M (2014) Mechanism of fertilization: a modern view. Exp Anim 63:357–365

Oresti GM, Reyes JG, Luquez JM, Osses N, Furland NE, Aveldaño MI (2010) Differentiation-related changes in lipid classes with long-chain and very long-chain polyenoic fatty acids in rat spermatogenic cells. J Lipid Res 51:2909–2921

Oresti GM, Luquez JM, Furland NE, Aveldaño MI (2011) Uneven distribution of ceramides, sphingomyelins and glycerophospholipids between heads and tails of rat spermatozoa. Lipids 46:1081–1090

Oresti GM, Penalva DA, Luquez JM, Antollini SS, Aveldaño MI (2015) Lipid biochemical and biophysical changes in rat spermatozoa during isolation and functional activation in vitro. Biol Reprod 93:140

Pang ZP, Sudhof TC (2010) Cell biology of Ca^{2+}-triggered exocytosis. Curr Opin Cell Biol 22:496–505

Park Y, Seo JB, Fraind A, Perez-Lara A, Yavuz H, Han K, Jung SR, Kattan I, Walla PJ, Choi M, Cafiso DS, Koh DS, Jahn R (2015) Synaptotagmin-1 binds to PIP2-containing membrane but not to SNAREs at physiological ionic strength. Nat Struct Mol Biol 22:815–823

Pavlos NJ, Gronborg M, Riedel D, Chua JJ, Boyken J, Kloepper TH, Urlaub H, Rizzoli SO, Jahn R (2010) Quantitative analysis of synaptic vesicle Rabs uncovers distinct yet overlapping roles for Rab3a and Rab27b in Ca^{2+}-triggered exocytosis. J Neurosci 30:13441–13453

Pelletan LE, Suhaiman L, Vaquer CC, Bustos MA, De Blas GA, Vitale N, Mayorga LS, Belmonte SA (2015) ADP Ribosylation Factor 6 (ARF6) Promotes Acrosomal Exocytosis by Modulating Lipid Turnover and Rab3A Activation. J Biol Chem 290:9823–9841

Petcoff DW, Holland WL, Stith BJ (2008) Lipid levels in sperm, eggs, and during fertilization in Xenopus laevis. J Lipid Res 49:2365–2378

Pocognoni CA, De Blas GA, Heuck AP, Belmonte SA, Mayorga LS (2013) Perfringolysin O as a useful tool to study human sperm physiology. Fertil Steril 99:99–106

Pocognoni CA, Berberian MV, Mayorga LS (2015) ESCRT (Endosomal Sorting Complex Required for Transport) machinery is essential for acrosomal exocytosis in human sperm. Biol Reprod 93:124

Porat-Shliom N, Milberg O, Masedunskas A, Weigert R (2013) Multiple roles for the actin cytoskeleton during regulated exocytosis. Cell Mol Life Sci 70:2099–2121

Pyenta PS, Holowka D, Baird B (2001) Cross-correlation analysis of inner-leaflet-anchored green fluorescent protein co-redistributed with IgE receptors and outer leaflet lipid raft components. Biophys J 80:2120–2132

Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Sudhof TC, Takahashi M, Rosenmund C, Brose N (2002) Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell 108:121–133

Rizo J, Sudhof TC (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices—guilty as charged? Annu Rev Cell Dev Biol 28:279–308

Rizo J, Xu J (2015) The synaptic vesicle release machinery. Annu Rev Biophys 44:339–367

Rizzoli SO, Jahn R (2007) Kiss-and-run, collapse and 'readily retrievable' vesicles. Traffic 8:1137–1144

Rodriguez F, Bustos MA, Zanetti MN, Ruete MC, Mayorga LS, Tomes CN (2011) alpha-SNAP prevents docking of the acrosome during sperm exocytosis because it sequesters monomeric syntaxin. PLoS ONE 6, e21925

Rodriguez F, Zanetti MN, Mayorga LS, Tomes CN (2012) Munc18-1 controls SNARE protein complex assembly during human sperm acrosomal exocytosis. J Biol Chem 287:43825–43839

Roggero CM, Tomes CN, De Blas GA, Castillo J, Michaut MA, Fukuda M, Mayorga LS (2005) Protein kinase C-mediated phosphorylation of the two polybasic regions of synaptotagmin VI regulates their function in acrosomal exocytosis. Dev Biol 285:422–435

Roggero CM, De Blas GA, Dai H, Tomes CN, Rizo J, Mayorga LS (2007) Complexin/synaptotagmin interplay controls acrosomal exocytosis. J Biol Chem 282:26335–26343

Roldan ER, Shi QX (2007) Sperm phospholipases and acrosomal exocytosis. Front Biosci 12:89–104

Ruete MC, Lucchesi O, Bustos MA, Tomes CN (2014) Epac, Rap and Rab3 act in concert to mobilize calcium from sperm inverted question marks acrosome during exocytosis. Cell Commun Signal 12:43

Sanchez-Cardenas C, Servin-Vences MR, Jose O, Trevino CL, Hernandez-Cruz A, Darszon A (2014) Acrosome reaction and Ca^{2+} imaging in single human spermatozoa: new regulatory roles of $[Ca^{2+}]_i$. Biol Reprod 91:67

Schweizer FE, Dresbach T, DeBello WM, O'Connor V, Augustine GJ, Betz H (1998) Regulation of neurotransmitter release kinetics by NSF. Science 279:1203–1206

Scott CC, Vacca F, Gruenberg J (2014) Endosome maturation, transport and functions. Semin Cell Dev Biol 31:2–10

Seven AB, Brewer KD, Shi L, Jiang QX, Rizo J (2013) Prevalent mechanism of membrane bridging by synaptotagmin-1. Proc Natl Acad Sci U S A 110:E3243–E3252

Shitara A, Weigert R (2015) Imaging membrane remodeling during regulated exocytosis in live mice. Exp Cell Res 337:219–225

Slochower DR, Wang YH, Tourdot RW, Radhakrishnan R, Janmey PA (2014) Counterion-mediated pattern formation in membranes containing anionic lipids. Adv Colloid Interface Sci 208:177–188

Sollner TH (2003) Regulated exocytosis and SNARE function. Mol Membr Biol 20:209–220

Sosa CM, Pavarotti MA, Zanetti MN, Zoppino FC, De Blas GA, Mayorga LS (2015) Kinetics of human sperm acrosomal exocytosis. Mol Hum Reprod 21:244–254

Sosa CM, Zanetti MN, Pocognoni CA, Mayorga LS (2016) Acrosomal swelling is triggered by cAMP downstream of the opening of store-operated calcium channels during acrosomal exocytosis in human sperm. Biol Reprod 94(3):57. doi:10.1095/biolreprod.115.133231

Stahl B, von Mollard GF, Walch-Solimena C, Jahn R (1994) GTP cleavage by the small GTP-binding protein Rab3A is associated with exocytosis of synaptic vesicles induced by alpha-latrotoxin. J Biol Chem 269:24770–24776

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513–525

Stith BJ (2015) Phospholipase C and D regulation of Src, calcium release and membrane fusion during Xenopus laevis development. Dev Biol 401:188–205

Sudhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. Science 323:474–477

Sumigama S, Mansell S, Miller M, Lishko PV, Cherr GN, Meyers SA, Tollner T (2015) Progesterone accelerates the completion of sperm capacitation and activates CatSper channel in spermatozoa from the Rhesus macaque. Biol Reprod 93:130

Tolmachova T, Anders R, Stinchcombe J, Bossi G, Griffiths GM, Huxley C, Seabra MC (2004) A general role for Rab27a in secretory cells. Mol Biol Cell 15:332–344

Tomes CN (2007) Molecular mechanisms of membrane fusion during acrosomal exocytosis. Soc Reprod Fertil Suppl 65:275–291

Tomes CN (2015) The proteins of exocytosis: lessons from the sperm model. Biochem J 465:359–370

Travis AJ, Merdiushev T, Vargas LA, Jones BH, Purdon MA, Nipper RW, Galatioto J, Moss SB, Hunnicutt GR, Kopf GS (2001) Expression and localization of caveolin-1, and the presence of membrane rafts, in mouse and Guinea pig spermatozoa. Dev Biol 240:599–610

Tsai PS, De Vries KJ, De Boer-Brouwer M, Garcia-Gil N, Van Gestel RA, Colenbrander B, Gadella BM, Van Haeften T (2007) Syntaxin and VAMP association with lipid rafts depends on cholesterol depletion in capacitating sperm cells. Mol Membr Biol 24:313–324

Tsai PS, Garcia-Gil N, Van Haeften T, Gadella BM (2010) How pig sperm prepares to fertilize: stable acrosome docking to the plasma membrane. PLoS ONE 5, e11204

Tsuboi T, Fukuda M (2005) The C2B domain of rabphilin directly interacts with SNAP-25 and regulates the docking step of dense core vesicle exocytosis in PC12 cells. J Biol Chem 280:39253–39259

van den Bogaart G, Lang T, Jahn R (2013) Microdomains of SNARE proteins in the plasma membrane. Curr Top Membr 72:193–230

van den Boggart G, Meyenberg K, Diederichsen U, Jahn R (2012) Phosphatidylinositol 4,5-bisphosphate increases Ca^{2+} affinity of synaptotagmin-1 by 40-fold. J Biol Chem 287:16447–16453

Verhage M, Sorensen JB (2008) Vesicle docking in regulated exocytosis. Traffic 9:1414–1424

Visconti PE (2009) Understanding the molecular basis of sperm capacitation through kinase design. Proc Natl Acad Sci U S A 106:667–668

Vitale N (2010) Synthesis of fusogenic lipids through activation of phospholipase D1 by GTPases and the kinase RSK2 is required for calcium-regulated exocytosis in neuroendocrine cells. Biochem Soc Trans 38:167–171

Xu T, Binz T, Niemann H, Neher E (1998) Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity. Nat Neurosci 1:192–200

Xue R, Zhao Y, Chen P (2009) Involvement of PKC alpha in PMA-induced facilitation of exocytosis and vesicle fusion in PC12 cells. Biochem Biophys Res Commun 380:371–376

Yunes R, Michaut M, Tomes C, Mayorga LS (2000) Rab3A triggers the acrosome reaction in permeabilized human spermatozoa. Biol Reprod 62:1084–1089

Zanetti N, Mayorga LS (2009) Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. Biol Reprod 81:396–405

Zanetti SR, de Los Angeles MM, Rensetti DE, Fornes MW, Aveldaño MI (2010) Ceramides with 2-hydroxylated, very long-chain polyenoic fatty acids in rodents: From testis to fertilization-competent spermatozoa. Biochimie 92:1778–1786

Zarelli VE, Ruete MC, Roggero CM, Mayorga LS, Tomes CN (2009) PTP1B dephosphorylates N-ethylmaleimide-sensitive factor and elicits SNARE complex disassembly during human sperm exocytosis. J Biol Chem 284:10491–10503

Zick M, Stroupe C, Orr A, Douville D, Wickner WT (2014) Membranes linked by trans-SNARE complexes require lipids prone to non-bilayer structure for progression to fusion. Elife 3, e01879

Zoppino FC, Halon ND, Bustos MA, Pavarotti MA, Mayorga LS (2012) Recording and sorting live human sperm undergoing acrosome reaction. Fertil Steril 97:1309–1315

Chapter 5
Sperm Capacitation and Acrosome Reaction in Mammalian Sperm

Cintia Stival, Lis del C. Puga Molina, Bidur Paudel, Mariano G. Buffone, Pablo E. Visconti, and Dario Krapf

5.1 Introduction

To acquire fertilizing capacity, mammalian sperm require two post-testicular maturation steps, one occurring in the male epididymis, known as epididymal maturation, and the other one occurring after ejaculation in the female tract known as capacitation. Capacitation was first observed independently by Chang (1951) and Austin (1952) in the 1950s. Their observations were crucial for future development of in vitro fertilization techniques, first in rabbit (Chang 1959) and later on in humans (Steptoe and Edwards 1978) with the birth of Louise Brown, the first "test-tube baby." At the cell biology level, capacitation induces changes in the sperm motility pattern known as hyperactivated movement and prepares the sperm to undergo an exocytotic process known as acrosome reaction. At the molecular level,

C. Stival • D. Krapf (✉)
Instituto de Biología Molecular y Celular de Rosario (CONICET-UNR) and Laboratorio de Especialidades Reproductivas (Facultad de Ciencias Bioquímicas y Farmacéuticas (UNR), 2000 Rosario, Argentina
e-mail: krapf@ibr-conicet.gov.ar

L.d.C. Puga Molina • M.G. Buffone
Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

B. Paudel • P.E. Visconti
Department of Veterinary and Animal Sciences, ISB, University of Massachusetts, 01003 Amherst, MA, USA

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_5

capacitation is associated with cholesterol loss from the sperm plasma membrane, increased membrane fluidity, changes in intracellular ion concentrations (Visconti et al. 2011), hyperpolarization of the sperm plasma membrane (Hernandez-Gonzalez et al. 2006), increased activity of the Protein Kinase A (PKA) (Krapf et al. 2010), and protein tyrosine phosphorylation (Arcelay et al. 2008). Although each of these events has been studied independently, information regarding how they interconnect to regulate sperm motility and to prepare the sperm to undergo the acrosome reaction is still unavailable. It is known that PKA plays a key role coordinating the majority of the events related to capacitation. Blockade of this kinase prevents the acrosome reaction, probably through inhibition of processes needed for the acquisition of acrosomal exocytotic competence (Visconti et al. 1995). Due to this essential role of PKA, it was surprising to find that the PKA catalytic subunit is absent in the head of the mouse sperm where all events related to acrosome reaction occur (Wertheimer et al. 2013). Interestingly, other molecules known to be essential for acrosomal reaction responsiveness such as SLO3 K^+ channels are also found exclusively in the sperm flagellum. These results suggest that activation of signaling pathways in the sperm tail plays a role in the regulation of events happening in the head compartment. The finding that injection of Lucifer Yellow dye promptly diffuses throughout the whole interior of the sperm indicates that molecules can pass from one compartment to the other (Navarro et al. 2007). Nevertheless, it should be considered that in the case of second messengers such as cAMP, their diffusion throughout the sperm is limited and highly controlled by hydrolyzing enzymes (i.e., phosphodiesterases). An alternative possibility is that changes in ion permeability and the consequent modification of the sperm membrane potential in the whole cell can orchestrate the activation of signaling pathways throughout the sperm. Therefore, both inter-compartmental diffusion and changes in the membrane potential could play a role in coordinating events in the tail with those occurring in the sperm head. Although diffusion of molecules cannot be discarded and is likely to be involved in energy transfer from the tail to the head, little experimental evidence of this process is available. On the other hand, it is clear that mammalian sperm undergo hyperpolarization of the sperm plasma membrane and that these changes appear to be involved in the preparation of sperm for exocytosis of their acrosomes (De La Vega-Beltran et al. 2012; Hernandez-Gonzalez et al. 2006). This review will focus on the signaling events involved in the regulation of the changes in the sperm membrane potential that occur during capacitation and their connection with the acrosome reaction. For more information regarding capacitation, see other reviews on this topic (Aitken and Nixon 2013; Buffone et al. 2014; Harayama 2013; Bailey 2010). Discussion of the acrosome reaction and the molecular mechanisms involved in this event can be found in different chapters of this book.

5.2 Signaling Pathways Connecting Capacitation with the Acrosome Reaction

Spermatozoa undergo significant changes in their ionic environment during their journey to the egg. In the epididymis, sperm are surrounded by epididymal fluid containing concentrations that differ significantly from those found in other biological milieus such as blood. Remarkably, the epididymal fluid contains low levels of Na^+ (~40 mM), Cl^- (~40 mM), and HCO_3^- (~4 mM) and high concentrations of K^+ ions (Pastor-Soler et al. 2005). In particular, low pH and low HCO_3^- concentrations are essential to maintain the sperm in a quiescent state before ejaculation (Pastor-Soler et al. 2005). Strikingly, the pH of the epididymis is kept low by the action of a battery of transporters in the epididymis epithelial cells including Na^+/H^+ antiporters, Na^+/HCO_3^- co-transporter, and a series of carbonic anhydrases and most importantly through the regulation of vacuolar H^+-ATPase (Pastor-Soler et al. 2005). Once ejaculated, sperm encounter first seminal fluid and later on other fluids secreted by the female tract epithelium. In their new environment, the pH and concentrations of Na^+, Cl^-, and HCO_3^- are increased while those of K^+ are decreased (Kavanagh 1985). Sperm decode these changes on environmental ions promoting the activation of signaling cascades leading to capacitation.

5.2.1 Role of HCO_3^- and cAMP

When sperm are exposed to higher HCO_3^- concentrations in the seminal fluid or in in vitro capacitation media, an increased transport of this anion is observed. One of the main targets of HCO_3^- inside the cell is the atypical adenylyl cyclase Adcy10 (aka sAC, aka SACY). This enzyme becomes stimulated upon binding of HCO_3^- (Kleinboelting et al. 2014; Chen et al. 2000) with the consequent increase in intracellular cAMP and activation of PKA. This whole signaling process occurs very fast (~1 min) and multiple evidence indicate that this initial PKA activation is upstream of most of the other known events associated with capacitation including increase in intracellular pH (Wang et al. 2003, 2007; Zeng et al. 1996), hyperpolarization of the plasma membrane (Demarco et al. 2003), actin polymerization (Romarowski et al. 2015), and the promotion of tyrosine phosphorylation (Visconti et al. 1995). In addition, pharmacological and genetic loss of function experiments showed that this initial activation of PKA prepares the sperm for the acrosome reaction. However, as mentioned above, the PKA catalytic subunit is localized to the flagellum and it is not found in the sperm head. This finding strongly suggests that PKA modulates the acrosome reaction indirectly through other downstream signaling pathways. Among them, we will concentrate on the regulation of the increase in intracellular pH (pHi) and on the hyperpolarization of the sperm membrane potential (Em) associated with capacitation.

5.2.2 Increase of Intracellular pH

As sperm leave the acidic vagina (pH ~5) and travel through the utero tubal junction, they face a significant increase of pH to a value of approximately 8 (Suarez and Pacey 2006). This change in extracellular pH has been proposed to promote sperm intracellular alkalinization (Hamamah and Gatti 1998). An increase in pHi is also observed in sperm incubated in conditions that support capacitation, despite the fact that extracellular pH is maintained constant. In this case, the intracellular alkalinization suggests the presence of active mechanisms controlling pHi. In sperm, three main molecular systems have been reported to control their pHi. First, direct electrophysiological recordings identified the voltage-gated channel Hv1 (Lishko et al. 2010). This channel is expressed in the flagellum of human sperm, suggesting a direct impact on Ca^{2+} homeostasis through modulation of pH-sensitive channels such as the Ca^{2+} channel CatSper and the K^+ channel SLO3, also located in the principal piece of the flagellum (see below). Hv1 is a H^+ transporter that promotes movement of protons across the membrane through voltage-gated mechanisms (Takeshita et al. 2014) and is highly sensitive to Zn^{2+}, an abundant ion in seminal plasma (Khan et al. 2011). It has been hypothesized that this high Zn^{2+} concentration plays a role in maintaining Hv1 closed until the seminal fluid is diluted in the female tract allowing its activation by still unknown mechanisms (Lishko et al. 2010). Added to the effect of Zn^{2+} removal, the endogenous cannabinoid anandamide found in the female tract (Gervasi et al. 2013) might facilitate Hv1 opening (Lishko et al. 2010). In contrast to human sperm, Hv1 is not found in mouse sperm. Consequently, the finding that Hv1 KO mice are fertile (Ramsey et al. 2009) is silent on the role of Hv1 in human sperm fertility.

A second possible mechanism for pHi alkalinization has been proposed in mouse. In particular, mice lacking the sperm-specific Na^+/H^+ exchanger (NHE-10 or sNHE) are sterile (Wang et al. 2007) and their sperm are unable to fertilize in vitro. In addition to the significant increase in HCO_3^- anions upon leaving the epididymis, sperm also encounter higher Na^+ concentrations in the seminal fluid and in the female tract. This increase in Na^+ would favor interchange of this cation for H^+ through sNHE. Interestingly, sNHE primary structure has a cyclic nucleotide binding domain, suggesting that it can respond to increased cAMP production during the initial steps of capacitation. Moreover, sperm from $sNHE^{-/-}$ have reduced levels of Adcy10 (Wang et al. 2003) and addition of permeable cAMP analogues restored protein tyrosine phosphorylation of sNHE-null sperm cells to a pattern similar to that of wild-type spermatozoa (Wang et al. 2007), suggesting a cross talk between cAMP synthesis and the regulation of sperm pHi.

Finally, as mentioned in the previous section, there is inward transport of HCO_3^- anions during capacitation. In addition to the well-established role in the regulation of Adcy10, HCO_3^- is also a weak base and its transport inside the sperm is predicted to alkalinize pHi. Addition of HCO_3^- to cells preincubated in the absence of this anion induced Em hyperpolarization accompanied by pHi increase (Demarco et al. 2003). Sodium replacement from the external media by the

non-permeant cation choline does not allow the hyperpolarization induced by HCO_3^- addition (Demarco et al. 2003), suggesting the presence of an electrogenic Na^+/HCO_3^- cotransporter in spermatozoa. Alternatively, it has also been proposed the presence of a neutral $Na^+/Cl^-/HCO_3^-$ cotransporter (Zeng et al. 1996) and the transport through CFTR (Chavez et al. 2012) or its coupled Cl^-/HCO_3^- exchangers (Chen et al. 2009). Equilibrium of HCO_3^-/CO_2 is modulated by the presence of sperm carbonic anhydrases, which catalyze the reversible hydration of CO_2 to HCO_3^-. In mouse sperm, carbonic anhydrases II and IV together contribute to almost all carbonic anhydrase activity detected (Wandernoth et al. 2015). When double knockout carbonic anhydrase II/IV male mice were mated with WT females, fertility was reduced by 90 % (Wandernoth et al. 2015).

Multiple evidence support the hypothesis that capacitation is accompanied by an increase in intracellular pH. However, how sperm alkalinization regulates other capacitation-associated events such as hyperactivation and the preparation for the acrosome reaction (AR) is not well established. Noteworthy, the activity of two of the best characterized sperm-specific ion channels is very sensitive to changes in pH. First, the sperm-specific Ca^{2+} channel CatSper, which is composed of multiple subunits including four pore-forming CatSper channel proteins, CatSper1–4, containing six transmembrane-spanning domains (Qi et al. 2007), and at least three auxiliary subunits β (Liu et al. 2007), γ (Wang et al. 2009), and δ (Chung et al. 2011). A His-rich domain found in the intracellular NH_2 terminus of CatSper1 (Ren et al. 2001) has been proposed to be responsible for the pHi sensitivity of the channel (Ren and Xia 2010). Patch clamp measurements indicate a significant activation of flagellar calcium channel that is strongly potentiated by intracellular alkalinization (Kirichok et al. 2006). This current is not present in CatSper1$^{-/-}$ knockout mice.

A second sperm-specific channel highly dependent on pHi is the K^+ channel SLO3 (Martinez-Lopez et al. 2009; Santi et al. 2010) which has an essential role in the regulation of the changes in plasma membrane potential associated with capacitation (see below). The possibility that these ion channels mediate the role of intracellular alkalinization in sperm is consistent with findings that CatSper and SLO3 functions are required for hyperactivation (Zeng et al. 2013) and that SLO3 is necessary for the acrosome reaction (Santi et al. 2010). Concurrent measurements of pHi, membrane potential, and intracellular Ca^{2+} concentrations using high-speed resolution and sperm from genetically modified knockout models will be required to conclusively test this hypothesis.

5.2.3 Changes in Membrane Potential

Capacitation is also associated with changes in the sperm plasma membrane potential (Em) (Zeng et al. 1995). Maintaining ionic gradients across their membranes requires considerable energy investment through modulation of pumps and ion transporters. As soon as cauda epididymal mouse sperm are suspended in a

culture media, they have a relatively depolarized Em of ~ −40 mV. However, this membrane potential hyperpolarizes to ~ −60 mV when capacitation proceeds, whereas no change is observed when sperm are incubated in conditions that do not support capacitation (Demarco et al. 2003; Zeng et al. 1995; Espinosa and Darszon 1995). These measurements of sperm Em have been conducted in populations using fluorimetric measurements and constitute an average value. On the other hand, when the sperm Em was monitored using single cell microscopy (Arnoult et al. 1999) or flow cytometry (Escoffier et al. 2015; Lopez-Gonzalez et al. 2014), two sperm subpopulations were observed: one with an Em closer to that of the non-capacitated sperm population (~ −40 mV) and the other with a more hyperpolarized Em (~ −80 mV) (Arnoult et al. 1999). These findings are consistent with the observation that only a fraction of the sperm population are capable to undergo the acrosome reaction (Salicioni et al. 2007).

The mechanisms that regulate hyperpolarization of the sperm plasma membrane during capacitation are poorly understood. Membrane potential at any point is given by the relative permeability of the plasma membrane to ions present in the capacitation media. The study of how these permeabilities change during capacitation is crucial for the understanding of the molecular basis of Em changes. Under normal conditions, sperm maintain an internal ion concentration markedly different from that in the extracellular medium, and these differences establish the resting plasma membrane potential. Therefore, Em changes that occur during capacitation reflect parallel changes in ion permeability. The most relevant ions involved in Em regulation are Na^+, K^+, and Cl^- which have equilibrium potentials of +40, −80, and −40 mV, respectively. Considering these ion's equilibrium Em, changes in Em from −40 mV to −80 mV that occur in sperm during capacitation can be explained by either closure of electrogenic Na^+ transport or by an opening of a K^+ channel. Regarding the first possibility, non-capacitated sperm are hyperpolarized when Na^+ ions are omitted from the incubation media. Moreover, hyperpolarization is also observed when sperm are treated with amiloride, a compound known to affect Na^+ transport by epithelial Na^+ channels (ENaC) (Hernandez-Gonzalez et al. 2006). ENaC channels contribute to the resting Em in many cell types by shifting the membrane potential toward the Na^+ equilibrium potential (Butterworth 2010), whereas closing of these channels during capacitation mediated by activation of the CFTR (Hernandez-Gonzalez et al. 2007; Xu et al. 2007) would explain the observed hyperpolarization. Consistent with this possibility, both mouse spermatogenic cells and testicular sperm display amiloride-sensitive inward Na^+ currents compatible with ENaC currents (Hernandez-Gonzalez et al. 2006), and fluorimetric measurements using Na^+ concentration dyes indicate that intracellular Na^+ concentrations decrease during capacitation (Escoffier et al. 2012).

Alternatively, hyperpolarization can also be explained by an increased outward K^+ current. In agreement with this hypothesis, sperm lacking SLO3, the aforementioned sperm-specific K^+ channel, do not undergo hyperpolarization during capacitation. Moreover, treatment of wild-type sperm with the SLO3 inhibitor clofilium blocks the capacitation-induced hyperpolarization (Sanchez-Carranza et al. 2015; Chavez et al. 2013). Although sperm from $SLO3^{-/-}$ mice cannot hyperpolarize

during capacitation, the hyperpolarization can be obtained when sperm are incubated in low Na^+ or in the presence of amiloride (Chavez et al. 2013; Hernandez-Gonzalez et al. 2006), indicating that an electrogenic Na^+ permeability is functionally present in mature mouse sperm and its elimination promotes hyperpolarization. These experiments suggest that a decrease in electrogenic Na^+ transport is sufficient to hyperpolarize the sperm Em. However, the lack of capacitation-induced hyperpolarization of sperm from $SLO3^{-/-}$ mice, while maintaining the ability to hyperpolarize when Na^+ transport is decreased, strongly argues in favor that an outward K^+ transport is the one responsible for changes in Em observed during capacitation. Consistently, recent electrophysiological recordings showed that although increasing extracellular [Na^+] (in a low sodium media) before capacitation produced larger depolarizations than after capacitation, this effect is not to be interpreted as changes of Na^+ permeability during capacitation. Rather, explanation to this is found in the observed permeability ratios of K^+ and Na^+: a P_K/P_{Na} of $1.6/.13 = 12.3$ before capacitation and P_K/P_{Na} of $4.38/.13 = 33$ after capacitation. These values indicate that adding external Na^+ after capacitation produces a much lower change in voltage than does the addition of external Na^+ before capacitation and that this difference is due to the large increase in K^+ permeability after capacitation, rather than a decrease in Na^+ permeability (Chavez et al. 2013).

Since capacitation prepares the sperm for the acrosome reaction (Zeng et al. 1995; Florman et al. 1998), it was proposed that the Em hyperpolarization that accompanies capacitation may regulate the sperm's ability to generate transient Ca^{2+} elevations in response to physiological agonists of the acrosome reaction (e.g., zona pellucida of the egg or progesterone). In support of this hypothesis, the presence of T-type Ca^{2+} channels has been documented in spermatogenic cells (Arnoult et al. 1996; Lievano et al. 1996) and in sperm (Escoffier et al. 2007). These channels were originally called low-voltage-activated (LVA) channels because they can be activated by a small depolarization of the plasma membrane. However, they are usually found in an inactive state at membrane potentials of ~ −40 mV that are observed in non-capacitated sperm. To release inactivation, Em should be hyperpolarized to potentials between −80 mV and −60 mV, from where they can be readily activated (Arnoult et al. 1996; Lievano et al. 1996). Accordingly, a depolarized membrane potential of sperm before capacitation would prevent a premature AR, provided that T-type Ca^{2+} channels are involved in the regulation of this event (Arnoult et al. 1996; Florman et al. 1998).

In recent years, patch clamp measurements became possible in sperm cells. These measurements were able to conclusively demonstrate using solid electrophysiological tools the presence of CatSper, SLO3, and Hv1 in mammalian sperm from different species (Kirichok et al. 2006; Lishko et al. 2010; Lishko and Kirichok 2010; Navarro et al. 2007; Strunker et al. 2011; Brenker et al. 2012, 2014; Sumigama et al. 2015). However, T channel currents from $Cav_{3.2}$ which are readily observed in testicular sperm cells (Arnoult et al. 1996; Lievano et al. 1996; Publicover and Barratt 1999; Martinez-Lopez et al. 2009) cannot be detected in epididymal sperm. Inward Ca^{2+} currents elicited by depolarization were fully abolished in spermatogenic cells from $Cav_{3.2}$-null mice revealing the molecular

identity of the T channel inward current in spermatogenic cells. However, mice lacking Ca$v_{3.2}$ channels are fertile (Escoffier et al. 2007) which argue against an essential role of this channel in sperm. Despite these results, antibodies against Ca$v_{3.2}$ stained the head of mouse sperm by immunofluorescence. This staining appears to be specific because it completely disappears in sperm from Ca$v_{3.2}$ KO mice (Escoffier et al. 2007). Why this channel is active in testicular sperm and then becomes inactivated during epididymal transit without being degraded is still not understood.

Despite the lack of solid electrophysiology evidence on the presence of Ca^{2+} T channels in mature sperm, hyperpolarization appears to be required for exocytosis of the sperm acrosome. As mentioned in the introduction, the ability of agonists to induce exocytosis is obtained only after sperm undergo capacitation (Buffone et al. 2012). Similarly, only capacitated sperm undergo acrosome reaction when exposed to high K^+ (60 mM for mouse sperm) (Florman et al. 1992). If sperm are incubated in the absence of both albumin and HCO_3^-, they do not acquire the ability to undergo acrosome reaction. However, if non-capacitated sperm are hyperpolarized with pharmacological agents such as valinomycin or with the aforementioned Na^+ transport blocker amiloride, they acquire the ability to react when exposed to high K^+, to solubilized ZP (De La Vega-Beltran et al. 2012), or to progesterone (Stival et al. 2015). Interestingly, the hyperpolarizing reagents did not induce other capacitation-associated processes such as PKA activation or the increase in tyrosine phosphorylation (De La Vega-Beltran et al. 2012). Moreover, inhibition of the tyrosine kinase cSrc, which prevents acrosome reaction (see below), could also be bypassed with K^+ ionophore, restoring Em hyperpolarization and the ability to undergo acrosome reaction (Stival et al. 2015). To analyze the necessity of hyperpolarization for the acrosome reaction, sperm were incubated in conditions that support capacitation (media containing both albumin and HCO_3^-) but in the presence of high K^+ concentrations (70 mM) to clamp the Em in a depolarized state. These sperm underwent normal PKA activation and the increase in tyrosine phosphorylation but were unable to undergo acrosome reaction when exposed to solubilized zona pellucida (De La Vega-Beltran et al. 2012). Finally, sperm from SLO3 KO mice do not hyperpolarize during capacitation and cannot undergo acrosome reaction (Santi et al. 2010). Altogether, these pharmacological and genetic approaches strongly suggest that hyperpolarization is essential to prepare the sperm for the acrosome reaction during capacitation.

5.3 Link Between Em Hyperpolarization and Other Capacitation Associated Events

At the starting of this review we asked how signaling events occurring during capacitation prepare mammalian sperm for a physiological acrosome reaction. As described in previous sections, a link between these processes is likely to be at the

level of the Em hyperpolarization observed during capacitation. One of the first signaling pathways observed during capacitation is the HCO_3^--dependent stimulation of cAMP synthesis which activates PKA. In turn, PKA activation appears to be necessary for Em hyperpolarization (Escoffier et al. 2015; Hernandez-Gonzalez et al. 2006). However, how PKA signals Em changes is not well understood. Considering the evidence mentioned above, hyperpolarization changes are due to SLO3 opening which increased K^+ permeability almost three times (Chavez et al. 2013). Therefore, to better understand the link between these processes, it will be necessary to investigate the connection between the cAMP pathway and SLO3 modulation. One possibility is that PKA directly phosphorylates SLO3 and induces its activation. Although this possibility cannot be discarded, there is no direct evidence of its occurrence. An alternative possibility is that the capacitation-associated increase in pHi mediates the action of cAMP on SLO3 activation. Consistently, the aforementioned sperm-specific NHE contains a cAMP-binding consensus sequence; it can be speculated that upon the increase in cAMP, this exchanger is activated, and therefore, the consequent intracellular alkalinization directly activates SLO3 (Lishko et al. 2012). This hypothesis is sound; however, conclusive demonstration will require further experimentation. In addition, this possibility does not take into account the role of PKA. A third alternative to explain the observed results is that SLO3 activation is mediated by other enzymes downstream of the cAMP/PKA pathway.

One of the possible candidates to mediate the connection between PKA and SLO3 activation is cSrc, a tyrosine kinase known to be present in mammalian sperm (Krapf et al. 2010; Baker et al. 2006; Lawson et al. 2008; Battistone et al. 2013; Krapf et al. 2012; Bragado et al. 2012). Although this enzyme was initially proposed to be the tyrosine kinase mediating the capacitation-associated increase in tyrosine phosphorylation (Baker et al. 2006), it was later shown that sperm from knockout mice models lacking cSrc display normal levels of tyrosine phosphorylation upon capacitation (Krapf et al. 2010). Despite not being involved in the regulation of the capacitation-associated increase in tyrosine phosphorylation, cSrc KO sperm are barely motile and they are unable to fertilize (Krapf et al. 2010), indicating an essential role of this enzyme for male reproduction. More recently, we used a combination of pharmacology and antibodies that detect the activated form Tyr(P)416-Src to evaluate the role of this tyrosine kinase in capacitation (Stival et al. 2015). Our results indicated that cSrc was activated downstream PKA in mouse sperm (Stival et al. 2015). In addition, we showed that cSrc inhibitors blocked Em hyperpolarization and the ability of progesterone to induce the acrosome reaction in capacitated sperm. Moreover, acrosome reaction responsiveness was rescued by inducing hyperpolarization with the K^+ ionophore valinomycin (Stival et al. 2015). Consistently, in human (Varano et al. 2008), bovine (Etkovitz et al. 2009), and porcine sperm (Bragado et al. 2012), cSrc was shown to be involved in the acquisition of acrosomal responsiveness, species in which cSrc inhibition completely blocked the progesterone-induced acrosome reaction. Altogether, these results support the hypothesis that cSrc is involved in the regulation of Em downstream of PKA activation. However, the extent by which the effect of cSrc

is mediated by SLO3 is still not known. Interestingly, electrophysiological recordings derived from *Xenopus* oocytes expressing mammalian SLO3 suggest that inhibition of cSrc leads to decrease of SLO3 currents (Stival et al. 2015). Whether this decrease is due to direct or indirect phosphorylation pathways will require direct electrophysiological assays of mature sperm.

5.4 Concluding Remarks

As stated in the introduction, how mammalian sperm acquire acrosome reaction responsiveness during capacitation is still not well understood. Although it is clear that two of the main physiological endpoints of capacitation are the ability to undergo acrosome reaction and the change in motility pattern known as hyperactivation, whether these processes are coordinately regulated or are independent is not known. Remarkably, PKA activation appears to be upstream most events associated with capacitation. However, this protein kinase has not been found in the sperm head, raising the question of how PKA could mediate events related to the acrosome reaction. In this review, we explored evidence suggesting a possible mechanism by which changes in the flagellum can signal the preparation for the acrosome reaction. Although it is not the only possible explanation, one hypothesis consistent with experimental observations is that PKA in the sperm tail regulates changes in the sperm membrane potential that will be felt in the complete cell including the plasma membrane surrounding the acrosome. As in all scientific inquiry, this hypothesis raises new questions. For example, it is not clear how a hyperpolarizing change in the sperm Em signals the preparation for the acrosome reaction. One possibility is that hyperpolarization mediates transformation of a Ca^{2+} channel from an inactive to a closed state as was proposed for the T channels (Arnoult et al. 1999). Another possibility is that these changes in Em favor contacts between the outer acrosomal membrane and the plasma membrane. This type of contacts has been observed using electronic microscopy (Zanetti and Mayorga 2009). At the molecular level, the identity of the molecules mediating these events has remained elusive. One of the reasons is the difficulties inherent to mammalian sperm which are not amenable to many relatively common techniques used in other cell types such as RNAi silencing or overexpression of molecules by cDNA transfection. In addition, many of the sperm compartments are at distances below the separation limits of normal immunofluorescence techniques. Promising new technical advances such as gene editing, super resolution, and helium ion microscopy are expected to significantly improve our understanding of those capacitation changes that regulate sperm exocytosis.

Acknowledgments This work was supported by Agencia Nacional de Promoción Científica y Tecnológica de Argentina PICT 2014-2702 (to DK) and PICT 2012-1175 (to MGB); NIH R01 HD44044 and HD038082 (to PEV), R01-TW008662 (to MGB); and the National Research Council of Argentina PIP 112-201101-00740 (to MGB and DK).

References

Aitken RJ, Nixon B (2013) Sperm capacitation: a distant landscape glimpsed but unexplored. Mol Hum Reprod 19:785–793

Arcelay E, Salicioni AM, Wertheimer E, Visconti PE (2008) Identification of proteins undergoing tyrosine phosphorylation during mouse sperm capacitation. Int J Dev Biol 52:463–472

Arnoult C, Cardullo RA, Lemos JR, Florman HM (1996) Activation of mouse sperm T-type Ca2+ channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci U S A 93:13004–13009

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM (1999) Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. Proc Natl Acad Sci U S A 96:6757–6762

Austin CR (1952) The capacitation of the mammalian sperm. Nature 170:326

Bailey JL (2010) Factors regulating sperm capacitation. Syst Biol Reprod Med 56:334–348

Baker MA, Hetherington L, Aitken RJ (2006) Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. J Cell Sci 119:3182–3192

Battistone MA, Da Ros VG, Salicioni AM, Navarrete FA, Krapf D, Visconti PE, Cuasnicu PS (2013) Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. Mol Hum Reprod 19:570–580

Bragado MJ, Gil MC, Martin-Hidalgo D, Hurtado DL, Bravo N, Moreno AD, Garcia-Marin LJ (2012) Src family tyrosine kinase regulates acrosome reaction but not motility in porcine spermatozoa. Reproduction 144:67–75

Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Krahling M, Muller A, Kaupp UB, Strunker T (2012) The CatSper channel: a polymodal chemosensor in human sperm. EMBO J 31:1654–1665

Brenker C, Zhou Y, Muller A, Echeverry FA, Trotschel C, Poetsch A, Xia XM, Bonigk W, Lingle CJ, Kaupp UB, Strunker T (2014) The Ca2 + -activated K+ current of human sperm is mediated by Slo3. Elife 3, e01438

Buffone MG, Ijiri TW, Cao W, Merdiushev T, Aghajanian HK, Gerton GL (2012) Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility. Mol Reprod Dev 79:4–18

Buffone MG, Wertheimer EV, Visconti PE, Krapf D (2014) Central role of soluble adenylyl cyclase and cAMP in sperm physiology. Biochim Biophys Acta 1842:2610–2620

Butterworth MB (2010) Regulation of the epithelial sodium channel (ENaC) by membrane trafficking. Biochim Biophys Acta 1802:1166–1177

Chang MC (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:697–698

Chang MC (1959) Fertilization of rabbit ova in vitro. Nature 184(Suppl 7):466–467

Chavez JC, Hernandez-Gonzalez EO, Wertheimer E, Visconti PE, Darszon A, Trevino CL (2012) Participation of the Cl-/HCO(3)- exchangers SLC26A3 and SLC26A6, the Cl- channel CFTR, and the regulatory factor SLC9A3R1 in mouse sperm capacitation. Biol Reprod 86:1–14

Chavez JC, De La Vega-Beltran JL, Escoffier J, Visconti PE, Trevino CL, Darszon A, Salkoff L, Santi CM (2013) Ion permeabilities in mouse sperm reveal an external trigger for SLO3-dependent hyperpolarization. PLoS One 8, e60578

Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR, Buck J (2000) Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 289:625–628

Chen WY, Xu WM, Chen ZH, Ni Y, Yuan YY, Zhou SC, Zhou WW, Tsang LL, Chung YW, Hoglund P, Chan HC, Shi QX (2009) Cl- is required for HCO3- entry necessary for sperm capacitation in guinea pig: involvement of a Cl-/HCO3- exchanger (SLC26A3) and CFTR. Biol Reprod 80:115–123

Chung JJ, Navarro B, Krapivinsky G, Krapivinsky L, Clapham DE (2011) A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa. Nat Commun 2:153

De La Vega-Beltran JL, Sanchez-Cardenas C, Krapf D, Hernandez-Gonzalez EO, Wertheimer E, Trevino CL, Visconti PE, Darszon A (2012) Mouse sperm membrane potential hyperpolarization is necessary and sufficient to prepare sperm for the acrosome reaction. J Biol Chem 287:44384–44393

Demarco IA, Espinosa F, Edwards J, Sosnik J, De La Vega-Beltran JL, Hockensmith JW, Kopf GS, Darszon A, Visconti PE (2003) Involvement of a Na+/HCO-3 cotransporter in mouse sperm capacitation. J Biol Chem 278:7001–7009

Escoffier J, Boisseau S, Serres C, Chen CC, Kim D, Stamboulian S, Shin HS, Campbell KP, De Waard M, Arnoult C (2007) Expression, localization and functions in acrosome reaction and sperm motility of Ca(V)3.1 and Ca(V)3.2 channels in sperm cells: an evaluation from Ca(V)3.1 and Ca(V)3.2 deficient mice. J Cell Physiol 212:753–763

Escoffier J, Krapf D, Navarrete F, Darszon A, Visconti PE (2012) Flow cytometry analysis reveals a decrease in intracellular sodium during sperm capacitation. J Cell Sci 125:473–485

Escoffier J, Navarrete F, Haddad D, Santi CM, Darszon A, Visconti PE (2015) Flow cytometry analysis reveals that only a subpopulation of mouse sperm undergoes hyperpolarization during capacitation. Biol Reprod 92:121

Espinosa F, Darszon A (1995) Mouse sperm membrane potential: changes induced by Ca2+. FEBS Lett 372:119–125

Etkovitz N, Tirosh Y, Chazan R, Jaldety Y, Daniel L, Rubinstein S, Breitbart H (2009) Bovine sperm acrosome reaction induced by G-protein-coupled receptor agonists is mediated by epidermal growth factor receptor transactivation. Dev Biol 334:447–457

Florman HM, Corron ME, Kim TD, Babcock DF (1992) Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. Dev Biol 152:304–314

Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM (1998) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. Biol Reprod 59:12–16

Gervasi MG, Marczylo TH, Lam PM, Rana S, Franchi AM, Konje JC, Perez-Martinez S (2013) Anandamide levels fluctuate in the bovine oviduct during the oestrous cycle. PLoS One 8, e72521

Hamamah S, Gatti JL (1998) Role of the ionic environment and internal pH on sperm activity. Hum Reprod 13(Suppl 4):20–30

Harayama H (2013) Roles of intracellular cyclic AMP signal transduction in the capacitation and subsequent hyperactivation of mouse and boar spermatozoa. J Reprod Dev 59:421–430

Hernandez-Gonzalez EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, Lopez-Gonzalez I, Demarco I, Wertheimer E, Darszon A, Visconti PE (2006) Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyperpolarization in mouse sperm. J Biol Chem 281:5623–5633

Hernandez-Gonzalez EO, Trevino CL, Castellano LE, De La Vega-Beltran JL, Ocampo AY, Wertheimer E, Visconti PE, Darszon A (2007) Involvement of cystic fibrosis transmembrane conductance regulator in mouse sperm capacitation. J Biol Chem 282:24397–24406

Kavanagh JP (1985) Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. J Reprod Fertil 75:35–41

Khan MS, Zaman S, Sajjad M, Shoaib M, Gilani G (2011) Assessment of the level of trace element zinc in seminal plasma of males and evaluation of its role in male infertility. Int J Appl Basic Med Res 1:93–96

Kirichok Y, Navarro B, Clapham DE (2006) Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. Nature 439:737–740

Kleinboelting S, Diaz A, Moniot S, van den Heuvel J, Weyand M, Levin LR, Buck J, Steegborn C (2014) Crystal structures of human soluble adenylyl cyclase reveal mechanisms of catalysis and of its activation through bicarbonate. Proc Natl Acad Sci U S A 111:3727–3732

Krapf D, Arcelay E, Wertheimer EV, Sanjay A, Pilder SH, Salicioni AM, Visconti PE (2010) Inhibition of Ser/Thr phosphatases induces capacitation-associated signaling in the presence of Src kinase inhibitors. J Biol Chem 285:7977–7985

Krapf D, Ruan YC, Wertheimer EV, Battistone MA, Pawlak JB, Sanjay A, Pilder SH, Cuasnicu P, Breton S, Visconti PE (2012) cSrc is necessary for epididymal development and is incorporated into sperm during epididymal transit. Dev Biol 369:43–53

Lawson C, Goupil S, Leclerc P (2008) Increased activity of the human sperm tyrosine kinase SRC by the cAMP-dependent pathway in the presence of calcium. Biol Reprod 79:657–666

Lievano A, Santi CM, Serrano CJ, Trevino CL, Bellve AR, Hernandez-Cruz A, Darszon A (1996) T-type Ca2+ channels and alpha1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. FEBS Lett 388:150–154

Lishko PV, Kirichok Y (2010) The role of Hv1 and CatSper channels in sperm activation. J Physiol 588:4667–4672

Lishko PV, Botchkina IL, Fedorenko A, Kirichok Y (2010) Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. Cell 140:327–337

Lishko PV, Kirichok Y, Ren D, Navarro B, Chung JJ, Clapham DE (2012) The control of male fertility by spermatozoan ion channels. Annu Rev Physiol 74:453–475

Liu J, Xia J, Cho KH, Clapham DE, Ren D (2007) CatSperbeta, a novel transmembrane protein in the CatSper channel complex. J Biol Chem 282:18945–18952

Lopez-Gonzalez I, Torres-Rodriguez P, Sanchez-Carranza O, Solis-Lopez A, Santi CM, Darszon A, Trevino CL (2014) Membrane hyperpolarization during human sperm capacitation. Mol Hum Reprod 20(7):619–629

Martinez-Lopez P, Santi CM, Trevino CL, Ocampo-Gutierrez AY, Acevedo JJ, Alisio A, Salkoff LB, Darszon A (2009) Mouse sperm K+ currents stimulated by pH and cAMP possibly coded by Slo3 channels. Biochem Biophys Res Commun 381:204–209

Navarro B, Kirichok Y, Clapham DE (2007) KSper, a pH-sensitive K+ current that controls sperm membrane potential. Proc Natl Acad Sci U S A 104:7688–7692

Pastor-Soler N, Pietrement C, Breton S (2005) Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. Physiology (Bethesda) 20:417–428

Publicover SJ, Barratt CL (1999) Voltage-operated Ca2+ channels and the acrosome reaction: which channels are present and what do they do? Hum Reprod 14:873–879

Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, Kirichok Y, Ramsey IS, Quill TA, Clapham DE (2007) All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc Natl Acad Sci U S A 104:1219–1223

Ramsey IS, Ruchti E, Kaczmarek JS, Clapham DE (2009) Hv1 proton channels are required for high-level NADPH oxidase-dependent superoxide production during the phagocyte respiratory burst. Proc Natl Acad Sci U S A 106:7642–7647

Ren D, Xia J (2010) Calcium signaling through CatSper channels in mammalian fertilization. Physiology (Bethesda) 25:165–175

Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham DE (2001) A sperm ion channel required for sperm motility and male fertility. Nature 413:603–609

Romarowski A, Battistone MA, La Spina FA, Puga Molina LC, Luque GM, Vitale AM, Cuasnicu PS, Visconti PE, Krapf D, Buffone MG (2015) PKA-dependent phosphorylation of LIMK1 and Cofilin is essential for mouse sperm acrosomal exocytosis. Dev Biol 405:237–249

Salicioni AM, Platt MD, Wertheimer EV, Arcelay E, Allaire A, Sosnik J, Visconti PE (2007) Signalling pathways involved in sperm capacitation. Soc Reprod Fertil Suppl 65:245–259

Sanchez-Carranza O, Torres-Rodriguez P, Darszon A, Trevino CL, Lopez-Gonzalez I (2015) Pharmacology of hSlo3 channels and their contribution in the capacitation-associated hyperpolarization of human sperm. Biochem Biophys Res Commun 466:554–559

Santi CM, Martinez-Lopez P, De La Vega-Beltran JL, Butler A, Alisio A, Darszon A, Salkoff L (2010) The SLO3 sperm-specific potassium channel plays a vital role in male fertility. FEBS Lett 584:1041–1046

Steptoe PC, Edwards RG (1978) Birth after the reimplantation of a human embryo. Lancet 2:366
Stival C, La Spina FA, Baro GC, Arcelay E, Arranz SE, Ferreira JJ Sr, Le Grand S, Santi CM, Visconti PE, Buffone MG, Krapf D (2015) Src is the connecting player between PKA activation and hyperpolarization through SLO3 regulation in mouse sperm. J Biol Chem 290 (30):18855–18864
Strunker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB (2011) The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature 471:382–386
Suarez SS, Pacey AA (2006) Sperm transport in the female reproductive tract. Hum Reprod Update 12:23–37
Sumigama S, Mansell S, Miller M, Lishko PV, Cherr GN, Meyers SA, Tollner T (2015) Progesterone accelerates the completion of sperm capacitation and activates CatSper channel in spermatozoa from the rhesus macaque. Biol Reprod 93:130
Takeshita K, Sakata S, Yamashita E, Fujiwara Y, Kawanabe A, Kurokawa T, Okochi Y, Matsuda M, Narita H, Okamura Y, Nakagawa A (2014) X-ray crystal structure of voltage-gated proton channel. Nat Struct Mol Biol 21:352–357
Varano G, Lombardi A, Cantini G, Forti G, Baldi E, Luconi M (2008) Src activation triggers capacitation and acrosome reaction but not motility in human spermatozoa. Hum Reprod 23:2652–2662
Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139–1150
Visconti PE, Krapf D, De La Vega-Beltran JL, Acevedo JJ, Darszon A (2011) Ion channels, phosphorylation and mammalian sperm capacitation. Asian J Androl 13:395–405
Wandernoth PM, Mannowetz N, Szczyrba J, Grannemann L, Wolf A, Becker HM, Sly WS, Wennemuth G (2015) Normal fertility requires the expression of carbonic anhydrases II and IV in sperm. J Biol Chem 290:29202–29216
Wang D, King SM, Quill TA, Doolittle LK, Garbers DL (2003) A new sperm-specific Na+/H+ exchanger required for sperm motility and fertility. Nat Cell Biol 5:1117–1122
Wang D, Hu J, Bobulescu IA, Quill TA, McLeroy P, Moe OW, Garbers DL (2007) A sperm-specific Na+/H+ exchanger (sNHE) is critical for expression and in vivo bicarbonate regulation of the soluble adenylyl cyclase (sAC). Proc Natl Acad Sci U S A 104:9325–9330
Wang H, Liu J, Cho KH, Ren D (2009) A novel, single, transmembrane protein CATSPERG is associated with CATSPER1 channel protein. Biol Reprod 81:539–544
Wertheimer E, Krapf D, De La Vega-Beltran JL, Sanchez-Cardenas C, Navarrete F, Haddad D, Escoffier J, Salicioni AM, Levin LR, Buck J, Mager J, Darszon A, Visconti PE (2013) Compartmentalization of distinct cAMP signaling pathways in mammalian sperm. J Biol Chem 288:35307–35320
Xu WM, Shi QX, Chen WY, Zhou CX, Ni Y, Rowlands DK, Yi LG, Zhu H, Ma ZG, Wang XF, Chen ZH, Zhou SC, Dong HS, Zhang XH, Chung YW, Yuan YY, Yang WX, Chan HC (2007) Cystic fibrosis transmembrane conductance regulator is vital to sperm fertilizing capacity and male fertility. Proc Natl Acad Sci U S A 104:9816–9821
Zanetti N, Mayorga LS (2009) Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. Biol Reprod 81:396–405
Zeng Y, Clark EN, Florman HM (1995) Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev Biol 171:554–563
Zeng Y, Oberdorf JA, Florman HM (1996) pH regulation in mouse sperm: identification of Na(+)-, Cl(-)-, and HCO3(-)-dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. Dev Biol 173:510–520
Zeng XH, Navarro B, Xia XM, Clapham DE, Lingle CJ (2013) Simultaneous knockout of Slo3 and CatSper1 abolishes all alkalization- and voltage-activated current in mouse spermatozoa. J Gen Physiol 142:305–313

Chapter 6
Lipid Regulation of Acrosome Exocytosis

Roy Cohen, Chinatsu Mukai, and Alexander J. Travis

6.1 Introduction to Membrane Lipids

Lipids are critical regulators of mammalian sperm function, first helping prevent premature acrosome exocytosis, then enabling sperm to become competent to fertilize at the right place/time through the process of capacitation, and ultimately triggering acrosome exocytosis. Yet because they do not fit neatly into the "DNA–RNA–protein" synthetic pathway, they are understudied and poorly understood. Here, we focus on three lipids or lipid classes—cholesterol, phospholipids, and the ganglioside G_{M1}—in context of the modern paradigm of acrosome exocytosis. We describe how these various species are precisely segregated into membrane macrodomains and microdomains, simultaneously preventing premature exocytosis while acting as foci for organizing regulatory and effector molecules that will enable exocytosis. Although the mechanisms responsible for these domains are poorly defined, there is substantial evidence for their composition and functions. We present diverse ways that lipids and lipid modifications regulate capacitation and acrosome exocytosis, describing in more detail how removal of cholesterol plays a master regulatory role in enabling exocytosis through at least two complementary pathways. First, cholesterol efflux leads to proteolytic activation of

R. Cohen • C. Mukai
Baker Institute for Animal Health, Cornell University, Ithaca, NY 14853, USA

A.J. Travis (✉)
Baker Institute for Animal Health, Cornell University, Ithaca, NY 14853, USA

Atkinson Center for a Sustainable Future, Cornell University, Ithaca, NY 14853, USA
e-mail: ajt32@cornell.edu

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_6

phospholipase B, which cleaves both phospholipid tails. The resultant changes in membrane curvature provide a mechanism for the point fusions now known to occur far before a sperm physically interacts with the zona pellucida. Cholesterol efflux also enables G_{M1} to regulate the voltage-dependent cation channel, CaV2.3, triggering focal calcium transients required for acrosome exocytosis in response to subsequent whole-cell calcium rises. We close with a model integrating functions for lipids in regulating acrosome exocytosis.

Excellent reviews of membrane lipids are available to provide a comprehensive introduction to these biological components (van Meer et al. 2008; Simons and Sampaio 2011). Here, we provide only the briefest of introductions to set the stage for the multiple functions of lipids in acrosome exocytosis. Cells produce an almost staggering diversity of lipid species to carry out widely varying functions. Lipids act as caches of energy. They provide a barrier between the cell and the outside world, organizing and segregating components within specific organelles versus the cytoplasm or within a cell versus the extracellular environment. These barriers are mutable, however, and lipids provide cells with the flexibility to allow movement between membranous compartments. Lipids can provide a means for focal organization of signaling or membrane fusion machinery. They can regulate the activity of ion channels and other components of signaling pathways, and they can act as first and second messengers in signaling pathways. Excepting energy storage, all of these functions are directly relevant to regulated exocytosis in sperm and will be touched upon in this review.

As in all cells, the sperm plasma membrane exists as a bilayer, with asymmetric distribution of both lipid and protein components. Phosphatidylcholine, sphingomyelin, and glycosphingolipids are typically in the outer leaflet, and phosphatidylethanolamine and phosphatidylserine are in the inner leaflet. Cholesterol is the major nonpolar lipid in mammalian cells. Sperm are noteworthy for also having desmosterol (Lin et al. 1993), though the vast majority of desmosterol is found in the sperm tail in primates (Connor et al. 1998). Cholesterol exists affiliated with both leaflets, and its relative abundance and organization play major regulatory roles in capacitation and acrosome exocytosis and are discussed further below. Sterol sulfates have a wide tissue distribution and have been suggested to play a stabilizing role in various cells, including the sperm plasma membrane overlying the acrosome (Langlais et al. 1981; Roberts 1987; Strott and Higashi 2003).

The gangliosides, glycosphingolipids with terminal sialic acids, are found in lower abundance, but are an excellent example of the combinatorial complexity that has kept our understanding of lipids at a nascent stage. There are at least 5 sphingoid bases, more than 20 species of fatty acid, and around 500 carbohydrate structures that define the gangliosides (Kolter et al. 2002; Futerman and Hannun 2004). Using

G_{M1} as a specific example of a glycosphingolipid, it is known that G_{M1} comprises a ceramide tail and has five sugars in its carbohydrate headgroup. Ceramide is itself made of a sphingoid base and a fatty acid chain that can vary in length and bond structure. The sugar residues that define the gangliosides (G_{M1} is a monosialic acid) can also differ in linkages; for example, G_{M1a} has the sialic acid attached to galactose nearest the ceramide, whereas G_{M1b} has the sialic acid attached to the terminal galactose. Therefore, when referring to G_{M1} in a cell, one is actually referring to a *group of molecules, or one of a possible number of slightly different molecules*. As studies have uncovered critical roles for lipids in sperm, discerning the functions associated with specific linkages or chain lengths might one day have a similar effect on lipid biology as study of posttranslational modifications did for our understanding of protein function.

6.2 Compartmentalization of the Plasma Membrane of the Sperm Head

The extreme compartmentalization of structure and function in mammalian sperm is one of the defining characteristics of these cells. Polarization into tail and head is the most obvious division. The flagellum is organized further into a midpiece, principal piece, and endpiece, and the head is also polarized with the presence of a single exocytotic vesicle, the acrosome, situated apically. The plasma membrane on the sperm head is similarly organized, with distinct macrodomains being the acrosomal plasma membrane (APM) and the post-acrosomal plasma membrane (PAPM). Compartmentalization extends further, with the APM being subdivided into the fusogenic apical acrosomal domain (AA) and the larger equatorial segment (ES). These domains are presented schematically for mouse and human sperm in Fig. 6.1 (a–b). Note that all these regions are macrodomains, stably existing at a micron scale in sperm from multiple species due to interactions between the membrane and underlying cytoskeletal or other structures. In this regard, sperm are similar to epithelial cells with defined apical versus basolateral compartments and cells with specialized macrodomains such as immune synapses.

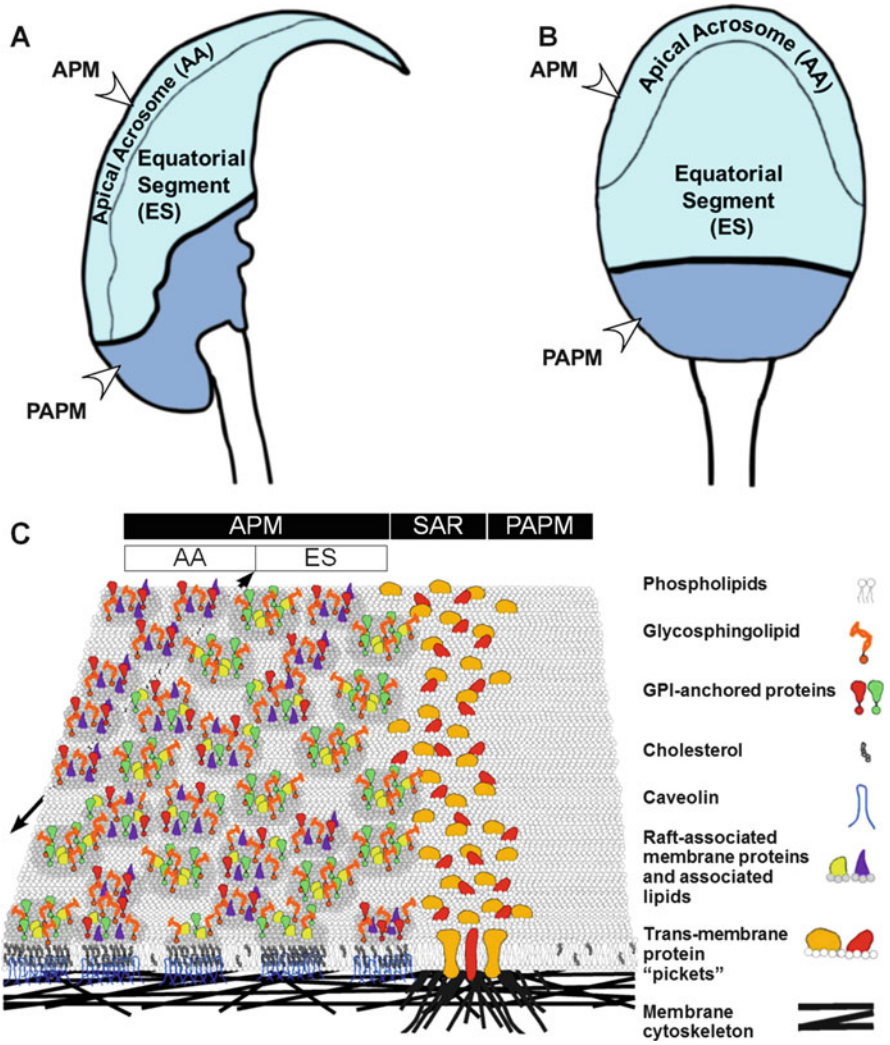

Fig. 6.1 Organization and maintenance of membrane macrodomains and microdomains in mammalian sperm. Schematic of murine (**a**) and human (**b**) sperm showing the plasma membrane overlying the acrosome (APM) in *light blue* and post-acrosomal plasma membrane (PAPM) in *darker blue*. The AA and ES macrodomains lie within the APM. Panel (**c**) presents a model for the existence and maintenance of membrane raft microdomains in the APM. In this model, interactions between underlying cytoskeletal elements and membrane proteins and membrane lipids constitute the sub-acrosomal ring (SAR), which acts as a "picket fence," allowing some lipid components to pass between the APM and PAPM freely, but which restricts different species of membrane raft to the APM. Note that the raft microdomains are themselves dynamic and move within the APM, but specific components differ between the AA and ES macrodomains, with the boundary between them delineated by the black arrow. Panels adapted from Selvaraj et al. (2009)

6.3 Membrane Rafts in Sperm: Visualization

The theme of compartmentalization extends to the molecular level in terms of how lipids are organized in the plasma membrane. Due to its functional importance and the clear organization of the macrodomains, multiple studies were performed to localize cholesterol. Most utilized the antibiotic filipin, which is both fluorescent and can be seen on freeze fracture electron microscopy because of the globular deformations it causes when complexed with sterols. Regardless of approach, these studies showed a remarkable, uniform enrichment in the APM vs the PAPM (Friend 1982; Pelletier and Friend 1983; Visconti et al. 1999b). The effect of cholesterol enrichment on membrane behavior is complex and depends upon the other lipids and proteins in the local membrane microenvironment. Biophysical models show that membranes have low fluidity with a phospholipid composition, and then increasing amounts of cholesterol lead to increases in fluidity, until sterol concentrations reach a point where the membrane enters a liquid-ordered, less fluid state (Feigenson 2009). These changes are known to affect membrane fusion and permeability to various ions, underscoring the potential relevance of these domains to our understanding of regulated exocytosis in sperm.

Indeed, the finding of cholesterol enrichment in the APM was contemporaneous with functional studies showing that removal of cholesterol during sperm transit through the female tract or in vitro was one of the primary stimuli for "capacitation" (Davis 1981; Go and Wolf 1985)—the process through which sperm become able to fertilize an egg (Travis and Kopf 2002). Sterol efflux, or reverse cholesterol transport, was later linked biochemically to stimulation of signaling cascades, such as those involving elevations in cAMP and protein tyrosine phosphorylation, which promote acquisition of a hyperactivated pattern of motility as well as ability to undergo acrosome exocytosis (Visconti et al. 1995, 1999a). Removal of hydrophobic sterols from a membrane to extracellular acceptors is not energetically favorable, and multiple molecules have been suggested to mediate this key regulatory step [Please see Leahy and Gadella for a recent review (Leahy and Gadella 2015)]. In vivo, these molecules pass membrane cholesterol to acceptors such as high-density lipoproteins and albumin in the oviductal fluid; interestingly, their capacity to accept sterols increases during estrus as opposed to other phases of the estrous cycle (Ehrenwald et al. 1990).

Together, these results led to a model of sperm function in which initial enrichment of sterols in the APM would provide two critical points of regulation: First, enrichment would provide a mechanism to prevent premature fusion of the APM with the underlying outer acrosomal membrane. Premature fusion would have devastating consequences on male fertility, whether during storage in the epididymides or transit through the distal female reproductive tract. Second, removal or efflux of sterols from the membrane would provide a mechanism to signal the sperm to become ready to fertilize an egg. However, the mechanism(s) by which this membrane change is transduced into changes in cellular function remained unclear until recently.

To address this issue of linking changes in membrane cholesterol to changes in sperm function, several laboratories including our own turned their attention to new models of membrane organization. Although the fluid-mosaic model governed our understanding of biological membranes for several decades, numerous studies over the past 20 years have provided evidence against the notion that membrane lipids merely provide a fluid "solvent" in which membrane proteins could move (Simons and Gerl 2010). Rather, this new model recognized that membrane lipids and proteins were often organized into focal enrichments or microdomains. The best-characterized example of membrane microdomain is the membrane raft, a nanometer-scale, dynamic focal enrichment of sterols, gangliosides, and associated proteins. Functionally, these microdomains are believed to provide foci for organization and regulation of signaling complexes. However, because of their small size and dynamic nature, the existence of rafts has been controversial for some time [reviewed in (Simons and Gerl 2010)]. Methods used to visualize rafts or raft components typically utilize fixation or other reagents that cross-link molecules, such as the B subunit of cholera toxin (CTB), which can bind up to five molecules of G_{M1}. Such approaches can induce patching, artifactually aggregating and stabilizing components in close proximity.

When used to localize putative rafts in sperm, these cell biological approaches provided results consistent with the localization of sterols—the APM was enriched in sterol-binding proteins such as caveolin-1 (Travis et al. 2001), and the ganglioside G_{M1} in heavily fixed cells (Selvaraj et al. 2006). Interestingly, these were also found in the membranes of the developing acrosome (Travis et al. 2001; Selvaraj et al. 2009). Cognizant of the risks associated with relying on fixed cells, we also sought to visualize rafts in live sperm. Demonstrating that the abovementioned problems with using fixed cells are in fact valid issues, we found that G_{M1} was localized throughout the APM in live mouse, bull, and human sperm, but upon cell death or in lightly fixed cells, the CTB induced rapid movement of the cross-linked G_{M1} to the PAPM in non-capacitated sperm (Buttke et al. 2006; Selvaraj et al. 2006, 2009). In fact, the combination of cross-linking due to the fixative and CTB, coupled with the biophysical state of the membrane reflecting sterol concentration, induced different localization patterns of G_{M1} (Selvaraj et al. 2007). That is, G_{M1} localization patterns were different in lightly fixed murine and bovine sperm in a matter dependent upon capacitation status (Selvaraj et al. 2007).

Despite the complexity associated with visualizing lipids, over a series of studies we demonstrated (1) that segregation of the G_{M1} and cholesterol in live cells was due to a selectively permeable "fence" at the sub-acrosomal ring that relied at least in part on disulfide-bonded proteins (Fig. 6.1c) (Selvaraj et al. 2006, 2009); (2) that exogenous lipid probes did not necessarily show the same behaviors as endogenous lipids (Selvaraj et al. 2009); (3) that the G_{M1} was dynamic within the APM macrodomain but did not cross the sub-acrosomal ring (Selvaraj et al. 2009); (4) that despite the apparently uniform labeling by filipin, in live sperm the sterols were not uniformly distributed and were either organized in focal enrichments and/or were associated differentially in the inner vs outer leaflet (Selvaraj et al. 2009); and (5) that transfer of

lipids could occur between the APM and outer acrosomal membrane without release of the contents of the acrosome (Selvaraj et al. 2006, 2009).

These findings underscored the perils of using fixed cells, cross-linking reagents, and exogenous probes to visualize membrane rafts in sperm. From our first characterization of rafts in mammalian sperm (Travis et al. 2001), we therefore complemented imaging techniques with membrane biochemistry.

6.4 Membrane Rafts in Sperm: Biochemical and Proteomic Characterizations

In a parallel to the difficulties in interpreting localization in fixed cells, efforts to isolate rafts biochemically based on solubility in cold Triton X-100 (so-called detergent resistant membranes or DRM) have equally been subject to criticism. Although membranes enriched in sterols and sphingolipids as opposed to phospholipids are inherently more resistant to solubilization in cold detergent, this is hardly a physiological approach. One could easily imagine how solubilization of some membrane components might cause others to coalesce artifactually. Therefore, we also utilized another property to isolate rafts biochemically—the fact that they have lighter buoyant densities than surrounding non-raft membranes. Using this approach, we identified caveolin-1 as well as membrane fusion proteins such as syntaxin-2 in the buoyant fraction without use of detergent, as well as showing them partitioning with DRM (Travis et al. 2001).

We took our non-detergent isolation method one step further, replacing the commonly used step gradient with a linear density gradient. With this method, we were able to demonstrate that murine sperm reproducibly possess at least three subtypes of membrane raft microdomain (Asano et al. 2009) (Fig. 6.2a–d). The most buoyant fraction is enriched in both sterols and G_{M1} and, interestingly, also has the highest protein:total lipid ratio. The next most buoyant fraction has a high sterol:phospholipid ratio but lower amounts of G_{M1}, and the last had relatively high G_{M1} but only a slight enrichment of sterol compared to phospholipid (Asano et al. 2009). These findings demonstrated unequivocally that one cannot make generalizations about "raft vs non-raft" in terms of composition.

In addition to different species of raft existing in non-capacitated sperm, it has also been reported that rafts *form* as part of membrane rearrangements during capacitation. Two separate sets of evidence have been provided to support this finding. In the first, it was shown that exposure to bicarbonate could activate phospholipid scramblases. Members of this enzyme family create membrane disorder (increasing fluidity) by disrupting the phospholipid asymmetry between inner and outer leaflets in sperm of several species (Gadella and Harrison 2000; Rathi et al. 2001; de Vries et al. 2003). This work led to a model in which the increased membrane fluidity caused by the scramblases generated formation of one or more sterol-enriched membrane rafts in the APM, and these rafts then facilitated efflux

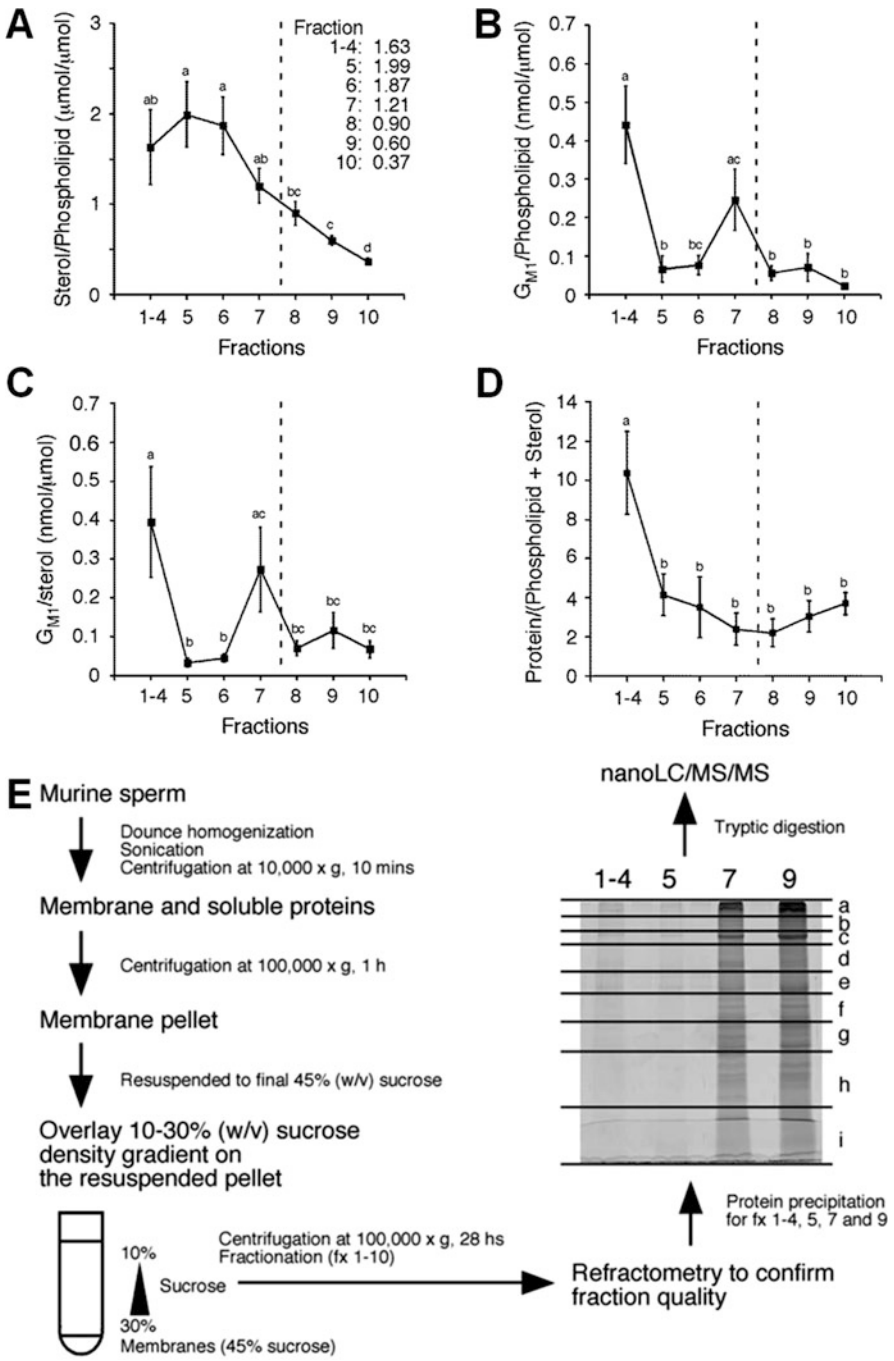

Fig. 6.2 Separation and quantitative biochemical characterization of membrane raft microdomains in murine sperm. Membrane vesicles were prepared from murine sperm and reproducibly separated in the absence of detergent by their inherent buoyancy in a linear density gradient. Sterols, phospholipids, G_{M1}, and total protein were quantified in these fractions and

(Flesch et al. 2001). In the second set of studies, it was shown that DRM from capacitated boar sperm had different properties than rafts isolated from non-capacitated sperm, including enhanced ability to bind the zona pellucida (Bou Khalil et al. 2006). These DRM were enriched in the male germ cell-specific sulfogalactosylglycerolipid (also known as seminolipid), which constitutes roughly 5 mol% of boar sperm lipids (Bou Khalil et al. 2006).

The concept of rafts as organizing foci for molecules involved in various aspects of sperm function is supported further by the results of proteomics studies (Fig. 6.2e). In a comparison of the three raft subtypes using shotgun proteomics, we found evidence that proteins such as apolipoprotein-A1 were added to sperm membrane rafts during epididymal transit (Asano et al. 2010). Indeed, many sperm raft proteins had high levels of epididymal expression, corroborating an earlier hypothesis that a function of sperm membrane rafts is to act as targets for selective acquisition of proteins (Sullivan et al. 2007). Of direct relevance to acrosome exocytosis, the third raft subtype, enriched in G_{M1} but having only slight enrichment in cholesterol and likely having large contributions from the acrosomal membranes (Asano et al. 2009, 2010), was found to contain a variety of calcium channels and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Asano et al. 2010). SNARE complex machinery is known to drive the membrane fusion events required for acrosome exocytosis (De Blas et al. 2005).

6.5 Membrane Rafts in Sperm: Dynamics During Capacitation

Beyond providing foci for cellular functions, these studies of membrane rafts and lipid order also present different, sometimes competing views about the temporal order of when rafts form and how sperm respond to stimuli for capacitation. Do rafts exist in sperm before capacitation or only arise after? Does bicarbonate influx lead to raft formation and sterol efflux, or is sterol efflux from preexisting rafts the primary upstream driver of capacitation? As is often the case with competing theories in biology, the truth with these questions is that both competing viewpoints have merit. Based on abundant biochemical and cell biological data, rafts clearly exist prior to capacitation. Yet this doesn't preclude new rafts from forming upon sterol efflux and phospholipid scrambling. Recall that our localization studies revealed that communication can occur between the APM and the outer acrosomal membrane without triggering full exocytosis. This finding is consistent with the studies driving the new paradigm of acrosome exocytosis, showing exposure of

Fig. 6.2 (continued) compared (**a-d**), clearly defining three subtypes of membrane raft microdomains in sperm, as well as at least one non-raft form of membrane. This same procedure (**e**) was employed to isolate the three raft subtypes for shotgun proteomics. Panels adapted from Asano et al. (2009, 2010)

acrosomal matrix components without full exocytosis (Kim and Gerton 2003; Kim et al. 2011), and that exocytosis can occur during transit through the cumulus cells long before physical contact with the zona pellucida (Jin et al. 2011). It is possible that communication between the APM and outer acrosomal membrane allows new rafts to form from components previously isolated in different membranes.

Regarding the primacy of bicarbonate versus sterol efflux in driving membrane changes associated with capacitation, our studies of how G_{M1} localization changed in response to stimuli for capacitation clearly showed that exposure to bicarbonate in the absence of a sterol acceptor caused a change in membrane organization in a subpopulation of cells (Selvaraj et al. 2007). This finding supports the work on scramblases and the ability for bicarbonate to change membrane properties without a requirement for sterol efflux. However, our same studies showed that exposure of sperm to a sterol acceptor in the absence of bicarbonate (in experiments performed under nitrogen to limit de novo formation of bicarbonate from carbon dioxide) also caused changes in membrane organization in a subpopulation of sperm (Selvaraj et al. 2007). Thus, both stimuli can independently promote membrane fluidity, which is necessary for exocytosis. Both stimuli also act on the intracellular signaling pathways important for capacitation, which enables the sperm to undergo acrosome exocytosis, providing positive feedback. Yet, these findings that phospholipid scrambling and cholesterol efflux regulate capacitation and therefore exocytosis don't in and of themselves provide the mechanisms for how changes in membrane lipids confer this ability upon capacitated sperm.

6.6 Membrane Rafts and Regulation of Acrosome Exocytosis

Before discussing how membrane raft lipids positively regulate and induce acrosome exocytosis, we must first step back and mention three other means by which lipids are involved in inhibition of premature exocytosis. The first is by having cholesterol sulfate present in the membrane; as noted above, sterol sulfates function widely as membrane stabilizers. In sperm, they also have been shown to be potent inhibitors of acrosin, a proteolytic enzyme found in the acrosomal matrix (Roberts 1987). It has been hypothesized that sulfatase activity in the female reproductive tract might release this inhibition (Roberts 1987). Addition of cholesterol sulfate has been shown to inhibit capacitation and acrosome exocytosis in sperm of several species (Cross 1996; Visconti et al. 1999b; Galantino-Homer et al. 2006), with potential applications for sperm cryopreservation and assisted reproductive technologies.

In addition to this built-in regulation, several aspects of sperm function are also modulated by interaction with components of seminal plasma. For example, seminal plasma has been found to have a "decapacitating" effect on sperm of multiple species. A specific component of murine seminal plasma, the protein SVS2, has been shown to exert this decapacitating effect by binding sperm surface G_{M1}

(Kawano and Yoshida 2007; Kawano et al. 2008). Loss of bound SVS2 as sperm move up the female tract then releases them from this inhibition. This interaction is quite potent in terms of preventing CTB from binding G_{M1} (Buttke et al. 2006) and was actually responsible for mistaken early assumptions that rafts were not initially present in sperm, but only appeared later during capacitation.

Another regulatory factor is the bovine seminal plasma (BSP) family of proteins, which bind sperm surface phosphatidylcholine and sphingomyelin. The BSPs have dual roles in helping promote viability, mobility, and sperm storage in the oviduct (Gwathmey et al. 2006), while also facilitating capacitation and sterol efflux (Manjunath and Therien 2002). One mechanism for this dual role is that in addition to binding sperm surface phospholipids, the BSPs also bind sterol acceptors, such as HDL (Manjunath and Therien 2002). In this way, they can help promote capacitation in the presence of appropriate sterol acceptors by bringing them near the membrane.

6.7 Sterol Efflux-Mediated Regulation of Sperm Phospholipids

In several respects, sterols are master regulators of capacitation and acrosome exocytosis, organizing signaling and effector proteins, inhibiting premature fusion, and also providing a release from that inhibition upon sterol efflux. Only in the past few years, as our understanding improved regarding the timing and nature of the initial point fusions that occur during capacitation and transit through the cumulus, have we begun to determine how that efflux actively promotes fusion. Prior models of acrosome exocytosis, including the long-dominant theory of the "acrosome reaction," relied upon contact between the plasma membrane of the sperm and specific molecules on the zona pellucida to induce fusion events between the AA macrodomain and underlying outer acrosomal membrane. Recent studies of sperm lipid function from several labs now provide insights into how fusion events can be stimulated long before contact with the zona, and they show interesting interactions between sterol modulation and downstream changes in plasma membrane phospholipids.

In our proteomic studies of sperm membrane rafts, we identified phospholipase B (PLB) from multiple specific peptide sequences (Asano et al. 2010). This membrane-anchored protein is found in a number of mammalian tissues, but is best known as a fungal virulence factor, where it is found in membrane rafts and, in at least one case, has its activity increased dramatically upon release from rafts due to sterol efflux, enabling adhesion and fusion with host cell membranes (Siafakas et al. 2006). Unlike other phospholipase family members, PLB can hydrolyze both the sn-1 and sn-2 acyl ester bonds of glycerophospholipids in a calcium-independent fashion (Fig. 6.3a). This gives it the activities of both phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2), and it also possesses lysophospholipase activity (Ghannoum 2000). These combined activities could potentially induce dramatic changes in membrane curvature, enhancing membrane fusion.

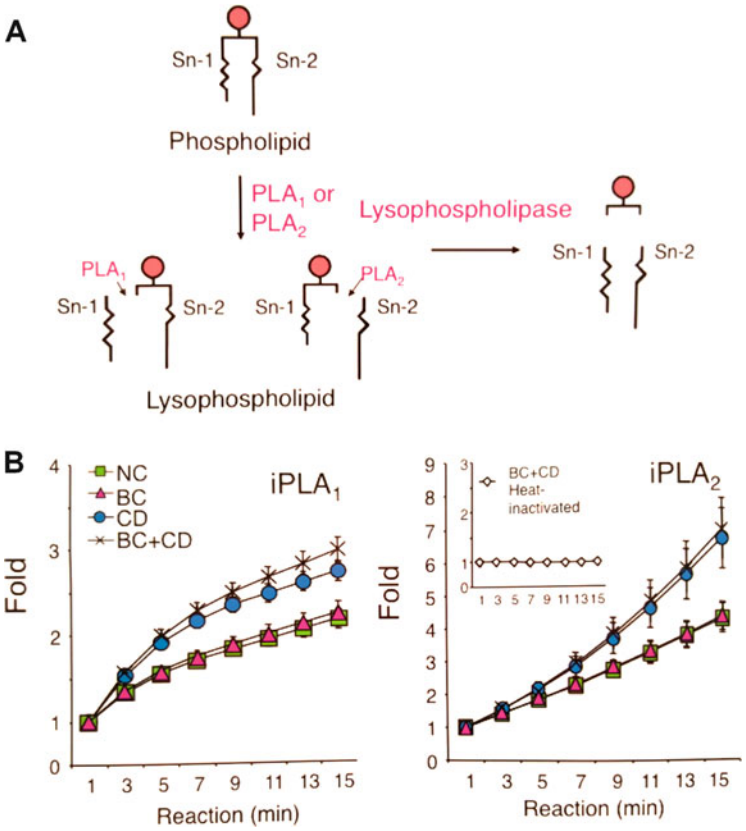

Fig. 6.3 Activities of phospholipase B (PLB). Schematic (**a**) shows PLB's PLA_1, PLA_2, and lysophospholipase activities. Both calcium-independent PLA_1 and PLA_2 activities were released from murine sperm (**b**) in response to sterol efflux mediated by 2-hydroxypropyl-β-cyclodextrin (CD), but not in response to bicarbonate (BC), which induced no change versus the non-capacitated state (NC). The inset shows that heat inactivated the calcium-independent PLA_2 activity released in response to capacitating conditions (BC + CD). Panel **b** adapted from Asano et al. (2013a)

Previously, PLB had been localized to the developing acrosome in rat spermatids (Takemori et al. 1998), but had not been studied in mature sperm. We identified it in both the APM and acrosomal membranes by indirect immunofluorescence and also confirmed an immunoreactive band of the appropriate size, 165 kDa (Asano et al. 2013b). Biochemical studies confirmed that sperm PLB is similar to other mammalian PLBs in being a transmembrane protein, as opposed to being GPI anchored as are the fungal forms (Asano et al. 2013b). Functionally, we found that both calcium-independent PLA_1 and PLA_2 activities were released from sperm during capacitation, in response to exposure to 2-hydroxypropyl-beta-cyclodextrin

(2-OHCD), a mediator of sterol efflux. Insignificant activity was released in the presence of bicarbonate alone or in non-capacitated sperm (Fig. 6.3b).

In examining the secretome, we identified a 50 kDa product that was immunoreactive with antibodies against PLB. A fraction containing this molecular weight range also had PLB activity (Asano et al. 2013b), suggesting that PLB is activated by proteolytic processing as has been shown in the intestine and epididymis (Delagebeaudeuf et al. 1998; Tojo et al. 1998). Corroborating this result, indirect immunofluorescence experiments showed PLB to be lost from the sperm surface in response to incubation with 2-OHCD (Asano et al. 2013b). Incubation of sperm with furin-specific inhibitors prevented release of enzyme activity and decreased the percentage of sperm undergoing acrosome exocytosis in response to progesterone down to levels seen in sperm incubated under non-capacitating conditions (Asano et al. 2013b). Unfortunately, no inhibitors are known to be specific for PLB that do not also inhibit PLA_1 or PLA_2. With that caveat in mind, incubation of sperm with DL-carnitine reduced both acrosome exocytosis and fertilization success (Asano et al. 2013b). To verify that an active fragment of PLB was in fact responsible, we demonstrated that the supernatant from capacitated sperm could induce acrosome exocytosis in other sperm to a similar extent as progesterone and that a recombinant domain of PLB could induce the same increase in acrosome exocytosis (Asano et al. 2013b). Together, these results show that PLB activation is involved in triggering early membrane fusion events in acrosome exocytosis and provide the first mechanism for promotion of fusion in response to sterol efflux.

However, other phospholipases also make substantial contributions to both physiological acrosome exocytosis and spontaneous exocytosis that can occur in capacitated sperm. Of great interest, different isoforms seem to be responsible, suggesting possible dysfunction in specific compartments of cells undergoing spontaneous exocytosis. For example, intracellular, calcium-independent $iPLA_2\beta$ is critical for spontaneous exocytosis, whereas both it and secreted group X $sPLA_2$ are involved in progesterone-induced acrosome exocytosis, to extents that differ depending on the concentration of progesterone and the kinetics of the sperm's response (Abi Nahed et al. 2015).

The majority of our knowledge about the relative contributions of different PLA family members results from the use of a growing array of inhibitors. In an elegant series of experiments with these inhibitors and different concentrations of progesterone, it was found that $iPLA_2\beta$ had more effect on early stages of exocytosis at lower, more physiological concentrations of 2 μM (Abi Nahed et al. 2015). Later stages of exocytosis relied more on X $sPLA_2$ (Abi Nahed et al. 2015); these results support the notion that lipid modulation is important at different stages of the regulated exocytotic event described by the acrosome exocytosis model. These results do not contradict the abovementioned findings with PLB. Indeed, some sperm were still able to undergo acrosome exocytosis in the presence of inhibitors of both $iPLA_2\beta$ and group X $sPLA_2$, suggesting that all three phospholipases likely participate (Abi Nahed et al. 2015). In much the same way that membrane fluidity induced by bicarbonate and sterol efflux are mutually reinforcing and can occur independently, it makes sense that sperm would have several reinforcing and potentially redundant mechanisms in place to promote membrane fusion.

6.8 Sterol Efflux-Mediated Regulation of Sperm Ion Channels

In addition to direct induction of changes in membrane curvature through phospholipase activity, changes in membrane lipids also influence sperm intracellular signaling via direct and indirect regulation of ion channels. Although review of ion channels in capacitation and acrosome exocytosis is beyond the scope of this work, we'll briefly highlight potential roles for lipids in regulating several channels involved in early events in capacitation, as these are critical for eventual ability to undergo exocytosis. In human sperm, membrane depolarization and sperm capacitation are physiologically relevant activators of the sperm proton channel, Hv1 (Lishko et al. 2010). In murine sperm, it appears that a sperm-specific sodium–hydrogen exchanger (sNHE) plays this role (Wang et al. 2003). Activity of these channels causes intracellular alkalinization. This in turn activates both the SLO3 potassium channel, responsible for hyperpolarization (Santi et al. 2010), and CATSPER channels responsible for large-scale calcium influx throughout the principal piece (Lishko and Kirichok 2010). It is possible that the BSA-induced calcium influx mediated by CATSPER might reflect a direct effect at the level of the membrane and/or that it might run through Hv1 and/or SLO3. While not precluding a direct effect, the latter is likely as it has been suggested that SLO3 might regulate CATSPER through complementary pathways (Chavez et al. 2014).

The interplay between membrane changes and the activities of these various channels is complex, with feedback from lipids at multiple points. For example, phosphatidylinositol 4,5-bisphosphate (PIP2) exerts a strong positive regulatory effect on SLO3 (Tang et al. 2010). As a recurring theme, it is likely that these pathways are redundant and mutually reinforcing—putting the sperm on a "one-way street" to capacitation and acrosome exocytosis. However, lipids provide at least one more critical point of regulation, so that the changes in ion flux originating from the flagellum are transduced into acrosomal responsiveness only in sperm heads that are ready for exocytosis.

Namely, we've shown that sterol efflux and focal enrichments of G_{M1} together promoted calcium influx through the $Ca_V2.3$ channel in the AA microdomain (Cohen et al. 2014). We first identified this lipid regulation fortuitously, observing that focal enrichment by cross-linking or addition of exogenous G_{M1} induced acrosome exocytosis. Neither the ceramide tail, asialo-G_{M1} (G_{M1} lacking the sialic acid residue), or free sialic acid mimicked this effect. These results suggested that the extracellular sugars on this glycosphingolipid were mechanistically important, corroborating that extracellular binding of SVS2 might exert a physiological inhibition (Kawano et al. 2008). Using various calcium-channel inhibitors, we identified $Ca_V2.3$, with an α_{1E} pore-forming subunit, as a candidate for mediating G_{M1}'s effect. Confirming its potential involvement, α_{1E} localized to the AA in murine sperm (Wennemuth et al. 2000; Cohen et al. 2014). Sperm from mice lacking α_{1E} failed to undergo exocytosis in response to progesterone, zona pellucida proteins, CTB, or G_{M1} (Cohen et al. 2014), although they were able to respond to

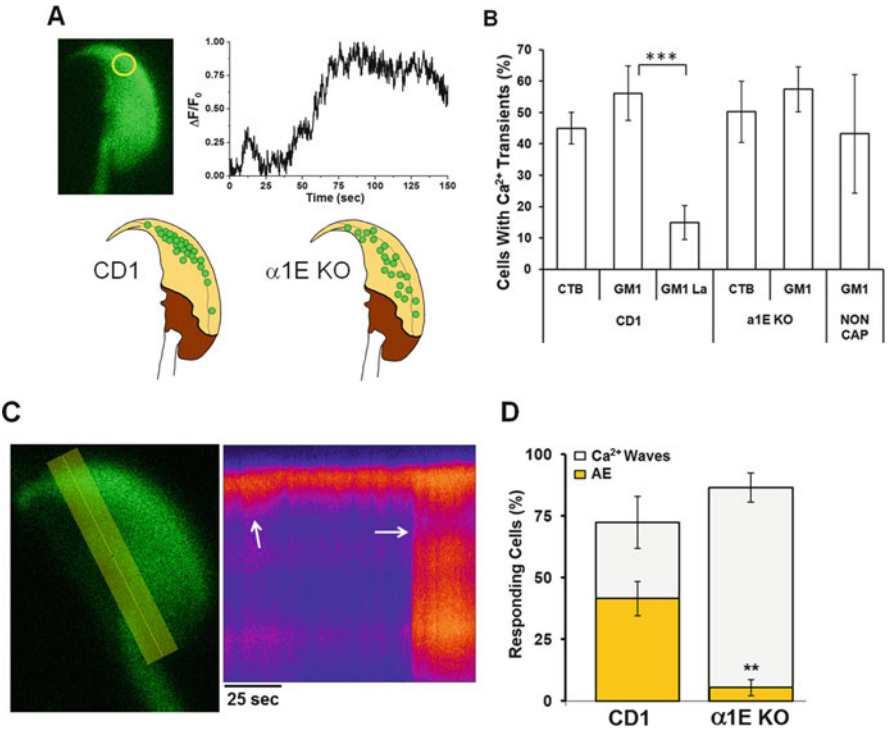

Fig. 6.4 Calcium transients differ in sperm from wild-type and α_{1E}-null mice and are necessary for acrosome exocytosis in response to calcium waves. Sperm were loaded with Fluo4-AM, and the occurrence and localization of calcium transients (**a**) identified. The *yellow circle* represents a region of interest shown in the trace. Transients from multiple sperm were compiled into single images to show that the localization of transients in sperm from CD1 mice tended to lie in the AA macrodomain, whereas the majority of those from α_{1E}-null mice occurred in the ES. Both wild-type and null sperm expressed transients in response to CTB and G_{M1} (**b**). Panel **c** shows a line scan over the sperm head. The *top of the line* corresponds to the top of the adjacent image, and the *bottom of the line* scan corresponds to the bottom of that image, in which brighter colors correspond to higher calcium concentrations. The *white arrow* pointing upward on the *left* indicates a calcium transient in the AA, followed by a lag until a subsequent calcium wave, arising distally and moving proximally, and indicated by the *arrow* pointing to the *right*. Panel **d** shows the relative occurrence of acrosome exocytosis (*yellow*) in capacitated sperm having calcium waves from CD1 versus α_{1E}-null mice. Figure adapted from Cohen et al. (2014)

calcium ionophore A23187 (Cohen et al. 2014). Furthermore, mating trials showed a strong male-factor subfertility phenotype with litter size halved. These findings led us to explore the molecular interactions and mechanisms for these effects.

Using calcium imaging of single sperm (Fig. 6.4a), we identified that wild-type sperm exhibited focal transient increases in calcium in the apical acrosome in response to sterol efflux, exogenous G_{M1}, or CTB. Sperm from mice null for α_{1E} still had transients, but these had greatly diminished integrated calcium flux and were mislocalized to the ES [(Cohen et al. 2014); Fig. 6.4a–b]. Of great interest, both

wild-type and knockout sperm exhibited large amplitude waves arising distally and moving apically that dwarfed the transient influx. However, dramatically fewer sperm underwent exocytosis if they did not first exhibit a transient in the apical acrosome [(Cohen et al. 2014); Fig. 6.4c–d]. Together, this combination of cell biological, pharmacological, and genetic approaches revealed that acrosome exocytosis depended on spatiotemporal information encoded by flux through $Ca_V2.3$ in the AA (Cohen et al. 2014). Exocytosis rarely occurred following calcium waves induced by sterol efflux, exogenous G_{M1}, or CTB in the absence of this priming calcium transient.

Knowing that G_{M1}'s extracellular sugars were critical for this interaction, that the $\alpha_2\delta$ subunits play a strong role in regulating Ca_V function, and that these subunits have highly charged extracellular sugars, we hypothesized that G_{M1}'s effect on flux through α_{1E} might involve the $\alpha_2\delta$ subunit. Subcellular localization and the inhibitory effects of gabapentin combined to suggest that these subunits are involved, and $\alpha_2\delta_1$ is the subunit responsible. Use of the voltage clamp technique in the *Xenopus* oocyte heterologous expression system provided mechanistic insight, with a significant left shift of the G/V relationship of $Ca_V2.3$ in the presence of G_{M1} and the $\alpha_2\delta$ subunit (Cohen et al. 2014). Importantly, G_{M1} exerted a moderate depolarization of the sperm head. This effect was also dependent on the sialic acid residue and was stronger in the more hyperpolarized capacitated sperm (Cohen et al. 2014).

6.9 Intracellular Lipid Signaling and Acrosome Exocytosis

After voltage-operated calcium entry, phospholipase Cδ4 is activated to produce inositol triphosphate (IP3) and diacylglycerol from PIP2 (Rice et al. 2000; Fukami et al. 2001). Intracellular IP3 then activates IP3 receptors on the acrosome (Walensky and Snyder 1995) to release calcium stores from the acrosome (Herrick et al. 2005). This increase in intracellular calcium mediates calcium influx from a store-operated plasma membrane calcium channel, likely a TRPC channel, although species differences in channels exist (Jungnickel et al. 2001; Sutton et al. 2004; Stamboulian et al. 2005). The sustained influx of calcium results in SNARE-mediated fusion of the AA and outer acrosomal membrane (De Blas et al. 2005). Intracellular lipid signaling in sperm is likely to be more complex than this simplistic picture and is fertile ground for future investigations.

6.10 Summary of Lipid Regulation of Acrosome Exocytosis

This brief review has touched upon multiple points at which sperm lipids negatively and positively regulate acrosome exocytosis. Indeed, *lipids exercise the major points of regulation* of sperm capacitation and acrosome exocytosis (Fig. 6.5). Recognizing these critical roles is not only important from a scientific perspective, but also from the standpoint of applied, clinical research. Can this improved

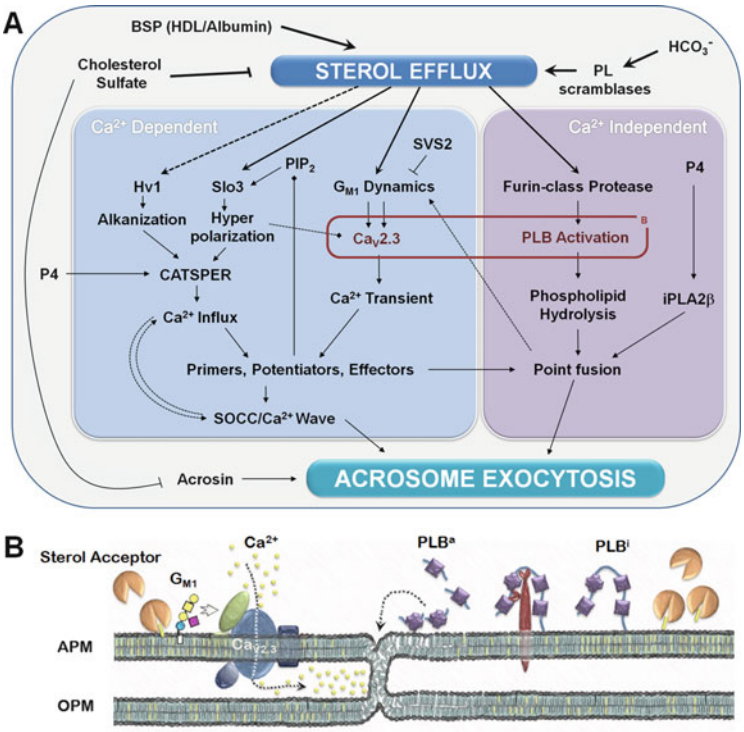

Fig. 6.5 Models summarizing lipid regulation of acrosome exocytosis. Panel **a** shows that sterol efflux is facilitated by BSP proteins that bind sterol acceptors, as well as phospholipid scramblase activity. Efflux is inhibited by cholesterol sulfate that also inhibits acrosin, believed to be involved in dispersion of the acrosomal matrix. Sterol efflux facilitates or triggers several calcium-dependent and independent pathways. Alkalinization is promoted through Hv1 in human sperm or sNHE in murine sperm, and hyperpolarization through SLO3, which is positively regulated by PIP2. These changes stemming from sterol efflux, as well as exposure to progesterone, lead to whole-cell calcium rises through CATSPER. As inhibitory SVS2 is lost from the sperm surface, sterol efflux also allows G_{M1} to enhance calcium flux through $Ca_V2.3$. This depends on the extracellular sugars of G_{M1}, particularly the sialic acid residue, and involves both the α_{1E} and $\alpha_2\delta$ subunits of the channel. G_{M1} might also exert a local depolarizing effect, which is more prominent in the more hyperpolarized, capacitated sperm. The resultant transients somehow prime the APM and/or outer acrosomal membrane, whether through unknown means, or stimulating potentiators such as PLCδ4, or by priming membrane fusion effectors such as SNAREs. The spatiotemporal information encoded in these priming events enables these cells to respond to subsequent waves, arising distally and believed to be initiated by CATSPER activity. Together, these focal changes lead to store-operated release culminating in acrosome exocytosis. Sterol efflux also facilitates exocytosis through calcium-independent pathways, such as by stimulating proteolytic activation of PLB. The hydrolysis of phospholipids causes point fusions that can sum to full exocytosis. They also can feed into the calcium-dependent pathways; for example, by facilitating communication with the outer acrosomal membrane, additional G_{M1} might become available in the plasma membrane to regulate $Ca_V2.3$. Progesterone also can induce early stages of exocytosis, presumably through promotion of point fusions, via calcium-independent $iPLA_2\beta$. Panel **a** adapted from Cohen et al. (2014). Activation of $Ca_V2.3$ and PLB is highlighted and magnified schematically in Panel **b**. In this diagram, sterol efflux facilitates lateral movement of

understanding of the functions of sperm membranes influence how we diagnose male infertility, how we cryopreserve sperm, and how we handle them in vitro prior to use in intrauterine insemination or in vitro fertilization? Could the extracellular points of regulation be targets for non-detergent-based topical spermicides or other contraceptives? Although the redundant pathways regulating sperm function can make our ability to enhance or inhibit that function seem an intractable problem, a new focus on lipids might provide more progress than our previous concentration on protein targets.

Acknowledgments This work was supported by Cornell's Baker Institute for Animal Health, the College of Veterinary Medicine, and Atkinson Center for a Sustainable Future. A.J.T. is a founder and an officer of Androvia LifeSciences, LLC, a biotechnology company investigating solutions for male infertility.

References

Abi Nahed R, Martinez G, Escoffier J et al (2015) Progesterone-induced acrosome exocytosis requires sequential involvement of calcium-independent iPLA2[beta] and group X sPLA2. J Biol Chem. doi:10.1074/jbc.M115.677799

Asano A, Selvaraj V, Buttke DE, Nelson JL, Green KM, Evans JE, Travis AJ (2009) Biochemical characterization of membrane fractions in murine sperm: identification of three distinct sub-types of membrane rafts. J Cell Physiol 218:537–548. doi:10.1002/jcp.21623

Asano A, Nelson JL, Zhang S, Travis AJ (2010) Characterization of the proteomes associating with three distinct membrane raft sub-types in murine sperm. Proteomics 10:3494–3505. doi:10.1002/pmic.201000002

Asano A, Nelson-Harrington JL, Travis AJ (2013a) Membrane rafts regulate phospholipase B activation in murine sperm. Commun Integr Biol 6, e27362. doi:10.4161/cib.27362

Asano A, Nelson-Harrington JL, Travis AJ (2013b) Phospholipase B is activated in response to sterol removal and stimulates acrosome exocytosis in murine sperm. J Biol Chem 288: 28104–28115. doi:10.1074/jbc.M113.450981

Bou Khalil M, Chakrabandhu K, Xu H et al (2006) Sperm capacitation induces an increase in lipid rafts having zona pellucida binding ability and containing sulfogalactosylglycerolipid. Dev Biol 290:220–235

Buttke DE, Nelson JL, Schlegel PN, Hunnicutt GR, Travis AJ (2006) Visualization of GM1 with cholera toxin B in live epididymal versus ejaculated bull, mouse, and human spermatozoa. Biol Reprod 74:889–895

Fig. 6.5 (continued) G_{M1}, such that it stimulates influx through $Ca_V2.3$. The focal calcium transient acts on primers, potentiators, and/or effectors as shown above, but left out of this drawing for simplicity. Sterol efflux also facilitates contact between inactive PLB (*purple*) and a furin protease (*deep red*). The fragments contain the active catalytic domains, leading to changes in membrane curvature and point fusions. Together, these findings show how lipids and lipid modifications can first negatively and then positively regulate capacitation and acrosome exocytosis in the complete absence of contact with the zona pellucida, consistent with new models of regulated acrosome exocytosis. Panel **b** adapted from Asano et al. (2013b)

Chavez JC, Ferreira JJ, Butler A et al (2014) SLO3 K+ channels control calcium entry through CATSPER channels in sperm. J Biol Chem 289:32266–32275. doi:10.1074/jbc.M114.607556
Cohen R, Buttke DE, Asano A et al (2014) Lipid modulation of calcium flux through CaV2.3 regulates acrosome exocytosis and fertilization. Dev Cell 28:310–321. doi:10.1016/j.devcel.2014.01.005
Connor WE, Lin DS, Wolf DP, Alexander M (1998) Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. J Lipid Res 39:1404–1411
Cross NL (1996) Effect of cholesterol and other sterols on human sperm acrosomal responsiveness. Mol Reprod Dev 45:212–217
Davis BK (1981) Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proc Natl Acad Sci U S A 78:7560–7564
De Blas GA, Roggero CM, Tomes CN, Mayorga LS (2005) Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. PLoS Biol 3, e323
de Vries KJ, Wiedmer T, Sims PJ, Gadella BM (2003) Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. Biol Reprod 68:2122–2134
Delagebeaudeuf C, Gassama-Diagne A, Nauze M et al (1998) Ectopic epididymal expression of guinea pig intestinal phospholipase B. Possible role in sperm maturation and activation by limited proteolytic digestion. J Biol Chem 273:13407–13414
Ehrenwald E, Foote RH, Parks JE (1990) Bovine oviductal fluid components and their potential role in sperm cholesterol efflux. Mol Reprod Dev 25:195–204
Feigenson GW (2009) Phase diagrams and lipid domains in multicomponent lipid bilayer mixtures. Biochim Biophys Acta 1788:47–52. doi:10.1016/j.bbamem.2008.08.014
Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B, Gadella BM (2001) Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. J Cell Sci 114:3543–3555
Friend DS (1982) Plasma-membrane diversity in a highly polarized cell. J Cell Biol 93:243–249
Fukami K, Nakao K, Inoue T et al (2001) Requirement of phospholipase Cdelta4 for the zona pellucida-induced acrosome reaction. Science 292:920–923
Futerman AH, Hannun YA (2004) The complex life of simple sphingolipids. EMBO Rep 5:777–782
Gadella BM, Harrison RA (2000) The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. Development 127:2407–2420
Galantino-Homer HL, Zeng WX, Megee SO, Dallmeyer M, Voelkl D, Dobrinski I (2006) Effects of 2-hydroxypropyl-beta-cyclodextrin and cholesterol on porcine sperm viability and capacitation status following cold shock or incubation. Mol Reprod Dev 73:638–650
Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 13:122–143, table of contents
Go KJ, Wolf DP (1985) Albumin-mediated changes in sperm sterol content during capacitation. Biol Reprod 32:145–153
Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS (2006) Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biol Reprod 75:501–507. doi:10.1095/biolreprod.106.053306
Herrick SB, Schweissinger DL, Kim SW, Bayan KR, Mann S, Cardullo RA (2005) The acrosomal vesicle of mouse sperm is a calcium store. J Cell Physiol 202:663–671. doi:10.1002/jcp.20172
Jin M, Fujiwara E, Kakiuchi Y et al (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci U S A 108:4892–4896. doi:10.1073/pnas.1018202108
Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM (2001) Trp2 regulates entry of Ca2+ into mouse sperm triggered by egg ZP3. Nat Cell Biol 3:499–502
Kawano N, Yoshida M (2007) Semen-coagulating protein, SVS2, in mouse seminal plasma controls sperm fertility. Biol Reprod 76:353–361

Kawano N, Yoshida K, Iwamoto T, Yoshida M (2008) Ganglioside GM1 mediates decapacitation effects of SVS2 on murine spermatozoa. Biol Reprod 79:1153–1159. doi:10.1095/biolreprod.108.069054

Kim KS, Gerton GL (2003) Differential release of soluble and matrix components: evidence for intermediate states of secretion during spontaneous acrosomal exocytosis in mouse sperm. Dev Biol 264:141–152

Kim KS, Foster JA, Kvasnicka KW, Gerton GL (2011) Transitional states of acrosomal exocytosis and proteolytic processing of the acrosomal matrix in guinea pig sperm. Mol Reprod Dev doi. doi:10.1002/mrd.21387

Kolter T, Proia RL, Sandhoff K (2002) Combinatorial ganglioside biosynthesis. J Biol Chem 277:25859–25862

Langlais J, Zollinger M, Plante L, Chapdelaine A, Bleau G, Roberts KD (1981) Localization of cholesteryl sulfate in human spermatozoa in support of a hypothesis for the mechanism of capacitation. Proc Natl Acad Sci U S A 78:7266–7270

Leahy T, Gadella BM (2015) New insights into the regulation of cholesterol efflux from the sperm membrane. Asian J Androl 17:561–567. doi:10.4103/1008-682X.153309

Lin DS, Connor WE, Wolf DP, Neuringer M, Hachey DL (1993) Unique lipids of primate spermatozoa: desmosterol and docosahexaenoic acid. J Lipid Res 34:491–499

Lishko PV, Kirichok Y (2010) The role of Hv1 and CatSper channels in sperm activation. J Physiol 588:4667–4672. doi:10.1113/jphysiol.2010.194142

Lishko PV, Botchkina IL, Fedorenko A, Kirichok Y (2010) Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. Cell 140:327–337. doi:10.1016/j.cell.2009.12.053

Manjunath P, Therien I (2002) Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 53:109–119

Pelletier RM, Friend DS (1983) Development of membrane differentiations in the guinea pig spermatid during spermiogenesis. Am J Anat 167:119–141

Rathi R, Colenbrander B, Bevers MM, Gadella BM (2001) Evaluation of in vitro capacitation of stallion spermatozoa. Biol Reprod 65:462–470

Rice A, Parrington J, Jones KT, Swann K (2000) Mammalian sperm contain a Ca(2+)-sensitive phospholipase C activity that can generate InsP(3) from PIP(2) associated with intracellular organelles. Dev Biol 228:125–135

Roberts KD (1987) Sterol sulfates in the epididymis; synthesis and possible function in the reproductive process. J Steroid Biochem 27:337–341

Santi CM, Martinez-Lopez P, de la Vega-Beltran JL, Butler A, Alisio A, Darszon A, Salkoff L (2010) The SLO3 sperm-specific potassium channel plays a vital role in male fertility. FEBS Lett 584:1041–1046. doi:10.1016/j.febslet.2010.02.005

Selvaraj V, Asano A, Buttke DE et al (2006) Segregation of micron-scale membrane sub-domains in live murine sperm. J Cell Physiol 206:636–646

Selvaraj V, Buttke DE, Asano A et al (2007) GM1 dynamics as a marker for membrane changes associated with the process of capacitation in murine and bovine spermatozoa. J Androl 28:588–599

Selvaraj V, Asano A, Buttke DE, Sengupta P, Weiss RS, Travis AJ (2009) Mechanisms underlying the micron-scale segregation of sterols and G(M1) in live mammalian sperm. J Cell Physiol 218:522–536. doi:10.1002/jcp.21624

Siafakas AR, Wright LC, Sorrell TC, Djordjevic JT (2006) Lipid rafts in Cryptococcus neoformans concentrate the virulence determinants phospholipase B1 and Cu/Zn superoxide dismutase. Eukaryot Cell 5:488–498

Simons K, Gerl MJ (2010) Revitalizing membrane rafts: new tools and insights. Nat Rev Mol Cell Biol 11:688–699. doi:10.1038/nrm2977

Simons K, Sampaio JL (2011) Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol 3:a004697. doi:10.1101/cshperspect.a004697

Stamboulian S, Moutin MJ, Treves S et al (2005) Junctate, an inositol 1,4,5-triphosphate receptor associated protein, is present in rodent sperm and binds TRPC2 and TRPC5 but not TRPC1 channels. Dev Biol 286:326–337

Strott CA, Higashi Y (2003) Cholesterol sulfate in human physiology: what's it all about? J Lipid Res 44:1268–1278. doi:10.1194/jlr.R300005-JLR200

Sullivan R, Frenette G, Girouard J (2007) Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian J Androl 9:483–491. doi:10.1111/j.1745-7262.2007.00281.x

Sutton KA, Jungnickel MK, Wang Y, Cullen K, Lambert S, Florman HM (2004) Enkurin is a novel calmodulin and TRPC channel binding protein in sperm. Dev Biol 274:426–435. doi:10.1016/j.ydbio.2004.07.031

Takemori H, Zolotaryov FN, Ting L et al (1998) Identification of functional domains of rat intestinal phospholipase B/lipase. Its cDNA cloning, expression, and tissue distribution. J Biol Chem 273:2222–2231

Tang QY, Zhang Z, Xia J, Ren D, Logothetis DE (2010) Phosphatidylinositol 4,5-bisphosphate activates Slo3 currents and its hydrolysis underlies the epidermal growth factor-induced current inhibition. J Biol Chem 285:19259–19266. doi:10.1074/jbc.M109.100156

Tojo H, Ichida T, Okamoto M (1998) Purification and characterization of a catalytic domain of rat intestinal phospholipase B/lipase associated with brush border membranes. J Biol Chem 273:2214–2221

Travis AJ, Kopf GS (2002) The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. J Clin Invest 110:731–736

Travis AJ, Merdiushev T, Vargas LA et al (2001) Expression and localization of caveolin-1, and the presence of membrane rafts, in mouse and Guinea pig spermatozoa. Dev Biol 240:599–610

van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9:112–124. doi:10.1038/nrm2330

Visconti PE, Moore GD, Bailey JL et al (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139–1150

Visconti PE, Galantino-Homer H, Ning X et al (1999a) Cholesterol efflux-mediated signal transduction in mammalian sperm. β-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. J Biol Chem 274:3235–3242

Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, Kopf GS (1999b) Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Dev Biol 214:429–443

Walensky LD, Snyder SH (1995) Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosome of mammalian sperm. J Cell Biol 130:857–869

Wang D, King SM, Quill TA, Doolittle LK, Garbers DL (2003) A new sperm-specific Na+/H+ exchanger required for sperm motility and fertility. Nat Cell Biol 5:1117–1122. doi:10.1038/ncb1072

Wennemuth G, Westenbroek RE, Xu T, Hille B, Babcock DF (2000) CaV2.2 and CaV2.3 (N- and R-type) Ca2+ channels in depolarization-evoked entry of Ca2+ into mouse sperm. J Biol Chem 275:21210–21217. doi:10.1074/jbc.M002068200[pii]

Chapter 7
Role of Actin Cytoskeleton During Mammalian Sperm Acrosomal Exocytosis

Ana Romarowski, Guillermina M. Luque, Florenza A. La Spina, Dario Krapf, and Mariano G. Buffone

7.1 Capacitation Prepares Sperm to Undergo Acrosomal Exocytosis

Mammalian sperm are not able to fertilize after ejaculation. They require to spend a limited period of time within the female reproductive tract in order to become fertilization competent in a process known as capacitation (Chang 1951; Austin 1951). Capacitation prepares sperm for fertilization by priming the cells for acrosomal exocytosis which has multiple similarities with other exocytotic events but also has unique properties: (1) sperm possess only one secretory vesicle, the acrosome; (2) multiple fusion points are established between the outer acrosomal membrane (OAM) and the plasma membrane (PM); (3) fusion of the OAM and the PM results in membrane loss due to the formation and release of hybrid membrane vesicles during exocytosis; and (4) membrane recycling does not take place, making acrosomal exocytosis irreversible (Mayorga et al. 2007).

Although for many years it was believed that this process occurred upon sperm interaction with the zona pellucida (ZP), recent reports provided evidence against this idea (Baibakov et al. 2007; Jin et al. 2011; Inoue et al. 2011). Nowadays, it is not clear what is the physiological stimulant of this process and the site where exocytosis takes place (Buffone et al. 2014a). What is absolutely clear is that acrosomal exocytosis is an absolute prerequisite for fertilization because its occurrence

A. Romarowski • G.M. Luque • F.A. La Spina • M.G. Buffone (✉)
Instituto de Biología y Medicina Experimental (IBYME), National Research Council of Argentina (CONICET), Vuelta de Obligado, 2490 (1428) Buenos Aires, Argentina
e-mail: mgbuffone@ibyme.conicet.gov.ar

D. Krapf
Institute of Molecular and Cell Biology of Rosario (CONICET-UNR), Buenos Aires, Argentina

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_7

produces the relocalization of Izumo1 (Inoue et al. 2005), which is essential for sperm–egg fusion (Sosnik et al. 2009; Satouh et al. 2012). As a consequence, acrosomal exocytosis must be tightly regulated to ensure successful fertilization.

In the past, several physiological events of the capacitation process have been dissected. However, what are the underlying mechanisms that convert the sperm cell from a state unresponsive to agonist-stimulated acrosomal exocytosis to one primed to respond to these stimuli? What is already known is that when sperm are incubated under capacitating conditions, they spontaneously transit through defined morphologically and biochemically intermediate stages of exocytosis that lead to the incremental exposure and eventual release of acrosomal components. The rate of this release is dependent upon their state within the acrosome: soluble or particulate (acrosomal matrix-associated) (Stock and Fraser 1987; Yudin et al. 1998; Kim and Gerton 2003; Buffone et al. 2008). Thus, during capacitation, sperm commence unresponsive to stimuli, but then certain molecular changes that occur within the female reproductive tract let these cells exit the "metastable" state, represented by intermediate stages of exocytosis during capacitation, and finally undergo acrosomal exocytosis upon interaction with the appropriate stimulus.

In this scenario, the tight regulation of acrosomal exocytosis to ensure successful fertilization is controlled in several ways: by activation of specific signaling pathways, biophysical changes at the level of plasma membrane, activation of the fusion machinery, and the structure of the actin cytoskeleton. Without precluding the participation of any of these molecular changes, this review is focused on how the actin cytoskeleton and its related proteins control acrosomal exocytosis in sperm. In other mammalian cells, the cortical actin network acts as a dominant negative clamp which blocks constitutive exocytosis (Muallem et al. 1995) but, at the same time, is necessary to prepare the cell to undergo regulated exocytosis. Thus, F-actin stabilizes structures generated by exocytosis and supports the physiological progression of this process. Is this also the case in mammalian sperm? The following sections summarize what is currently known about actin and its related proteins in spermatozoa, with particular emphasis on their participation in the acrosome reaction.

7.2 The Actin Cytoskeleton as a Key Component in Eukaryotic Exocytosis

In all eukaryotic cells, a cytoskeleton is present and constitutes an essential structure for cell biology. The cytoskeleton is a complex network of interlinking filaments throughout the cell and is composed of three major components: the microfilaments, the microtubules, and the intermediate filaments. Actin is a globular ATPase protein that forms microfilaments. The actin protein's relative mass is around 42,000 Mr, and it is the monomeric subunit of two types of filaments in cells: microfilaments (one of the major components of the cytoskeleton) and thin filaments (part of the contractile apparatus in muscle cells). Actin is present as a

monomer (globular or G-actin) or as part of a polymer named F-actin (from filamentous). The actin cytoskeleton participates in several important cellular events, such as cell motility, muscle contraction, cell division, cytokinesis, signaling, organelle movement, control of cell junctions and shape, and endo/exocytosis. Some of these processes are mediated by close interactions of actin with other proteins in the plasma membrane.

Cells simultaneously assemble, maintain, and disassemble different F-actin networks to facilitate a particular process (Chhabra and Higgs 2007; Blanchoin et al. 2014). F-actin networks with specified organization and dynamics are controlled through the action of different actin-binding proteins with a variety of functions that include assembly, end capping, actin monomer binding, bundling, and severing/disassembling (Blanchoin et al. 2014). F-actin network assembly, organization, and dynamics are therefore controlled by the spatial and temporal regulation of the activity of actin-binding proteins.

It is well accepted that the final steps of regulated exocytosis such as vesicle docking, triggering, and membrane fusion are regulated by a constitutively operating fusion machinery shared by many vesicular trafficking processes and specialized clamps to prevent fusion until the appropriate signals are received (Muallem et al. 1995; Malacombe et al. 2006). The actin cytoskeleton that is in close interaction with the plasma membrane has long been proposed as a physical barrier to granule docking because it transiently depolymerizes during exocytosis. However, it was also reported that certain drugs that cause actin depolymerization do not result in exocytosis but can potentiate agonist-evoked responses (Lelkes et al. 1986; Sontag et al. 1988; Matter et al. 1989), suggesting that dissolution of the actin cytoskeleton is a necessary but not sufficient part of regulated exocytosis.

The participation of actin cytoskeleton in the processes of controlled secretion was first demonstrated in pancreatic β-cells. The insulin-containing granules present in these cells were found to co-sediment with filamentous actin (Malaisse et al. 1971; Orci et al. 1972). This co-sedimentation was reduced upon addition of calcium (Howell and Tyhurst 1979). Later studies indicated that F-actin was organized as a dense network beneath the plasma membrane, which would impede the access of insulin-containing granules to the cell periphery (Orci et al. 1972; Somers et al. 1979; Howell and Tyhurst 1979; Snabes and Boyd 1982; Wang et al. 1990). It is also reported that F-actin-disrupting agents such as cytochalasins showed enhanced secretagogue-induced insulin secretion, suggesting that F-actin blocks granule movement (van Obberghen et al. 1973; Malaisse et al. 1975; Li et al. 1994). However, other authors demonstrated the existence of cytochalasin-insensitive pools of actin filaments (Cassimeris et al. 1990).

The actin cytoskeleton is also associated with the process of endocytosis, which is related with changes in membrane shape as a result of fusions and invaginations. These events are properly coordinated in space and time in part by cytoskeletal elements in most of these events if not in all. The interplay between the basal exocytotic and endocytic machineries suggests that these processes may have evolved as one instead of two distinct processes for controlling intracellular membrane traffic (Jaiswal et al. 2009).

7.3 Actin and Its Binding Proteins in Mammalian Sperm

In mammalian sperm, actin is present in its monomeric form as well as filamentous actin (Flaherty et al. 1988; Moreno-Fierros et al. 1992; Vogl et al. 1993; de las Heras et al. 1997; Hernández-González et al. 2000; Delgado-Buenrostro et al. 2005; Oikonomopoulou et al. 2009). The sperm regions reported to contain actin include the acrosomal matrix (Zepeda-Bastida et al. 2011), the equatorial and post-acrosomal regions, and the tail (Flaherty et al. 1988). The presence of actin in the tail might be important for the regulation of motility, and its presence in the head suggests a possible involvement in acrosomal exocytosis as well as for sperm–egg fusion. In particular, the presence of actin in the matrix of the acrosome might be associated with the differential release of certain acrosomal components (Kim and Gerton 2003) and with the organized progression of exocytosis (Buffone et al. 2009). Additional experiments are necessary to validate this hypothesis. The expression of several actin-related proteins in mammalian sperm (some of which are testis-specific) suggests that actin polymerization and depolymerization might be involved in certain aspects of sperm function. A summary of the actin-binding proteins reported in mammalian sperm and the possible function is shown in Table 7.1.

7.4 Actin Polymerization During Sperm Capacitation

Evidence from different laboratories indicates that actin polymerization occurs during capacitation in several mammalian species such as boar, guinea pig, mouse, rabbit, bovine, and human sperm (Castellani-Ceresa et al. 1993; Brener et al. 2003; Cabello-Agüeros et al. 2003; Baltiérrez-Hoyos et al. 2012; Romarowski et al. 2015; Lee et al. 2015). The increase in F-actin was observed in the sperm head as well as in the flagellum.

It is well established that in most secretory cells, vesicles or granules are at certain distance from the plasma membrane, implying that they must be transported to access exocytotic sites (Malacombe et al. 2006). The traffic between these two granule pools is subjected to a fine regulation by the actin cytoskeleton that includes the local disassembly of actin filaments, which permits secretory granules to gain access to the plasma membrane, where docking and fusion occur at exocytotic sites (Orci et al. 1972; Sontag et al. 1988; Vitale et al. 1995). Although the sperm acrosome is a single vesicle, the situation may be similar. In recent reports, Zanetti and coworkers (Zanetti and Mayorga 2009) demonstrated the coexistence of different stages of acrosomal docking to the plasma membrane in the course of capacitation as a result of acrosomal swelling and, possibly, by the dynamic changes in the actin cytoskeleton. While in some areas, the plasma membrane and the outer acrosomal membrane are not in contact or “docked,” other regions displayed docking of both membranes. The first situation would represent or be

Table 7.1 Actin-related proteins in mammalian sperm

Protein	Specie	Possible function in sperm	References
Alfa-actinin	Bovine	Structure and stabilization of the cytoskeleton	Yagi and Paranko (1992)
Tropomyosin	Bovine	Structure and stabilization of the cytoskeleton	Yagi and Paranko (1992)
Spectrin	Human, rabbit, guinea pig	Structure and stabilization of the cytoskeleton	Virtanen et al. (1984), Camatini et al. (1991), Ocampo et al. (2005)
Calicin	Human, boar, guinea pig, hamster, rat, mouse, bull	Morphogenic cytoskeletal element in spermiogenic differentiation. Structure and stabilization of the cytoskeleton	von Bülow et al. (1995), (Longo et al. (1987), (Paranko et al. (1988)
CPbeta3	Human, bovine	Components of the cytoskeletal calyx of the mammalian sperm head	von Bülow et al. (1997)
CPa3	Mouse	Components of the cytoskeletal calyx of the mammalian sperm head	Tanaka et al. (1994)
Arp-T1	Bull	Components of the cytoskeletal calyx of the mammalian sperm head. Nucleation of actin filaments	Heid et al. (2002)
Arp-T2	Bull	Components of the cytoskeletal calyx of the mammalian sperm head. Nucleation of actin filaments	Heid et al. (2002)
Myosin	Human, bovine	Molecular motors	Clarke and Yanagimachi (1978), Tamblyn (1983), Oikonomopoulou et al. (2009), Sosnik et al. (2009)
Gelsolin	Ram, human, guinea pig	Severe actin filaments during acrosomal exocytosis	de las Heras et al. (1997), Cabello-Agüeros et al. (2003), Finkelstein et al. (2010)
Scinderin	Bovine	Calcium-dependent actin filament-severing protein	Pelletier et al. (1999)
Destrin	Bovine	Severs actin filaments (F-actin) and binds to actin monomers (G-actin)	Howes et al. (2001)
Cofilin	Bovine, mouse, human	Actin nucleation or depolymerization in sperm	Fiedler et al. (2008), Romarowski et al. (2015), Megnagi et al. (2015)
CAPZA3	Mouse	Sperm-specific plus end actin capping protein maintains polymerized actin during spermiogenesis	Sosnik et al. (2010), Geyer et al. (2009)

(continued)

Table 7.1 (continued)

Protein	Specie	Possible function in sperm	References
LIMK1/2	Mouse	Phosphorylation of Cofilin in the control of actin polymerization and acrosomal exocytosis	Romarowski et al. (2015)
Arc	Mouse	Possible role in the acrosome formation and the sperm acrosome reaction	Maier et al. (2003)
Arp2/3	Mouse, guinea pig	Generation of branched actin networks	Lee et al. (2015), Chiquete-Felix et al. (2009)

analogous to the pool of vesicles that are not ready to undergo exocytosis, while the second one represents the granules that were transported to the exocytotic sites.

What are the signaling pathways involved in mammalian sperm actin polymerization? The following sections summarize what is currently proposed in mouse, human, guinea pig, and bovine sperm.

7.4.1 Cross Talk Between PKA and PKC and Regulation of PLD1

The role of phospholipase D (PLD) in the induction of actin polymerization during capacitation of bovine spermatozoa was demonstrated (Cohen et al. 2004). PLD activity increases steadily during capacitation. Actin polymerization is significantly inhibited by the PLD inhibitors butan-1-ol and C2-ceramide but not by butan-2-ol. In addition, exogenous PLD or PA (phosphatidic acid) promotes actin polymerization that is not inhibited by butan-1-ol (Cohen et al. 2004).

PLD1 is activated by a cross talk between protein kinase A and C during capacitation. Protein kinase A (PKA) is known to be rapidly activated while sperm are incubated in capacitating conditions (Buffone et al. 2014b), and protein kinase C (PKC), which was previously involved in the acrosomal exocytosis (Liu and Baker 1997; Chen et al. 2000), can activate both PLD and actin polymerization (Cohen et al. 2004). One possibility is that PKA inhibition toward the end of the capacitation would cause PKC activation to complete acrosomal exocytosis (Cohen et al. 2004). Further experimentation is necessary to clarify this pathway.

Phosphatidylinositol 4,5-bisphosphate (PIP2) is required as a cofactor for the activation of PLD. The activation of PLC results in PIP2 production. The increases in PIP2 and F-actin intracellular levels during sperm capacitation are mediated by PI3K activity (Etkovitz et al. 2007). Activation of PKA by dibutyryl cAMP enhanced PIP2, PIP3, and F-actin formation, and these effects were mediated through PI3K. On the other hand, activation of PKC by phorbol myristate acetate enhanced PIP2 and F-actin formation mediated by PI4K activity, while the PI3K activity and intracellular PIP3 levels were reduced.

In addition, it was recently reported that diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate (Lopez et al. 2012). Roldan and Dawes (1993) suggested that PLD does not make a substantial contribution to the events leading to acrosomal exocytosis. Moreover, knockout mice for *pld1* are fertile, suggesting that this protein can be functionally compensated by PLD2 or any of the other enzymes that increase the levels of phosphatidic acid (Jenkins and Frohman 2005).

7.4.2 *Small GTPAses and the Activation of LIMK1 and Cofilin*

In somatic cells, small GTPases of the Rho family are widely known as master regulators of actin dynamics (Heasman and Ridley 2008). However, the role of these proteins in sperm has not been studied in detail since the targeted deletion of these proteins or any of their downstream effectors results in embryonic lethality. In mammals, the Rho family is composed of the small GTPases Rho, Rac, and Cdc42. All of them switch between an active GTP-bound and an inactive GDP-bound form. The cycling of Rho GTPases between these two states is regulated by three sets of proteins: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). Small GTPases activate downstream effector proteins and stimulate a variety of cellular processes. The activation of Rho, Rac, and Cdc42 and the resulting signal transduction through downstream effectors result in the phosphorylation of LIM kinases (LIMKs; composed of LIMK1 and LIMK2 in mammals) (Ohashi et al. 2000). The activation of LIMKs results in the phosphorylation of Cofilin (Bernstein and Bamburg 2010). Cofilin is a family of actin-binding proteins that regulate assembly and disassembly of actin filaments. The activity of Cofilin is regulated by several mechanisms being phosphorylation the best characterized. LIMKs specifically phosphorylate Cofilin at Serine 3 and thereby inhibit the actin binding, severing, and depolymerizing activities of Cofilin. Thus, protein kinases and phosphatases related to Cofilin phosphorylation and dephosphorylation at Ser-3 are expected to play a central role in the regulation of actin dynamics.

Previous reports have demonstrated the presence of small GTPases and some of the downstream effectors in mammalian sperm (Freeman et al. 2002; Ducummon and Berger 2006; Fiedler et al. 2008; Baltiérrez-Hoyos et al. 2012; Romarowski et al. 2015). In particular, it was demonstrated the expression of RhoA, RhoC, Rac1, and Cdc42 and the effector proteins ROCK1, PAK1, LIMK1, LIMK2 (only the testis-specific isoform of 52,000 Mr), and Cofilin. All the necessary components for an active signaling pathway leading to actin polymerization are present in mouse sperm (Romarowski et al. 2015). In guinea pig sperm, it was suggested that small

GTPases RhoA, RhoB, and Cdc42 may participate in acrosomal exocytosis (Delgado-Buenrostro et al. 2005).

In mouse sperm, LIMK1 is phosphorylated on Thr508 (pLIMK1) as early as 10 min of incubation under capacitating conditions. Phosphorylated LIMK1 is only detected in the Triton-insoluble fraction, suggesting that this protein is associated with the polymerized actin cytoskeleton.

In the presence of esterified C4 (a specific Rho GTPases inhibitor) as well as with a specific ROCK1 kinase inhibitor, reduced levels of pLIMK1 were observed. Similar results were obtained with a specific inhibitor of RAC1, suggesting that both RhoA/C and Rac1 participate in the phosphorylation of LIMK1 through activation of ROCK1. However, it is still unknown how RAC1 activates LIMK1 since specific inhibition of PAK1 did not result in low levels of pLIMK1 (Romarowski et al. 2015).

Following phosphorylation, LIMK1 becomes activated and phosphorylates Cofilin on Ser3 (pCofilin) that, according to its phosphorylation status, it promotes actin polymerization or F-actin cleavage. Cofilin was phosphorylated on Ser3 (pCofilin) in a transient manner in mouse and human sperm (Romarowski et al. 2015; Megnagi et al. 2015). Inhibition of pLIMK with BMS-3 resulted in a decrease of pCofilin (Romarowski et al. 2015) and, as a consequence, lower levels of actin polymerization during capacitation and strong inhibition of acrosomal exocytosis (Romarowski et al. 2015; Megnagi et al. 2015).

Cofilin is also regulated by phosphatases. In mammals, two distinct Cofilin-selective phosphatases have been described: the Slingshot family of phosphatases (SSH1) and Chronophin (Niwa et al. 2002). The phosphatase PP2B (aka Calcineurin) was observed to dephosphorylate and activate SSH1 (Soosairajah et al. 2005). In addition, SSH1 activity was inhibited by PAK4-mediated phosphorylation and possibly others including ROCK and MRCKα (Soosairajah et al. 2005). Other reports demonstrated that SSH1 phosphatase might dephosphorylate and inactivate LIMK1 as well (Soosairajah et al. 2005). The participation of these phosphatases in the regulation is critical to understand how this process is controlled. Changes in pCofilin levels could be generated by direct SSH1 dephosphorylation, SSH1-mediated LIMK inactivation, or a combination of both.

LIMK1/ Cofilin signaling pathway interacts with PLD1, which, as previously mentioned, was shown to be essential for actin polymerization in bovine sperm. One interesting possibility is that pCofilin stimulates PLD1 activity as demonstrated recently (Han et al. 2007). However, this possibility requires future experimentation.

7.4.3 *Actin-Related Protein 2/3 Complex-Based Actin Polymerization*

The dynamic functions of the actin cytoskeleton require the generation of branched actin networks. The actin-related proteins 2 and 3 (Arp2/3) complex is the molecular machine that nucleates these branched actin networks (Mullins et al. 1998). The Arp2/3 complex consists of Arp2, Arp3, and five Arp subunits—Arpc1, Arpc2, Arpc3, Arpc4, and Arpc5 (Goley and Welch 2006; Campellone and Welch 2010). When the Arp2/3 complex is engaged by nucleation-promoting factor proteins, it is activated to initiate the formation of a new filament that emerges from an existing filament in a branch configuration with a regular 70° branch angle (Amann and Pollard 2001). The coupling of nucleation and branching by the Arp2/3 complex is known as autocatalytic branching and is central to its functions in vivo (Goley et al. 2006).

In sperm, little is known about the role of these proteins. Recently, Lee and coworkers (Lee et al. 2015) reported that inhibition of the Arp2/3 complex using high concentrations of CK-636 induced hyperactivated motility and acrosomal exocytosis. Moreover, inhibition of the Arp2/3 complex with CK-636 decreased the ratio of F-actin/G-actin in a dose-dependent manner. Further investigations are needed to understand these observations.

In guinea pig sperm, it was claimed that aldolase A is tightly associated with cytoskeletal structures where it interacts with actin and Arp2/3 (Chiquete-Felix et al. 2009), suggesting that aldolase A functions as a bridge between filaments of actin and the actin-polymerizing machinery.

7.5 Actin Depolymerization During Acrosomal Exocytosis

Extensive data obtained by many investigators using actin-perturbing drugs indicated that stabilization of the actin network inhibits exocytosis, whereas depolymerization of this network increases the number of docked secretory granules and thereby enhances the exocytotic response (Muallem et al. 1995; Chowdhury et al. 1999, 2000; Gasman et al. 2004; Ehre et al. 2005). However, some observations do not completely support this housekeeping function for actin, but suggest a more active role in the exocytotic process (Matter et al. 1989; Norman et al. 1994; Muallem et al. 1995; Pendleton and Koffer 2001). In general, these authors observed that low doses of actin-depolymerizing drugs promoted exocytosis while higher doses completely inhibit the process, suggesting that the actin cortex is not simply a barrier that hinders the movements of vesicles to the plasma membrane, but it also plays a role in the exocytotic machinery.

Using a cell-free system to study membrane fusion during sperm exocytosis, it was postulated that a rapid depolymerization of the actin network is necessary for exocytosis to occur (Spungin et al. 1995). The authors showed that cortical F-actin

functions as a physical barrier to membrane fusion which is removed by the increases in intracellular calcium and pH, events that precede fusion (Spungin et al. 1995). These data are consistent with findings showing that actin is lost from the acrosome following exocytosis (Hernández-González et al. 2000) since actin in the cytoplasmic space between the OAM and PM would be shed within the hybrid membranes vesicles. Moreover, inhibition of actin polymerization blocks the ZP-induced acrosomal exocytosis, as well as sperm penetration into zona-free eggs and the in vitro fertilization ability of sperm (Rogers et al. 1989; Castellani-Ceresa et al. 1993; Brener et al. 2003).

How is the actin network disassembled at the onset of acrosomal exocytosis? In specialized cell types, like neurons and endocrine cells, exocytosis of neurotransmitters and hormones occurs in response to a specific stimulus, usually an elevation of intracellular free calcium. Some actin-binding proteins such as Gelsolin and Scinderin sever assembled actin filaments and cap the fast-growing plus end of free or newly severed filaments in response to calcium ions. Gelsolin is expressed in several mammalian species (Cabello-Agüeros et al. 2003; Finkelstein et al. 2010). Recently, it was demonstrated that Gelsolin is inactive during capacitation and is activated prior to the acrosome reaction. The activation of Gelsolin by its release from PIP2 by activation of PLC, which hydrolyzes PIP2, caused rapid calcium-dependent F-actin depolymerization and acrosomal exocytosis. However, it is possible that other actin-severing proteins may also participate in these final steps of exocytosis because mice with targeted deletion of the *gelsolin* gene did not show any alteration in their reproductive outcome (Witke et al. 1995). Scinderin, which is also expressed in mammalian sperm (Pelletier et al. 1999), is an example of these proteins.

7.6 Future Perspectives

Nowadays, it seems evident that there are dynamic changes in the actin cytoskeleton during capacitation and acrosomal exocytosis. However, it is not clear how these changes led to exocytosis. It is possible that the actin network may control the docking of the outer acrosomal membrane and the plasma membrane as well as at the same time stop the progression of the exocytosis until the appropriate stimuli, as observed in other somatic cells (Fig. 7.1). However, because of the unique properties of the acrosome, this possibility needs to be further elucidated.

There are two important limitations to study actin and its related proteins in mammalian sperm: (i) Because there are no efficient ways to generate "sperm-specific" knockout mice, it is not possible to use this technology to study the role of these proteins in mature sperm since most of these knockout are embryonic lethal. (ii) Another problem with the transgenic approach is that actin and its related proteins are very important during spermatogenesis resulting in a complicated strategy to study their role in mature sperm.

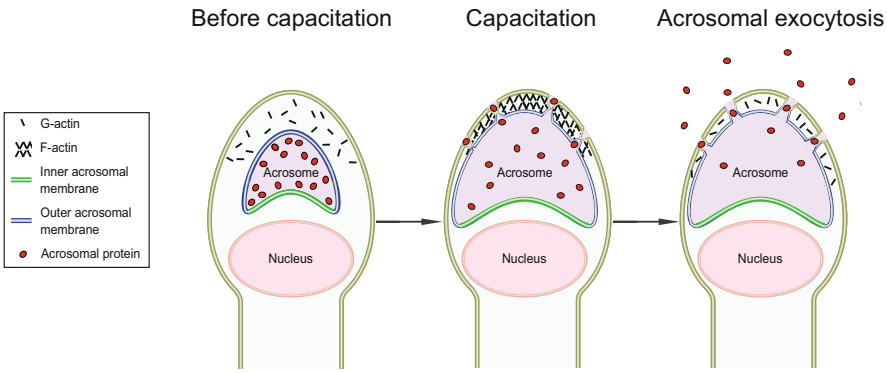

Fig. 7.1 Proposed model for actin dynamics during capacitation and acrosomal exocytosis in mammalian sperm

In addition, most of our knowledge about actin in mammalian sperm comes from experiments using fixed (such as phalloidin staining) or disrupted cell (ultracentrifugation to separate G- and F-actin). New technologies such as transgenic mice containing GFP-Lifeact (a protein that can bind to F-actin without perturbing its function) may be useful to study changes in the actin cytoskeleton in real time using live cells.

Acknowledgments We would like to thank the National Institutes of Health (NIH, R01TW008662), *Agencia Nacional de Promoción Científica y Tecnológica* (PICT 2012-1175), and CONICET (PIP 740) for funding support.

References

Amann KJ, Pollard TD (2001) Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy. Proc Natl Acad Sci U S A 98:15009–15013. doi:10.1073/pnas.211556398

Austin CR (1951) Observations on the penetration of the sperm in the mammalian egg. Aust J Sci Res Ser B Biol Sci 4:581–596

Baibakov B, Gauthier L, Talbot P et al (2007) Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. Development 134:933–943. doi:10.1242/dev.02752

Baltiérrez-Hoyos R, Roa-Espitia AL, Hernández-González EO (2012) The association between CDC42 and caveolin-1 is involved in the regulation of capacitation and acrosome reaction of guinea pig and mouse sperm. Reproduction 144:123–134. doi:10.1530/REP-11-0433

Bernstein BW, Bamburg JR (2010) ADF/cofilin: a functional node in cell biology. Trends Cell Biol 20:187–195. doi:10.1016/j.tcb.2010.01.001

Blanchoin L, Boujemaa-Paterski R, Sykes C, Plastino J (2014) Actin dynamics, architecture, and mechanics in cell motility. Physiol Rev 94:235–263. doi:10.1152/physrev.00018.2013

Brener E, Rubinstein S, Cohen G et al (2003) Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. Biol Reprod 68:837–845

Buffone MG, Foster JA, Gerton GL (2008) The role of the acrosomal matrix in fertilization. Int J Dev Biol 52:511–522. doi:10.1387/ijdb.072532mb

Buffone MG, Rodriguez-Miranda E, Storey BT, Gerton GL (2009) Acrosomal exocytosis of mouse sperm progresses in a consistent direction in response to zona pellucida. J Cell Physiol 220:611–620. doi:10.1002/jcp.21781

Buffone MG, Hirohashi N, Gerton GL (2014a) Unresolved questions concerning mammalian sperm acrosomal exocytosis. Biol Reprod 90:112. doi:10.1095/biolreprod.114.117911

Buffone MG, Wertheimer EV, Visconti PE, Krapf D (2014b) Central role of soluble adenylyl cyclase and cAMP in sperm physiology. Biochim Biophys Acta 1842:2610–2620. doi:10.1016/j.bbadis.2014.07.013

Cabello-Agüeros JF, Hernández-González EO, Mújica A (2003) The role of F-actin cytoskeleton-associated gelsolin in the guinea pig capacitation and acrosome reaction. Cell Motil Cytoskeleton 56:94–108. doi:10.1002/cm.10135

Camatini M, Colombo A, Bonfanti P (1991) Identification of spectrin and calmodulin in rabbit spermiogenesis and spermatozoa. Mol Reprod Dev 28:62–69. doi:10.1002/mrd.1080280110

Campellone KG, Welch MD (2010) A nucleator arms race: cellular control of actin assembly. Nat Rev Mol Cell Biol 11:237–251. doi:10.1038/nrm2867

Cassimeris L, McNeill H, Zigmond SH (1990) Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. J Cell Biol 110:1067–1075

Castellani-Ceresa L, Mattioli M, Radaelli G et al (1993) Actin polymerization in boar spermatozoa: fertilization is reduced with use of cytochalasin D. Mol Reprod Dev 36:203–211. doi:10.1002/mrd.1080360211

Chang MC (1951) Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:697–698

Chen WY, Yuan YY, Shi QX, Zhang XY (2000) Effect of protein kinase C on guinea pig sperm acrosome reaction induced by progesterone. Acta Pharmacol Sin 21:787–791

Chhabra ES, Higgs HN (2007) The many faces of actin: matching assembly factors with cellular structures. Nat Cell Biol 9:1110–1121. doi:10.1038/ncb1007-1110

Chiquete-Felix N, Hernández JM, Méndez JA et al (2009) In guinea pig sperm, aldolase A forms a complex with actin, WAS, and Arp2/3 that plays a role in actin polymerization. Reproduction 137:669–678. doi:10.1530/REP-08-0353

Chowdhury HH, Popoff MR, Zorec R (1999) Actin cytoskeleton depolymerization with clostridium spiroforme toxin enhances the secretory activity of rat melanotrophs. J Physiol 521 (Pt 2):389–395

Chowdhury HH, Popoff MR, Zorec R (2000) Actin cytoskeleton and exocytosis in rat melanotrophs. Pflügers Arch Eur J Physiol 439:R148–R149

Clarke GN, Yanagimachi R (1978) Actin in mammalian sperm heads. J Exp Zool 205:125–132. doi:10.1002/jez.1402050115

Cohen G, Rubinstein S, Gur Y, Breitbart H (2004) Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. Dev Biol 267:230–241. doi:10.1016/j.ydbio.2003.10.034

de las Heras MA, Valcarcel A, Pérez LJ, Moses DF (1997) Actin localization in ram spermatozoa: effect of freezing/thawing, capacitation and calcium ionophore-induced acrosomal exocytosis. Tissue Cell 29:47–53

Delgado-Buenrostro NL, Hernández-González EO, Segura-Nieto M, Mújica A (2005) Actin polymerization in the equatorial and postacrosomal regions of guinea pig spermatozoa during the acrosome reaction is regulated by G proteins. Mol Reprod Dev 70:198–210. doi:10.1002/mrd.20192

Ducummon CC, Berger T (2006) Localization of the Rho GTPases and some Rho effector proteins in the sperm of several mammalian species. Zygote 14:249–257. doi:10.1017/S0967199406003790

Ehre C, Rossi AH, Abdullah LH et al (2005) Barrier role of actin filaments in regulated mucin secretion from airway goblet cells. Am J Physiol Cell Physiol 288:C46–C56. doi:10.1152/ajpcell.00397.2004

Etkovitz N, Rubinstein S, Daniel L, Breitbart H (2007) Role of PI3-kinase and PI4-kinase in actin polymerization during bovine sperm capacitation. Biol Reprod 77:263–273. doi:10.1095/biolreprod.106.056705

Fiedler SE, Bajpai M, Carr DW (2008) Identification and characterization of RHOA-interacting proteins in bovine spermatozoa. Biol Reprod 78:184–192. doi:10.1095/biolreprod.107.062943

Finkelstein M, Etkovitz N, Breitbart H (2010) Role and regulation of sperm gelsolin prior to fertilization. J Biol Chem 285:39702–39709. doi:10.1074/jbc.M110.170951

Flaherty SP, Winfrey VP, Olson GE (1988) Localization of actin in human, bull, rabbit, and hamster sperm by immunoelectron microscopy. Anat Rec 221:599–610. doi:10.1002/ar.1092210206

Freeman EA, Jani P, Millette CE (2002) Expression and potential function of Rho family small G proteins in cells of the mammalian seminiferous epithelium. Cell Commun Adhes 9:189–204

Gasman S, Chasserot-Golaz S, Malacombe M et al (2004) Regulated exocytosis in neuroendocrine cells: a role for subplasmalemmal Cdc42/N-WASP-induced actin filaments. Mol Biol Cell 15:520–531. doi:10.1091/mbc.E03-06-0402

Geyer CB, Inselman AL, Sunman JA et al (2009) A missense mutation in the Capza3 gene and disruption of F-actin organization in spermatids of repro32 infertile male mice. Dev Biol 330:142–152. doi:10.1016/j.ydbio.2009.03.020

Goley ED, Welch MD (2006) The ARP2/3 complex: an actin nucleator comes of age. Nat Rev Mol Cell Biol 7:713–726. doi:10.1038/nrm2026

Goley ED, Ohkawa T, Mancuso J et al (2006) Dynamic nuclear actin assembly by Arp2/3 complex and a baculovirus WASP-like protein. Science 314:464–467. doi:10.1126/science.1133348

Han L, Stope MB, de Jesús ML et al (2007) Direct stimulation of receptor-controlled phospholipase D1 by phospho-cofilin. EMBO J 26:4189–4202. doi:10.1038/sj.emboj.7601852

Heasman SJ, Ridley AJ (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol 9:690–701. doi:10.1038/nrm2476

Heid H, Figge U, Winter S et al (2002) Novel actin-related proteins Arp-T1 and Arp-T2 as components of the cytoskeletal calyx of the mammalian sperm head. Exp Cell Res 279:177–187

Hernández-González EO, Lecona-Valera AN, Escobar-Herrera J, Mújica A (2000) Involvement of an F-actin skeleton on the acrosome reaction in guinea pig spermatozoa. Cell Motil Cytoskeleton 46:43–58. doi:10.1002/(SICI)1097-0169(200005)46:1<43::AID-CM5>3.0.CO;2-1

Howell SL, Tyhurst M (1979) Interaction between insulin-storage granules and F-actin in vitro. Biochem J 178:367–371

Howes EA, Hurst SM, Jones R (2001) Actin and actin-binding proteins in bovine spermatozoa: potential role in membrane remodeling and intracellular signaling during epididymal maturation and the acrosome reaction. J Androl 22:62–72

Inoue N, Ikawa M, Isotani A, Okabe M (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434:234–238. doi:10.1038/nature03362

Inoue N, Satouh Y, Ikawa M et al (2011) Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. Proc Natl Acad Sci U S A 108:20008–20011. doi:10.1073/pnas.1116965108

Jaiswal JK, Rivera VM, Simon SM (2009) Exocytosis of post-Golgi vesicles is regulated by components of the endocytic machinery. Cell 137:1308–1319. doi:10.1016/j.cell.2009.04.064

Jenkins GM, Frohman MA (2005) Phospholipase D: a lipid centric review. Cell Mol Life Sci 62:2305–2316. doi:10.1007/s00018-005-5195-z

Jin M, Fujiwara E, Kakiuchi Y et al (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci U S A 108:4892–4896. doi:10.1073/pnas.1018202108

Kim K-S, Gerton GL (2003) Differential release of soluble and matrix components: evidence for intermediate states of secretion during spontaneous acrosomal exocytosis in mouse sperm. Dev Biol 264:141–152

Lee JS, Kwon WS, Rahman MS et al (2015) Actin-related protein 2/3 complex-based actin polymerization is critical for male fertility. Andrology 3:937–946. doi:10.1111/andr.12076
Lelkes PI, Friedman JE, Rosenheck K, Oplatka A (1986) Destabilization of actin filaments as a requirement for the secretion of catecholamines from permeabilized chromaffin cells. FEBS Lett 208:357–363
Li G, Rungger-Brändle E, Just I et al (1994) Effect of disruption of actin filaments by Clostridium botulinum C2 toxin on insulin secretion in HIT-T15 cells and pancreatic islets. Mol Biol Cell 5:1199–1213
Liu DY, Baker HW (1997) Protein kinase C plays an important role in the human zona pellucida-induced acrosome reaction. Mol Hum Reprod 3:1037–1043
Longo FJ, Krohne G, Franke WW (1987) Basic proteins of the perinuclear theca of mammalian spermatozoa and spermatids: a novel class of cytoskeletal elements. J Cell Biol 105:1105–1120
Lopez CI, Pelletán LE, Suhaiman L et al (2012) Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate. Biochim Biophys Acta 1821:1186–1199. doi:10.1016/j.bbalip.2012.05.001
Maier B, Medrano S, Sleight SB et al (2003) Developmental association of the synaptic activity-regulated protein arc with the mouse acrosomal organelle and the sperm tail. Biol Reprod 68:67–76
Malacombe M, Bader M-F, Gasman S (2006) Exocytosis in neuroendocrine cells: new tasks for actin. Biochim Biophys Acta 1763:1175–1183. doi:10.1016/j.bbamcr.2006.09.004
Malaisse WJ, Malaisse-Lagae F, Walker MO, Lacy PE (1971) The stimulus-secretion coupling of glucose-induced insulin release. V. The participation of a microtubular-microfilamentous system. Diabetes 20:257–265
Malaisse WJ, Herchuelz A, Levy J et al (1975) The stimulus-secretion coupling of glucose-induced insulin release XIX. The insulinotropic effect of glyceraldehyde. Mol Cell Endocrinol 4:1–12
Matter K, Dreyer F, Aktories K (1989) Actin involvement in exocytosis from PC12 cells: studies on the influence of botulinum C2 toxin on stimulated noradrenaline release. J Neurochem 52:370–376
Mayorga LS, Tomes CN, Belmonte SA (2007) Acrosomal exocytosis, a special type of regulated secretion. IUBMB Life 59:286–292. doi:10.1080/15216540701222872
Megnagi B, Finkelstein M, Shabtay O, Breitbart H (2015) The role and importance of cofilin in human sperm capacitation and the acrosome reaction. Cell Tissue Res 362:665–675. doi:10.1007/s00441-015-2229-1
Moreno-Fierros L, Hernández EO, Salgado ZO, Mújica A (1992) F-actin in guinea pig spermatozoa: its role in calmodulin translocation during acrosome reaction. Mol Reprod Dev 33:172–181. doi:10.1002/mrd.1080330209
Muallem S, Kwiatkowska K, Xu X, Yin HL (1995) Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. J Cell Biol 128:589–598
Mullins RD, Heuser JA, Pollard TD (1998) The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc Natl Acad Sci U S A 95:6181–6186
Niwa R, Nagata-Ohashi K, Takeichi M et al (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 108:233–246
Norman JC, Price LS, Ridley AJ et al (1994) Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. J Cell Biol 126:1005–1015
Ocampo J, Mondragón R, Roa-Espitia AL et al (2005) Actin, myosin, cytokeratins and spectrin are components of the guinea pig sperm nuclear matrix. Tissue Cell 37:293–308. doi:10.1016/j.tice.2005.03.003
Ohashi K, Hosoya T, Takahashi K et al (2000) A Drosophila homolog of LIM-kinase phosphorylates cofilin and induces actin cytoskeletal reorganization. Biochem Biophys Res Commun 276:1178–1185. doi:10.1006/bbrc.2000.3599

Oikonomopoulou I, Patel H, Watson PF, Chantler PD (2009) Relocation of myosin and actin, kinesin and tubulin in the acrosome reaction of bovine spermatozoa. Reprod Fertil Dev 21:364–377

Orci L, Gabbay KH, Malaisse WJ (1972) Pancreatic beta-cell web: its possible role in insulin secretion. Science 175:1128–1130

Paranko J, Longo F, Potts J et al (1988) Widespread occurrence of calicin, a basic cytoskeletal protein of sperm cells, in diverse mammalian species. Differ Res Biol Divers 38:21–27

Pelletier R, Trifaro JM, Carbajal ME et al (1999) Calcium-dependent actin filament-severing protein scinderin levels and localization in bovine testis, epididymis, and spermatozoa. Biol Reprod 60:1128–1136

Pendleton A, Koffer A (2001) Effects of latrunculin reveal requirements for the actin cytoskeleton during secretion from mast cells. Cell Motil Cytoskeleton 48:37–51. doi:10.1002/1097-0169(200101)48:1<37::AID-CM4>3.0.CO;2-0

Rogers BJ, Bastias C, Coulson RL, Russell LD (1989) Cytochalasin D inhibits penetration of hamster eggs by guinea pig and human spermatozoa. J Androl 10:275–282

Roldan ER, Dawes EN (1993) Phospholipase D and exocytosis of the ram sperm acrosome. Biochim Biophys Acta 1210:48–54

Romarowski A, Battistone MA, La Spina FA et al (2015) PKA-dependent phosphorylation of LIMK1 and Cofilin Is essential for mouse sperm acrosomal exocytosis. Dev Biol. doi:10.1016/j.ydbio.2015.07.008

Satouh Y, Inoue N, Ikawa M, Okabe M (2012) Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. J Cell Sci 125:4985–4990. doi:10.1242/jcs.100867

Snabes MC, Boyd AE (1982) Increased filamentous actin in islets of Langerhans from fasted hamsters. Biochem Biophys Res Commun 104:207–211

Somers G, Blondel B, Orci L, Malaisse WJ (1979) Motile events in pancreatic endocrine cells. Endocrinology 104:255–264. doi:10.1210/endo-104-1-255

Sontag JM, Aunis D, Bader MF (1988) Peripheral actin filaments control calcium-mediated catecholamine release from streptolysin-O-permeabilized chromaffin cells. Eur J Cell Biol 46:316–326

Soosairajah J, Maiti S, Wiggan O et al (2005) Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin. EMBO J 24:473–486. doi:10.1038/sj.emboj.7600543

Sosnik J, Miranda PV, Spiridonov NA et al (2009) Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. J Cell Sci 122:2741–2749. doi:10.1242/jcs.047225

Sosnik J, Buffone MG, Visconti PE (2010) Analysis of CAPZA3 localization reveals temporally discrete events during the acrosome reaction. J Cell Physiol 224:575–580

Spungin B, Margalit I, Breitbart H (1995) Sperm exocytosis reconstructed in a cell-free system: evidence for the involvement of phospholipase C and actin filaments in membrane fusion. J Cell Sci 108(Pt 6):2525–2535

Stock CE, Fraser LR (1987) The acrosome reaction in human sperm from men of proven fertility. Hum Reprod 2:109–119

Tamblyn TM (1983) A bovine seminal plasma inhibitor of actin-stimulated myosin adenosine triphosphatase. Biol Reprod 29:725–732

Tanaka H, Yoshimura Y, Nishina Y et al (1994) Isolation and characterization of cDNA clones specifically expressed in testicular germ cells. FEBS Lett 355:4–10

van Obberghen E, Somers G, Devis G et al (1973) Dynamics of insulin release and microtubular-microfilamentous system. I. Effect of cytochalasin B. J Clin Invest 52:1041–1051. doi:10.1172/JCI107269

Virtanen I, Badley RA, Paasivuo R, Lehto VP (1984) Distinct cytoskeletal domains revealed in sperm cells. J Cell Biol 99:1083–1091

Vitale ML, Seward EP, Trifaró JM (1995) Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. Neuron 14:353–363

Vogl AW, Genereux K, Pfeiffer DC (1993) Filamentous actin detected in rat spermatozoa. Tissue Cell 25:33–48

von Bülow M, Heid H, Hess H, Franke WW (1995) Molecular nature of calicin, a major basic protein of the mammalian sperm head cytoskeleton. Exp Cell Res 219:407–413. doi:10.1006/excr.1995.1246

von Bülow M, Rackwitz HR, Zimbelmann R, Franke WW (1997) CP beta3, a novel isoform of an actin-binding protein, is a component of the cytoskeletal calyx of the mammalian sperm head. Exp Cell Res 233:216–224

Wang JL, Easom RA, Hughes JH, McDaniel ML (1990) Evidence for a role of microfilaments in insulin release from purified beta-cells. Biochem Biophys Res Commun 171:424–430

Witke W, Sharpe AH, Hartwig JH et al (1995) Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. Cell 81:41–51

Yagi A, Paranko J (1992) Localization of actin, alpha-actinin, and tropomyosin in bovine spermatozoa and epididymal epithelium. Anat Rec 233:61–74. doi:10.1002/ar.1092330109

Yudin AI, Cherr GN, Vandevoort CA, Overstreet JW (1998) Rearrangement of the PH-20 protein on the surface of macaque spermatozoa following exposure to anti-PH-20 antibodies or binding to zona pellucida. Mol Reprod Dev 50:207–220. doi:10.1002/(SICI)1098-2795(199806)50:2<207::AID-MRD12>3.0.CO;2-3

Zanetti N, Mayorga LS (2009) Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. Biol Reprod 81:396–405. doi:10.1095/biolreprod.109.076166

Zepeda-Bastida A, Chiquete-Felix N, Uribe-Carvajal S, Mujica A (2011) The acrosomal matrix from guinea pig sperm contains structural proteins, suggesting the presence of an actin skeleton. J Androl 32:411–419. doi:10.2164/jandrol.110.011122

Chapter 8
Site of Mammalian Sperm Acrosome Reaction

Noritaka Hirohashi

8.1 Introduction

Until recently, no special attention has been paid to the question of the site of mammalian sperm acrosome reaction (AR) in the female reproductive tract. Because AR is an essential process that enables the spermatozoon to fertilize, it is generally believed that it occurs at a specific step during sperm–egg interaction. It is generally thought that "the site of action coincides with the site of commitment." Thus, understanding the roles of AR and acrosomal substances is needed to gain insight into the site of the sperm commitment to undergo AR.

IZUMO1 is an integral membrane protein with an extracellular immunoglobulin-like domain and a short cytoplasmic tail (Inoue et al. 2015). It is located on the inner acrosomal membrane (IAM) and outer acrosomal membrane (OAM) and externalized upon AR. Then, IZUMO1 translocates to the equatorial segment where fusion occurs with the oolemma (Sosnik et al. 2010). Izumo1$^{(-/-)}$ knockout male mice were infertile and spermatozoa lacking IZUMO1 protein accumulated in the perivitelline space of the oocyte (Inoue et al. 2015). Hence, IZUMO1 is essential for sperm–egg fusion but not for AR or for sperm penetration through the zona pellucida (ZP). In other words, AR is a prerequisite for sperm–egg fusion to occur. Similarly, AR appears to be essential for penetration of the ZP.

Since the first descriptions of the acrosome by Waldeyer and von Lenhossek in the late nineteenth century, and more detailed observations with electron microscopy by Dan in marine invertebrates (Dan 1952), and by Barros in mammals (Barros et al. 1967), there has been a consistent belief that acrosomal substances

N. Hirohashi (✉)
Oki Marine Biological Station, Education and Research Center for Biological Resources, Shimane University, 194 Kamo, Okinoshima-cho, Oki, Shimane 685-0024, Japan
e-mail: hiro@life.shimane-u.ac.jp

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_8

play fundamental roles in fertilization. Loeb proposed that spermatozoa (and perhaps their acrosomes) contain specific substances that cause cytolysis of the cortical layer of the egg, leading to parthenogenetic egg activation without entry of the spermatozoon itself (Loeb 1901). This concept was taken over in the following decades in efforts aimed at identifying acrosomal enzymes that could activate the unfertilized egg. It should be noted that fine structural studies using spermatozoa of echinoderms clearly demonstrated that well-defined changes occur in the acrosome when spermatozoa make contact with the egg coat (jelly layer): findings that had a significant impact on mammalian studies.

In parallel with these studies, the theory of sperm lysins—sperm-borne lytic agents involved in egg coat lysis during sperm penetration—was developed after the initial study by Schenk who found that sperm suspensions caused dispersal of the follicle cells surrounding mammalian eggs (Schenk 1878). Yaname was the first to demonstrate that mammalian spermatozoa have proteolytic activity that could disperse the egg vestments (Yamane 1930). These initial works led to the discovery of sperm hyaluronidases (Leonard and Kurzrok 1946) and acrosin (Zaneveld et al. 1969) in mammals (see Sect. 8.3.3). Although various hydrolases were isolated from mammalian sperm acrosomes, evidence for them acting as sperm lysins in vivo remains to be confirmed. Moreover, the originally proposed mammalian sperm lysins have been discredited by gene knockout experiments; however, the concept remains active (see below). In addition, although lysins of many animals are contained in the acrosome, some are already present at the sperm surface before AR, which led us to rethink the actual sequence of events.

Historically, mammalian spermatozoa have been assumed to start AR when and where the acrosomal contents are needed. Because marine invertebrates (e.g., sea urchins) have greatly contributed to establishing the paradigm of fertilization, and because fertilization should follow general principles, the mammalian egg coats (the cumulus oophorus and ZP) have been the major sites where mammalian spermatozoa have been shown to undergo AR. However, we must be aware of the complexity of the mammalian reproductive system because most knowledge regarding the mammalian sperm AR has been based on information based on spermatozoa collected directly from the male reproductive tract (epididymis or vas deferens) and because pharmacological evidence has largely relied on in vitro fertilization (IVF) assays. In addition, because there is a high degree of interspecies variation in the female reproductive system as well as in the structure of the female reproductive tract, the site of AR might also differ between species.

The last matter to be considered is sperm capacitation: the complex processes that occur in spermatozoa after ejaculation into the female's reproductive tract, involving morphological, physiological, and behavioral changes in spermatozoa leading to the acquisition of full competence for fertilization. Capacitation can be induced in vitro in a suitable medium containing calcium and bicarbonate ions and serum albumin. However, not all spermatozoa become competent for fertilization under these conditions and only a small fraction of them can exhibit AR in response to natural or chemical stimuli such as progesterone, solubilized ZP factors, or

calcium ionophores. Consequently, IVF is carried out using a heterogeneous population of spermatozoa.

8.2 Dissection of the Sperm AR into Multiple Steps

The sperm AR is an essential process for fertilization, perhaps in all mammalian species. As well as other somatic cells capable of exocytosis, AR involves fusion of a secretory vesicle (the acrosome) with the sperm plasma membrane (PM), release of the acrosomal contents, and exposure of the inner membrane of the acrosomal vesicle. The molecular machinery and mechanisms involved in AR are believed to be similar to those of secretory vesicles in general. Key molecules involved in membrane fusion such as Rab3A (Garde and Roldan 1996; Iida et al. 1999), SNAREs (Ramalho-Santos et al. 2000; Tomes et al. 2002), synaptotagmins (Michaut et al. 2001; Hutt et al. 2002), α-SNAP (Tomes et al. 2005; Rodriguez et al. 2011), and NSF (Ramalho-Santos and Schatten 2004) are also found in the spermatozoa of several mammalian species. However, the morphology and capacity of spermatozoa become altered drastically after AR, which is unlike typical exocytotic cells. The hallmark of regulated exocytosis such as seen in neurons and neurosecretory cells involves rapid membrane retrieval (membrane recycling or endocytosis) following exocytosis; however, the sperm AR does not involve an endocytotic process. The acrosome typically consists of a large singular granule and fusion occurs between the sperm PM and the OAM at multiple points over the acrosomal region (Yanagimachi 1994). This facilitates loss of the acrosome from the anterior head and the release of soluble components rapidly or gradually from the acrosomal matrix, depending on the diffusion/dissociation characteristics of each component (Kim et al. 2001), and finally the generation of hybrid lipid vesicles comprising the PM and OAM (Flaherty and Olson 1988). Thus, the sperm AR comprises several distinguishable steps; however, the definition of AR is ambiguous in terms of when, in the succession of cellular and molecular changes, it can be termed "initiated" or "completed." Historically, AR was thought to occur in an all-or-none fashion, and this should not be problematic provided the distinction between "intact" and "reacted" is defined in physiological terms. Undoubtedly, this view has been changing after observations of the steady state of AR transitions by multiple investigators (Kligman et al. 1991; Kim et al. 2001; Kim et al. 2011; Kongmanas et al. 2015).

The early processes in AR are a series of membrane fusion events. The first step is membrane "tethering" or "docking," which indicates an initial interaction between the OAM and the PM. The second step is "prefusion" or "priming," which indicates a status awaiting membrane fusion to occur following specific stimuli such as calcium ion influx. The final step is "fusion" and "fusion pore opening" that allow the release of soluble acrosomal contents. The "docking" and "priming" steps are distinguishable by detecting *cis*- and *trans*-SNARE protein complexes, respectively (Abou-Haila and Tulsiani 2003; Tsai et al. 2010). Using

this approach, noncapacitated human spermatozoa showed a *cis* complex, suggesting that they were arrested at an early stage of fusion (De Blas et al. 2005). In addition, it was postulated that the entire fusion process must be completed after receiving a stimulus that evokes AR. However, in pig spermatozoa, *trans*-SNARE was detected in those incubated in capacitating medium (Tsai et al. 2010). It remains unknown at which step of the fusion process spermatozoa undergo AR in normal circumstances in vivo in the oviduct.

There is increasing evidence suggesting that a particular subset of acrosomal proteins becomes externalized or transported to the sperm surface before substantial membrane fusion is executed. In earlier studies, a fluorescence dye, chlortetracycline (the "CTC assay"), was used to detect three basic stages of AR in the mouse: thus, acrosome-intact (capacitated), acrosome-reacting, and acrosome-reacted spermatozoa display a banded (B), spotty (S), and fully reacted (AR) pattern, respectively. The S pattern is considered as one or more intermediate states of the membrane vesiculation process, so spermatozoa displaying this pattern might represent the early process of post-fusion events. Theoretically, AR drives a sequential transition of $B \rightarrow S \rightarrow AR$. However, this transition is speculative because CTC is not suitable for real-time live imaging. A recently developed transgenic mouse line carrying the gene encoding acrosomal enhanced green fluorescent protein (EGFP; *Acr-EGFP*) opens new avenues in this regard. The EGFP fluorescence in the acrosome disappears at ~7 s after stimulation of capacitated spermatozoa with a calcium ionophore (Nakanishi et al. 1999), suggesting that this loss of EGFP fluorescence represents an early stage of membrane fusion (i.e., pore forming and/or pore expansion).

Mouse ZP3R/sp56 is a 67-kDa intra-acrosomal component, initially identified as a potential adhesion molecule mediating sperm–ZP binding (Cheng et al. 1994). ZP3R/sp56 appears on the sperm surface in a subpopulation of spermatozoa after incubating them in a capacitating medium. These spermatozoa are still acrosome intact as they still express the soluble acrosomal marker protein, EGFP, suggesting that ZP3R/sp56 is capable of being transported through the OAM and PM before AR (Kim et al. 2001). Zonadhesin is an acrosomal protein, facilitating species specificity in sperm–ZP binding (Gao and Garbers 1998; Tardif et al. 2010), also showed cell-surface localization during sperm capacitation in a porcine model (Kongmanas et al. 2015). The mechanism by which such selective transport is enabled remains unknown.

The release of acrosomal matrix contents continues for a considerable time: up to 2 h for sp56/ZP3R (Buffone et al. 2009), after the onset of initial membrane fusion. Hence, events after the completion of membrane fusion are considered as a part of AR. Acrosin (Acr) might facilitate disassembling of the acrosomal matrix and its release from the acrosome, because a significant delay in dispersal of the matrix was observed in spermatozoa from $Acr^{-/-}$mice compared with wild-type spermatozoa (Yamagata et al. 1998). Accordingly, Acr-null spermatozoa exhibited a ~30 min delay in ZP penetration and fertilization in an IVF assay, albeit with normal fertility and fecundity ultimately (Adham et al. 1997). These results indicate that dispersal of the acrosomal matrix is necessary for spermatozoa to penetrate the

ZP. It is uncertain how long it takes for spermatozoa to become competent for this after initiation of AR; however, in one case, duration between the initial AR (loss of EGFP) and initial ZP penetration was estimated to be no longer than 2–3 min [Supplemental movie 6 in Jin et al. (2011)]. Thus, although a relatively slow mode of protein dispersal takes place during AR, most of the important changes must be completed within a few minutes of PM–OAM fusion.

8.3 Roles of Acrosomal Substances

8.3.1 Dispersal of the Cumulus Matrix

Ovulated oocytes are normally covered by a mass of cumulus cells with an extracellular matrix of hyaluronic acid (the cumulus oophorus). In most eutherian mammals, with a few known exceptions such as the cow and sheep (Yanagimachi 1994), spermatozoa must pass through this matrix to reach the ZP. In early studies, hyaluronidase in the acrosome was considered a possible lytic agent with which spermatozoa can penetrate the cumulus oophorus. However, later studies identified a glycosylphosphatidylinositol (GPI)-anchored hyaluronidase, SPAM/PH-20, on the surface of acrosome-intact spermatozoa. More recently, Hyal5, another GPI-anchored hyaluronidase that localizes to both the sperm PM and the OAM, was claimed to hydrolyze the hyaluronan complex of the cumulus matrix. However, spermatozoa from mice lacking *SPAM* or *Hyal5* expression exhibited no phenotypic defects in overall fertility or in sperm penetration of the cumulus mass (Kimura et al. 2009). Although both SPAM and Hyal5 are capable of dispersing the cumulus matrix, fertilization can be completed before the cumulus is substantially dispersed in vitro as well as in vivo. Hence, genetic evidence for requirement of hyaluronidase is still lacking (Kang et al. 2010). However, a recent study using prostaglandin E receptor EP2-deficient female mice showed that enhanced chemokine signaling in the cumulus cells can make the matrix resistant to sperm hyaluronidase, thereby preventing sperm penetration and fertilization (Tamba et al. 2008). Nonetheless, it is now clear that AR is not a mandatory event to occur for spermatozoa to penetrate the cumulus oophorus. In fact, when an IVF assay using intact cumulus–oocyte complexes was performed, spermatozoa with intact acrosomes, proved by the presence of acrosomal EGFP, were capable of reaching the ZP.

8.3.2 Adhesion to the ZP

Spermatozoa must attach to the surface of the ZP before they can penetrate it. -Acrosome-reacted spermatozoa were shown to have greater affinity for the ZP than

did acrosome-intact spermatozoa; so acrosomal components are thought to act as molecular "glue" to stick to the ZP. So far, dozens of molecules have been characterized as having adhesive properties to the ZP, yet there has been no case in which genetic ablation caused infertility attributed solely to the failure of sperm–ZP interaction. It has been hypothesized that these acrosomal proteins must work together by forming a complex (Foster et al. 1997; Kongmanas et al. 2015), so the deletion of any single gene does not impair this critical step of fertilization. Also, there is a huge distinction between IVF and fertilization in vivo regarding the chemical and physical environments surrounding spermatozoa, such as the presence of seminal vesicle secretions (Kawano et al. 2014), selective passage through the cervix and the uterotubal junction (Ikawa et al. 2010), the establishment of a sperm reservoir in the lower isthmus of the oviduct (Suarez 2015), specific oviductal secretions (Killian 2011), and selective transport through the female reproductive tract (Hunter and Gadea 2014). Because IVF bypasses all such interactions with the female reproductive tract, the results from IVF experiments do not cover all aspects of naturally occurring fertilization. Nonetheless, IVF is still a promising approach for discovering new molecular models for sperm–ZP interactions (Visconti and Florman 2010; Redgrove et al. 2012; Nagdas et al. 2015). A recent study using gene rescue experiments clearly demonstrated that human spermatozoa could recognize the human zona pellucida protein ZP2 (Avella et al. 2014). The N-terminal domains (spanning amino acid residues 39–154 or 39–267) of human ZP2 exhibited high affinity for spermatozoa and enabled sperm binding to the ZP matrix. However, the counterpart(s) of these short peptides in the acrosome await discovery.

8.3.3 Penetration of the ZP

It is most likely that the spermatozoa of most mammals must trigger AR before penetration of the ZP (Austin and Bishop 1958). The acrosome is thought to contain hydrolase activity that dissolves the ZP matrix. Besides this hypothesis, dozens of enzymes have been reported as candidate zona lysins. The best-studied acrosomal enzyme is acrosin (EC 3.4.21.10.), one of the major acrosomal constituents in all mammalian species examined. This becomes active when exposed to a neutral pH (proacrosin/acrosin processing) and hydrolyzes proteins and synthetic peptides with substrate specificity similar to trypsin. Alkalization of the acrosomal lumen is evident during sperm capacitation (see below), so proacrosin is activated just prior to and/or during AR. Experiments using small compounds or antibodies that block acrosin's enzymatic activity suggested that this enzyme is involved in AR and in ZP penetration. However, male mice with a targeted mutation of the gene for acrosin were found to be fertile (Baba et al. 1994), so this enzyme is unlikely to play a direct role in ZP penetration. Acrosin is a diffusible protein that readily disappears from the acrosomal region after AR. If acrosin or other molecules that exhibit diffusing characteristics play a significant role in ZP lysis, then AR should occur in

the vicinity of the ZP. Alternatively, if enzymes anchored in the sperm membranes are involved in ZP lysis, AR does not necessarily occur at the periphery of the ZP.

The mammalian sperm 26S proteasome has been proposed to be a zona lysin (Yi et al. 2007). First, it is present in the acrosomal matrix as well as on the acrosomal membranes. Second, even after AR, proteasomes are detectable on the surface of the IAM (Yi et al. 2010), the only acrosomal structure that remains patent during ZP penetration (Yanagimachi 1994). Third, blocking the ubiquitin–proteasome system (depletion of ATP and inhibition of 20S core proteasome activity) or promoting it (inhibition of deubiquitination) produced opposite effects on IVF outcomes (Sutovsky et al. 2004; Yi et al. 2007). This is consistent with this proteasome's involvement in zona lysis. It remains unknown how this proteasome is associated with the sperm membrane.

It has been thought that the perforatorium, the sharply pointed subacrosomal structure of the apical head of the spermatozoon exposed following the AR, facilitates physical thrusting of spermatozoa through the ZP matrix, powered by active flagellation (Bedford 1998). Although it is difficult to prove or disprove this hypothesis, some mammalian species with a large acrosome, such as the guinea pig, may require AR, at least passively, to reduce physical friction during ZP penetration. Nonetheless, it is still difficult to imagine that purely physical force can make a hole in the ZP with its viscoelastic properties (Kim and Kim 2013). We saw many cases where spermatozoa became stacked and ceased their forward movement while traversing the ZP even though flagellar beating remained vigorous. Zona hardening cannot explain this because subsequent spermatozoa successfully penetrated the same ZP. At least in mice, sperm–ZP adhesion seems required for penetrating the ZP. Real-time observations revealed that spermatozoa adhered tightly to the zona matrix, but occasionally this loosened, resulting in sperm detachment from the ZP within several minutes. This phenomenon was more obvious after another spermatozoon had initiated fusion with the oolemma, when additional spermatozoa penetrating the ZP tended to detach.

In this context, continuous interactions between acrosome-reacted spermatozoa and the internal matrix of the ZP might be needed for penetration. Initially, ZP2, a major component of the ZP matrix, was proposed to serve as a ligand for secondary sperm binding (for acrosome-reacted spermatozoa) to the surface of the ZP. Recent work using mouse oocytes expressing "humanized" zona proteins by replacing mouse ZP1-3 with human ZP1-4 clearly showed that ZP2 but not ZP3 is necessary and sufficient for binding and penetration of human spermatozoa to the humanized ZP matrix (Avella et al. 2014). Thus, the same pair of molecules might keep working through the entire process, from initial contact until final penetration of the ZP.

8.4 ZP-Induced Sperm AR

Although direct evidence for zona lysis by acrosomal components awaits confirmation, over the last three decades AR has been believed to occur at the surface of the ZP, based on a series of in vitro observations: (1) the cumulus is dispensable for fertilization to occur, (2) spermatozoa are capable of penetrating the cumulus without undergoing AR, (3) only acrosome-intact spermatozoa bind to the ZP (which turned out not to be the case), (4) the proportion of acrosome-reacted spermatozoa on the ZP increases with time, (5) the acrosomal membranous "ghost" shed after AR remains on the zona surface, (6) ZP3, but not ZP1 or ZP2, is capable of inducing AR significantly more than spontaneous AR, and (7) sperm fertilization potential after AR might be time limited because of the loss of acrosomal enzymes.

Simply observing these processes directly in vitro can help identify the missing links in this model. For this purpose, mouse spermatozoa carrying two fluorescence tags (EGFP in the acrosome and Ds-Red2 in the midpiece) were found to be suitable for detecting the status of the acrosome as well as for tracking the path of the fertilizing spermatozoon (Hasuwa et al. 2010). However, it was difficult to observe real-time commitment of AR in the spermatozoa that remain attached to the ZP surface under fluorescent microscopy (Nakanishi et al. 1999; Baibakov et al. 2007). Even through some spermatozoa triggered AR on the ZP, they were unable to penetrate the ZP (Yamashita et al. 2007). Thus, it was difficult to predict which spermatozoon would eventually fertilize the oocyte. Instead, retrospective video tracking proved useful in detecting the acrosomal status of a fertilizing spermatozoon at any moment during sperm–egg interaction. By this method, we found that $>90\,\%$ (12/13) of "fertilizing" spermatozoa were already acrosome reacted before making contact with the ZP, whereas only $<10\,\%$ (1/13) of such spermatozoa triggered AR after binding to the ZP (Jin et al. 2011). These results together with previous findings (Huang et al. 1981; Gahlay et al. 2010) call the "ZP-inducer" model into question.

8.5 Sperm AR in the Cumulus Oophorus

Presence of the cumulus matrix facilitates fertilization in vitro (Salustri et al. 2004; Shimada et al. 2008; Tamba et al. 2008; Tanii et al. 2011). Paradoxically, the matrix can also prevent sperm entry until it undergoes postovulatory cumulus expansion, which is tightly regulated by follicle-stimulating hormone (Nagyova 2012). The cumulus cells also release progesterone that can potentiate or trigger AR (Roldan et al. 1994). This release of progesterone can be promoted through interaction with a human sperm glycosylphosphatidylinositol (GPI)-anchored membrane protein (NYD-SP8) liberated from the sperm surface (Yin et al. 2009). The cumulus oophorus also contains capacitation-promoting factors (Suzuki et al. 2002). Furthermore, not only chemical factors but also the physical properties of the matrix such as its sperm trapping/sequestering capacities (Bronson and Hamada 1977;

Bedford and Kim 1993) might enhance fertilization success. Sperm lacking a disintegrin and metallopeptidase domain 3 (ADAM3) or its molecular chaperone, a testis-specific protein disulfide isomerase homolog (PDILT), exhibited loss of zona-binding capacity and failed to fertilize cumulus-free oocytes. However, these spermatozoa effectively fertilized oocytes when the cumulus was allowed to remain intact (Tokuhiro et al. 2012), suggesting the importance of the extracellular matrix in mediating fertilization. Overall, the cumulus oophorus secures fertility by controlling sperm–ZP encounters. Additional, detailed investigations on why spermatozoa cannot enter the immature cumulus could offer new insights into the physiology of fertilizing spermatozoa.

8.6 Sperm AR in the Female Reproductive Tract

Although much research has been based on IVF, little is known about what really occurs in the oviduct in vivo. In earlier works, high proportions of spermatozoa that had undergone the AR were found in the ampulla of the oviduct in the guinea pig (Yanagimachi and Mahi 1976), rabbit (Overstreet and Cooper 1979), and cow (Herz et al. 1985). In mice, spermatozoa with intact acrosomes were able to reach the ampullary region of the oviduct following copulation (Chang and Suarez 2012), while acrosome-reacted spermatozoa were also present within the oviductal ampulla and the upper isthmus before and around the time of fertilization (Hirohashi et al. 2015). However, it was uncertain whether the acrosome-reacted spermatozoa found in the ampulla had already contacted the cumulus. More importantly, it is unknown how long acrosome-reacted spermatozoa can maintain their fertilizing competence in vivo. Our preliminary observations indicated that most acrosome-reacted sperm entering the oviduct continued vigorous flagellar beating, and in one case, a spermatozoon followed a convoluted spiral pathway through the cumulus around the ovulated oocyte (Fig. 8.1). Sustained longevity and fertilization competence in spermatozoa that had undergone the AR were verified by experiments in which spermatozoa were recovered from the perivitelline space of oocytes retrieved from the ampulla and used to reinseminate fresh cumulus-enclosed oocytes in vitro. These acrosome-reacted mouse spermatozoa were still capable of fertilization (Inoue et al. 2011), as previously reported for the rabbit (Kuzan et al. 1984).

Half a century has passed since Yanagimachi first raised the question of where sperm AR occurs in the female reproductive tract in vivo (Yanagimachi 1966), and there is still no unambiguous answer. During this period, models of sperm AR have been refined (Fig. 8.2). It was once thought that the initiation of the AR coincides with the end of sperm capacitation, and current research has been highlighting many unsolved issues related to this. Thus, better understanding of the molecular mechanisms involved in sperm capacitation will lead to the development of new methods and models for understanding the complex biology of mammalian reproduction using both innovative and retrospective approaches.

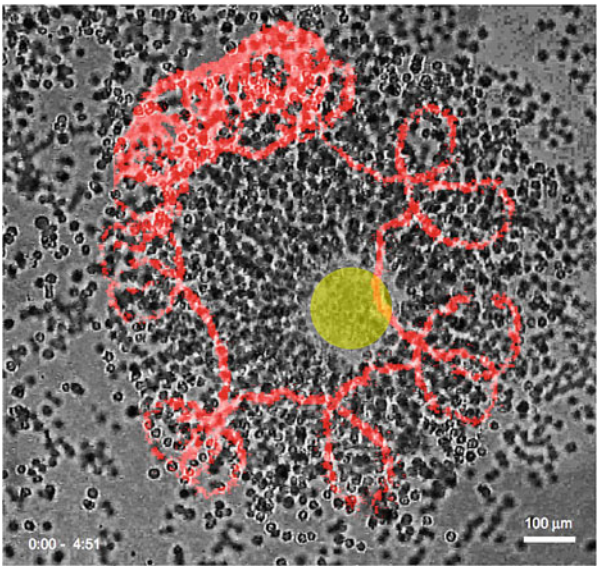

Fig. 8.1 Sperm behavior in the oviduct. Shown is a representative trajectory (*red*) by a spermatozoon that had undergone the AR and was found near the ovulated oocyte (*yellow*) in the ampulla. It exhibited two modes of circular swimming paths: in tight circles (with a clockwise motion) and around the periphery of the cumulus oophorus (with an anticlockwise motion). Duration: 4 min 51 s (The trajectory was drawn by SA Baba)

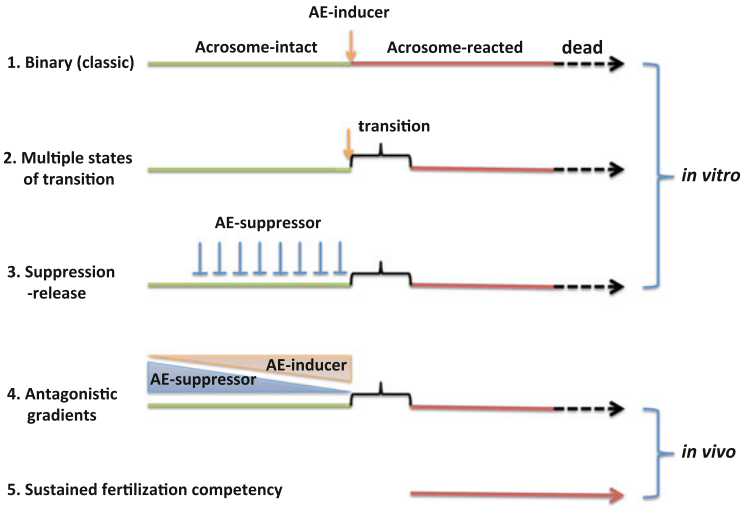

Fig. 8.2 Revised models of mammalian sperm acrosome reaction (AR). The model has developed from a simple binary one (1) to more complex ones (2–4). Major discoveries are steady-state intermediates (2), AR suppression by decapacitation factor (3), and the presence of antagonistic gradients (e.g., progesterone stimulation versus seminal vesicle protein 2 suppression) in the oviduct (4). The survival time of spermatozoa after completing AR is longer than initially thought (5)

References

Abou-Haila A, Tulsiani DR (2003) Evidence for the capacitation-associated membrane priming of mouse spermatozoa. Histochem Cell Biol 119:179–187. doi:10.1007/s00418-003-0504-9

Adham IM, Nayernia K, Engel W (1997) Spermatozoa lacking acrosin protein show delayed fertilization. Mol Reprod Dev 46:370–376. doi:10.1002/(SICI)1098-2795(199703)46:3<370::AID-MRD16>3.0.CO;2-2

Austin CR, Bishop MW (1958) Some features of the acrosome and perforatorium in mammalian spermatozoa. Proc R Soc Lond B Biol Sci 149:234–240

Avella MA, Baibakov B, Dean J (2014) A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. J Cell Biol 205:801–809. doi:10.1083/jcb.201404025

Baba T, Azuma S, Kashiwabara S, Toyoda Y (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. J Biol Chem 269:31845–31849

Baibakov B, Gauthier L, Talbot P, Rankin TL, Dean J (2007) Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. Development 134:933–943. doi:10.1242/dev.02752

Barros C, Bedford J, Franklin L, Austin C (1967) Membrane vesiculation as a feature of the mammalian acrosome reaction. J Cell Biol 34:1–5

Bedford JM (1998) Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. Biol Reprod 59:1275–1287

Bedford JM, Kim HH (1993) Cumulus oophorus as a sperm sequestering device, in vivo. J Exp Zool 265:321–328. doi:10.1002/jez.1402650314

Bronson R, Hamada Y (1977) Gamete interactions in vitro. Fertil Steril 28:570–576

Buffone MG, Kim KS, Doak BJ, Rodriguez-Miranda E, Gerton GL (2009) Functional consequences of cleavage, dissociation and exocytotic release of ZP3R, a C4BP-related protein, from the mouse sperm acrosomal matrix. J Cell Sci 122:3153–3160. doi:10.1242/jcs.052977

Chang H, Suarez SS (2012) Unexpected flagellar movement patterns and epithelial binding behavior of mouse sperm in the oviduct. Biol Reprod 86(140):141–148. doi:10.1095/biolreprod.111.096578

Cheng A, Le T, Palacios M, Bookbinder LH, Wassarman PM, Suzuki F, Bleil JD (1994) Sperm-egg recognition in the mouse: characterization of sp56, a sperm protein having specific affinity for ZP3. J Cell Biol 125:867–878

Dan J (1952) Study on the acrosome. I. Reaction to egg-water and other stimuli. Biol Bull 103:54–66

De Blas GA, Roggero CM, Tomes CN, Mayorga LS (2005) Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. PLoS Biol 3:e323. doi:10.1371/journal.pbio.0030323

Flaherty SP, Olson GE (1988) Membrane domains in guinea pig sperm and their role in the membrane fusion events of the acrosome reaction. Anat Rec 220:267–280. doi:10.1002/ar.1092200307

Foster JA, Friday BB, Maulit MT et al (1997) AM67, a secretory component of the guinea pig sperm acrosomal matrix, is related to mouse sperm protein sp56 and the complement component 4-binding proteins. J Biol Chem 272:12714–12722

Gahlay G, Gauthier L, Baibakov B, Epifano O, Dean J (2010) Gamete recognition in mice depends on the cleavage status of an egg's zona pellucida protein. Science 329:216–219. doi:10.1126/science.1188178

Gao Z, Garbers DL (1998) Species diversity in the structure of zonadhesin, a sperm-specific membrane protein containing multiple cell adhesion molecule-like domains. J Biol Chem 273:3415–3421

Garde J, Roldan ER (1996) rab 3-peptide stimulates exocytosis of the ram sperm acrosome via interaction with cyclic AMP and phospholipase A2 metabolites. FEBS Lett 391:263–268

Hasuwa H, Muro Y, Ikawa M, Kato N, Tsujimoto Y, Okabe M (2010) Transgenic mouse sperm that have green acrosome and red mitochondria allow visualization of sperm and their acrosome reaction in vivo. Exp Anim 59:105–107

Herz Z, Northey D, Lawyer M, First NL (1985) Acrosome reaction of bovine spermatozoa in vivo: sites and effects of stages of the estrous cycle. Biol Reprod 32:1163–1168

Hirohashi N, Spina FA, Romarowski A, Buffone MG (2015) Redistribution of the intra-acrosomal EGFP before acrosomal exocytosis in mouse spermatozoa. Reproduction 149:657–663. doi:10.1530/REP-15-0017

Huang TT, Fleming AD, Yanagimachi R (1981) Only acrosome-reacted spermatozoa can bind to and penetrate zona pellucida: a study using the guinea pig. J Exp Zool 217:287–290. doi:10.1002/jez.1402170215

Hunter RH, Gadea J (2014) Cross-talk between free and bound spermatozoa to modulate initial sperm:egg ratios at the site of fertilization in the mammalian oviduct. Theriogenology 82:367–372. doi:10.1016/j.theriogenology.2014.04.015

Hutt DM, Cardullo RA, Baltz JM, Ngsee JK (2002) Synaptotagmin VIII is localized to the mouse sperm head and may function in acrosomal exocytosis. Biol Reprod 66:50–56

Iida H, Yoshinaga Y, Tanaka S, Toshimori K, Mori T (1999) Identification of Rab3A GTPase as an acrosome-associated small GTP-binding protein in rat sperm. Dev Biol 211:144–155. doi:10.1006/dbio.1999.9302

Ikawa M, Inoue N, Benham AM, Okabe M (2010) Fertilization: a sperm's journey to and interaction with the oocyte. J Clin Invest 120:984–994. doi:10.1172/JCI41585

Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R (2011) Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. Proc Natl Acad Sci USA 108:20008–20011. doi:10.1073/pnas.1116965108

Inoue N, Hagihara Y, Wright D, Suzuki T, Wada I (2015) Oocyte-triggered dimerization of sperm IZUMO1 promotes sperm-egg fusion in mice. Nat Commun 6:8858. doi:10.1038/ncomms9858

Jin M, Fujiwara E, Kakiuchi Y et al (2011) Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proc Natl Acad Sci USA 108:4892–4896. doi:10.1073/pnas.1018202108

Kang W, Zhou C, Koga Y, Baba T (2010) Hyaluronan-degrading activity of mouse sperm hyaluronidase is not required for fertilization? J Reprod Dev 56:140–144

Kawano N, Araki N, Yoshida K et al (2014) Seminal vesicle protein SVS2 is required for sperm survival in the uterus. Proc Natl Acad Sci USA 111:4145–4150. doi:10.1073/pnas.1320715111

Killian G (2011) Physiology and endocrinology symposium: evidence that oviduct secretions influence sperm function: a retrospective view for livestock. J Anim Sci 89:1315–1322. doi:10.2527/jas.2010-3349

Kim J, Kim J (2013) Viscoelastic characterization of mouse zona pellucida. IEEE Trans Biomed Eng 60:569–575. doi:10.1109/TBME.2012.2230444

Kim KS, Foster JA, Gerton GL (2001) Differential release of guinea pig sperm acrosomal components during exocytosis. Biol Reprod 64:148–156

Kim KS, Foster JA, Kvasnicka KW, Gerton GL (2011) Transitional states of acrosomal exocytosis and proteolytic processing of the acrosomal matrix in guinea pig sperm. Mol Reprod Dev 78:930–941. doi:10.1002/mrd.21387

Kimura M, Kim E, Kang W et al (2009) Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in fertilization. Biol Reprod 81:939–947. doi:10.1095/biolreprod.109.078816

Kligman I, Glassner M, Storey BT, Kopf GS (1991) Zona pellucida-mediated acrosomal exocytosis in mouse spermatozoa: characterization of an intermediate stage prior to the completion of the acrosome reaction. Dev Biol 145:344–355

Kongmanas K, Kruevaisayawan H, Saewu A et al (2015) Proteomic Characterization of Pig Sperm Anterior Head Plasma Membrane Reveals Roles of Acrosomal Proteins in ZP3 Binding. J Cell Physiol 230:449–463. doi:10.1002/jcp.24728

Kuzan FB, Fleming AD, Seidel GE Jr (1984) Successful fertilization in vitro of fresh intact oocytes by perivitelline (acrosome-reacted) spermatozoa of the rabbit. Fertil Steril 41:766–770

Leonard S, Kurzrok R (1946) Inhibitors of hyaluronidase in blood sera and their effect on follicle cell dispersal. Endocrinology 39:85–90

Loeb J (1901) Experiments on artificial parthenogenesis in annelids (Chaetopterus) and the nature of the process at fertilization. Am J Physiol 4:423–459

Michaut M, De Blas G, Tomes CN, Yunes R, Fukuda M, Mayorga LS (2001) Synaptotagmin VI participates in the acrosome reaction of human spermatozoa. Dev Biol 235:521–529. doi:10.1006/dbio.2001.0316

Nagdas SK, Smith L, McNamara A, Hernandez-Encarnacion L, Medina-Ortiz I (2015) Identification and characterization of a bovine sperm acrosomal matrix protein and its mechanism of interaction with acrosomal hydrolases. Mol Cell Biochem 410:11–23. doi:10.1007/s11010-015-2534-8

Nagyova E (2012) Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation. Endocr Regul 46:225–235

Nakanishi T, Ikawa M, Yamada S, Parvinen M, Baba T, Nishimune Y, Okabe M (1999) Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein. FEBS Lett 449:277–283

Overstreet JW, Cooper GW (1979) The time and location of the acrosome reaction during sperm transport in the female rabbit. J Exp Zool 209:97–104. doi:10.1002/jez.1402090112

Ramalho-Santos J, Schatten G (2004) Presence of N-ethyl maleimide sensitive factor (NSF) on the acrosome of mammalian sperm. Arch Androl 50:163–168. doi:10.1080/01485010490425502

Ramalho-Santos J, Moreno RD, Sutovsky P et al (2000) SNAREs in mammalian sperm: possible implications for fertilization. Dev Biol 223:54–69. doi:10.1006/dbio.2000.9745

Redgrove KA, Nixon B, Baker MA, Hetherington L, Baker G, Liu DY, Aitken RJ (2012) The molecular chaperone HSPA2 plays a key role in regulating the expression of sperm surface receptors that mediate sperm-egg recognition. PLoS One 7:e50851. doi:10.1371/journal.pone.0050851

Rodriguez F, Bustos MA, Zanetti MN, Ruete MC, Mayorga LS, Tomes CN (2011) alpha-SNAP prevents docking of the acrosome during sperm exocytosis because it sequesters monomeric syntaxin. PLoS One 6:e21925. doi:10.1371/journal.pone.0021925

Roldan ER, Murase T, Shi QX (1994) Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science 266:1578–1581

Salustri A, Garlanda C, Hirsch E et al (2004) PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in vivo fertilization. Development 131:1577–1586. doi:10.1242/dev.01056

Schenk S (1878) Das Saugethierei kunstlich, befruchtet ausserhalb des Mutterthieres. Mitt Embryol Inst K K Univ Wien 1:107–118

Shimada M, Yanai Y, Okazaki T, Noma N, Kawashima I, Mori T, Richards JS (2008) Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. Development 135:2001–2011. doi:10.1242/dev.020461

Sosnik J, Buffone MG, Visconti PE (2010) Analysis of CAPZA3 localization reveals temporally discrete events during the acrosome reaction. J Cell Physiol 224:575–580. doi:10.1002/jcp.22211

Suarez SS (2015) Mammalian sperm interactions with the female reproductive tract. Cell Tissue Res 363(1):185–194. doi:10.1007/s00441-015-2244-2

Sutovsky P, Manandhar G, McCauley TC, Caamano JN, Sutovsky M, Thompson WE, Day BN (2004) Proteasomal interference prevents zona pellucida penetration and fertilization in mammals. Biol Reprod 71:1625–1637. doi:10.1095/biolreprod.104.032532

Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H (2002) Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. Int J Androl 25:84–93

Tamba S, Yodoi R, Segi-Nishida E, Ichikawa A, Narumiya S, Sugimoto Y (2008) Timely interaction between prostaglandin and chemokine signaling is a prerequisite for successful fertilization. Proc Natl Acad Sci USA 105:14539–14544. doi:10.1073/pnas.0805699105

Tanii I, Aradate T, Matsuda K, Komiya A, Fuse H (2011) PACAP-mediated sperm-cumulus cell interaction promotes fertilization. Reproduction 141:163–171. doi:10.1530/REP-10-0201

Tardif S, Wilson MD, Wagner R et al (2010) Zonadhesin is essential for species specificity of sperm adhesion to the egg zona pellucida. J Biol Chem 285:24863–24870. doi:10.1074/jbc.M110.123125

Tokuhiro K, Ikawa M, Benham AM, Okabe M (2012) Protein disulfide isomerase homolog PDILT is required for quality control of sperm membrane protein ADAM3 and male fertility [corrected]. Proc Natl Acad Sci USA 109:3850–3855. doi:10.1073/pnas.1117963109

Tomes CN, Michaut M, De Blas G, Visconti P, Matti U, Mayorga LS (2002) SNARE complex assembly is required for human sperm acrosome reaction. Dev Biol 243:326–338. doi:10.1006/dbio.2002.0567

Tomes CN, De Blas GA, Michaut MA, Farre EV, Cherhitin O, Visconti PE, Mayorga LS (2005) alpha-SNAP and NSF are required in a priming step during the human sperm acrosome reaction. Mol Hum Reprod 11:43–51. doi:10.1093/molehr/gah126

Tsai PS, Garcia-Gil N, van Haeften T, Gadella BM (2010) How pig sperm prepares to fertilize: stable acrosome docking to the plasma membrane. PLoS One 5:e11204. doi:10.1371/journal.pone.0011204

Visconti PE, Florman HM (2010) Mechanisms of sperm-egg interactions: between sugars and broken bonds. Sci Signal 3:pe35. doi:10.1126/scisignal.3142pe35

Yamagata K, Murayama K, Okabe M, Toshimori K, Nakanishi T, Kashiwabara S, Baba T (1998) Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. J Biol Chem 273:10470–10474

Yamane J (1930) The proteolytic action of mammalian spermatozoa and its bearing upon the second maturation division of ova. Cytologia 1:349–403

Yamashita M, Yamagata K, Tsumura K, Nakanishi T, Baba T (2007) Acrosome reaction of mouse epididymal sperm on oocyte zona pellucida. J Reprod Dev 53:255–262

Yanagimachi R (1966) Time and process of sperm penetration into hamster ova in vivo and in vitro. J Reprod Fertil 11:359–370

Yanagimachi R (1994) Mammalian fertilization. In: Knobil E, Neill J (eds) The physiology of reproduction, 10th edn. Raven, New York, pp 135–185

Yanagimachi R, Mahi CA (1976) The sperm acrosome reaction and fertilization in the guinea-pig: a study in vivo. J Reprod Fertil 46:49–54

Yi YJ, Manandhar G, Oko RJ, Breed WG, Sutovsky P (2007) Mechanism of sperm-zona pellucida penetration during mammalian fertilization: 26S proteasome as a candidate egg coat lysin. Soc Reprod Fertil Suppl 63:385–408

Yi YJ, Manandhar G, Sutovsky M et al (2010) Interference with the 19S proteasomal regulatory complex subunit PSMD4 on the sperm surface inhibits sperm-zona pellucida penetration during porcine fertilization. Cell Tissue Res 341:325–340. doi:10.1007/s00441-010-0988-2

Yin L, Chung CM, Huo R et al (2009) A sperm GPI-anchored protein elicits sperm-cumulus cross-talk leading to the acrosome reaction. Cell Mol Life Sci 66:900–908. doi:10.1007/s00018-009-8482-2

Zaneveld LJ, Srivastava PN, Williams WL (1969) Relationship of a trypsin-like enzyme in rabbit spermatozoa to capacitation. J Reprod Fertil 20:337–339

Chapter 9
Acrosome Reaction as a Preparation for Gamete Fusion

Patricia S. Cuasnicú, Vanina G. Da Ros, Mariana Weigel Muñoz, and Débora J. Cohen

9.1 Introduction

Fertilization strategies in Eutheria markedly differ from those of other metazoans (Bedford 2004, 2014). In most animals studied, this process involves a brief binding of spermatozoa to the egg coat, followed by the induction of the acrosome reaction (AR), a rapid and enzymatic penetration of the egg coat by the acrosome-reacted spermatozoa, and, finally, fusion of spermatozoa with the egg plasma membrane via the exposed inner acrosomal membrane. In Eutheria, however, fertilization involves the development of a special vigorous sperm motility known as hyperactivation that confers the cell the strength to penetrate the egg coats, followed by penetration of the cumulus mass by an either intact or acrosome-reacted spermatozoa, binding to and penetration of the unusually resilient zona pellucida (ZP) by hyperactivated spermatozoa mainly through mechanical mechanisms, and, finally, fusion of spermatozoa with the oolemma via a specific region of the head unique to eutherian mammals known as the equatorial segment (ES) (Bedford et al. 1979).

Underlying this fertilization mode in which physical factors seem to play a key role, eutherian spermatozoa display special characteristics such as a flattened form, a keratinoid nucleus and perinuclear theca, an insoluble acrosomal matrix, and a very stable inner acrosomal membrane (Bedford 2004). These structural characteristics seem to be linked to the unique features of the eutherian AR. In this regard, whereas throughout the animal kingdom the AR just implicates the fusion of the plasma and external acrosomal membranes leading to the release of the intra-

P.S. Cuasnicú (✉) • V.G. Da Ros • M. Weigel Muñoz • D.J. Cohen
Instituto de Biología y Medicina Experimental (IBYME-CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina
e-mail: pcuasnicu@ibyme.conicet.gov.ar

M.G. Buffone (ed.), *Sperm Acrosome Biogenesis and Function During Fertilization*, Advances in Anatomy, Embryology and Cell Biology 220,
DOI 10.1007/978-3-319-30567-7_9

acrosomal contents, in Eutheria the AR has an additional important consequence which is the acquisition of fusogenicity of the ES. Due to the unusual stability of the inner acrosomal membrane of eutherian sperm, likely related to the special nature of the ZP (Bedford 2004), this membrane does not have the fusogenic properties that it exhibits in other groups, and, as a consequence, the fusogenic role is substituted by the plasma membrane preserved over the ES. Unlike the rest of the plasma membrane of the acrosomal region, the ES does not vesiculate during the AR and becomes fusogenic only after completion of the AR (Takano et al. 1993; Yanagimachi 1994). Thus, the ES can be seen as an adaptation to preserve the fusogenicity of a membrane region until key moments in fertilization. This chapter focuses on the different molecular mechanisms involved in the acquisition of the fusogenicity of the ES after the AR.

9.2 Molecular Mechanisms Involved in the Acquisition of Fusogenicity of the Equatorial Segment

As the ES becomes fusogenic only after the AR, it is likely that some molecules involved in gamete fusion are not functional or available in the ES prior to the AR and activate and/or relocalize to this region as a consequence of this functional event. In this regard, all efforts have failed to make acrosome-intact spermatozoa fusion competent (Yanagimachi 1994). Although the mechanisms by which the ES becomes fusogenic are not yet clear, the information available has significantly contributed to a better understanding of this process. The following sections describe several proteins proposed to be involved in the acquisition of ES fusogenicity through different molecular mechanisms.

9.2.1 Proteins that Modify Other Molecules to Make the ES Fusogenic

One possible mechanism by which the ES might become fusogenic after the AR is that some acrosomal proteins released as a result of this exocytotic event activate or detach other molecules present in the ES. Other mechanisms could involve the modification of cytoskeletal molecules relevant for ES integrity and/or for protein relocalization to this region during the AR.

9.2.1.1 Acrosin

The serine protease acrosin, one of the major intra-acrosomal components, has been proposed to be responsible for changes in the ES based on the finding that acrosin inhibitors prevent spermatozoa from becoming fusogenic but not if the cells have

already undergone the AR (Takano et al. 1993). Acrosin could either activate fusion proteins already present in the ES or remove steric/charge barriers in that region. Based on several observations, it was concluded that, in addition to the changes that occur in the ES during the AR, the released acrosin further alters the ES plasma membrane to make it competent to fuse with the oolemma (Takano et al. 1993). Nevertheless, as acrosin inhibitors could not completely block gamete fusion and acrosin-null mice are fertile (Baba et al. 1994), it is likely that other acrosomal molecules are involved in the ES changes that occur concomitantly with the AR.

9.2.1.2 SPESP1

SPESP1 (Sperm Equatorial Segment Protein 1) is a post-meiotically expressed protein that first appears in the nascent acrosomal vesicle in early round spermatids and subsequently segregates to the periphery of the expanding acrosomal vesicle, defining a peripheral ES compartment within the flattened acrosomal vesicle (Wolkowicz et al. 2003). In view of this, SPESP1 has been proposed as a marker of the ES during acrosome biogenesis (Wolkowicz et al. 2003). Functional studies revealed that SPESP1 is exposed and surface accessible after the AR in human (Wolkowicz et al. 2003, 2008) and mouse (Lv et al. 2010) spermatozoa, supporting a potential role for this protein in gamete interaction. In accordance with this, antibodies against SPESP1 inhibit binding and fusion of human spermatozoa to hamster zona-free eggs (Wolkowicz et al. 2008) as well as mouse in vitro gamete fusion (Lv et al. 2010), and mouse SPESP1 binds to complementary sites in the oolemma (Lv et al. 2010). SPESP1-null spermatozoa present a reduced ability to fuse with zona-free eggs in vitro (Fujihara et al. 2010). In this case, it has been shown that the lack of SPESP1 affects the correct amount and localization of several other sperm proteins involved in gamete fusion (i.e., ADAMs, equatorin, and IZUMO1; see Sect. 9.2.2.1) and causes the loss of the equatorial membrane of acrosome-reacted spermatozoa. Therefore, SPESP1 has been proposed as a candidate molecule to be involved in the maintenance of the ES integrity and function after the AR has occurred.

9.2.1.3 TSSK6

TSSK6 is a Serine/Threonine protein kinase almost exclusively expressed in the testis, which localizes to a sperm region that includes the posterior head and the perforatorium and which is enriched with polymerized actin (Sosnik et al. 2009). *Tssk6*-null mice are infertile (Spiridonov et al. 2005) and their spermatozoa exhibit normal levels of spontaneous AR but are incapable of fusing with eggs in vitro (Sosnik et al. 2009). Interestingly, actin polymerization is compromised in *Tssk6*-null spermatozoa, and IZUMO1, a protein essential for gamete fusion (see Sect. 9.2.2.1), does not relocalize to the ES after the AR as observed in wild-type

spermatozoa (Sosnik et al. 2009). Taken together, these data suggest that TSSK6 is needed to maintain the sperm structural integrity essential for gamete fusion.

9.2.1.4 Calpains

Calpains belong to a family of non-lysosomal Ca^{2+}-dependent cysteine proteases widely expressed in a variety of tissues and cells (Molinari and Carafoli 1997; Goll et al. 2003). The most ubiquitous and well-characterized isoforms are calpain-1 and calpain-2, both of which are located in the acrosomal region of mouse (Ben-Aharon et al. 2005), human (Rojas et al. 1999), boar (Schollmeyer 1986), macaque (Yudin et al. 2000), and guinea pig spermatozoa (Bastián et al. 2010), between the plasma and outer acrosomal membranes (Yudin et al. 2000). Calpains play important roles related to cell adhesion, cell–cell fusion, and motility by processing adhesion and cytoskeletal proteins such as spectrin (Kwak et al. 1993; Franco and Huttenlocher 2005; Lebart and Benyamin 2006). Interestingly, it has been shown that sperm capacitation in the presence of calpain inhibitors results in a reduction of spectrin cleavage (Bastián et al. 2010), AR (Aoyama et al. 2001; Ben-Aharon et al. 2005; Bastián et al. 2010), and gamete fusion (Rojas and Moretti-Rojas 2000). Moreover, in agreement with the proposed role of calpain in cytoskeletal reorganization in other systems (Kwak et al. 1993), it has been proposed that the main role of calpain-1 during sperm capacitation is to cleave spectrin causing a disruption of the cytoskeleton (Bastián et al. 2010) and, thus, allowing the redistribution of proteins and lipids to the ES.

9.2.1.5 CD46

CD46, a complement regulatory protein present in spermatozoa (Anderson et al. 1989; Okabe et al. 1992), was originally proposed to be involved in the protection of these cells during their transit through the female tract (Anderson et al. 1993; Okabe et al. 1990; Seya et al. 1986). Whereas in humans CD46 is expressed throughout the body (Cervoni et al. 1992; Seya et al. 1993), in rodents its expression is limited to the testis, suggesting that although CD46 could function as a complement regulating factor in primates, its original role would have been in sperm–egg interaction (Inoue et al. 2003). In this regard, CD46 is exposed on the sperm surface only after the AR (Anderson et al. 1989; Fenichel et al. 1989; Simpson and Holmes 1994) and incubation of gametes with antibodies to CD46 significantly inhibited penetration of zona-free hamster eggs by human spermatozoa (Okabe et al. 1990; Anderson et al. 1993). The use of a CD46-deficient mouse model revealed that $CD46^{-/-}$ spermatozoa were fertilization competent but exhibited an accelerated spontaneous AR (Inoue et al. 2003). Thus, although the proposed role of CD46 in gamete fusion cannot be ruled out, these studies support the idea that its main function may be the stabilization of the acrosomal membranes (Johnson et al. 2007).

9.2.2 *Proteins that Localize to the ES After the AR and Mediate Gamete Fusion*

Many of the proteins involved in gamete fusion are not located in the ES before the AR and relocalize to this region shortly before or with the occurrence of this exocytotic event. The possible mechanisms that could explain the redistribution of these proteins depend on their original localization in spermatozoa. For those molecules located in domains of the plasma membrane other than the ES before the AR, it is possible that the dramatic reorganizations that take place in the plasma membrane during capacitation promote the breakdown of the diffusion barriers between domains, allowing protein migration to the ES (Topfer-Petersen et al. 1990; Flesch and Gadella 2000; Gadella et al. 2008). Proteins located in the acrosome, on the other hand, could either migrate to the ES via the "hairpin" structure formed by the acrosomal and plasma membranes in the equatorial sheath after the AR and/or reassociate with the ES after acrosomal vesiculation (Okabe 2015).

9.2.2.1 IZUMO1

IZUMO1, a sperm protein originally identified as an antigen recognized by the OBF13 antibody (Okabe et al. 1987), is a member of the immunoglobulin superfamily (Inoue et al. 2005). In permeabilized mouse spermatozoa, IZUMO1 can be detected in the dorsal portion of the anterior head and, after the AR, it spreads, enters the post-acrosomal compartment, and permeates the borders of different domains (Sosnik et al. 2009). Live imaging localization studies of IZUMO1 in gene-manipulated mice revealed that IZUMO1 moves from the inner and outer acrosomal membranes to the plasma membrane of the entire head, tending to mainly associate with the ES (Satouh et al. 2012). This translocation of IZUMO1 is blocked when actin dynamics is affected pharmacologically (Sosnik et al. 2009) or when spermatozoa lack TSSK6 (see Sect. 9.2.1.3). Although the localization of IZUMO1 after the AR is not limited to the ES, *Izumo*$^{-/-}$ males are infertile because their spermatozoa cannot fuse with the eggs (Inoue et al. 2005), identifying IZUMO1 as the only sperm protein found so far to be essential for gamete fusion. Accordingly, addition of the antibody against human or mouse IZUMO1 impedes spermatozoa to fuse with zona-free eggs (Okabe et al. 1987; Inoue et al. 2005). Recently, a folate receptor 4 (Fol4) protein that associates by GPI with the membrane of the oocyte has been described as the IZUMO1 egg-complementary site and named Juno (Bianchi et al. 2014). Females deficient in Juno are infertile and their eggs are unable to fuse with spermatozoa. These findings led to postulate IZUMO1 and Juno as the first pair of molecules essential for gamete fusion. Interestingly, more recent studies of cell–cell adhesion between oocytes exhibiting Juno and somatic cells overexpressing IZUMO show that the presence of these proteins is necessary but not sufficient for membrane fusion (Chalbi et al. 2014), reinforcing

the need for understanding more deeply the various mechanisms and molecules involved in gamete fusion.

9.2.2.2 CRISP1

CRISP1, the first identified member of the Cysteine-RIch Secretory Protein (CRISP) family, is an androgen-dependent glycoprotein synthetized by the epididymis in the rat (Cameo and Blaquier 1976; Garberi et al. 1979, 1982), mouse (Mizuki and Kasahara 1992; Haendler et al. 1993), human (Hayashi et al. 1996; Kratzschmar et al. 1996), and monkey (Sivashanmugam et al. 1999; Ellerman et al. 2010). This secretory protein associates with two different affinities with the surface of spermatozoa during epididymal maturation (Kohane et al. 1980a, b). Based on this, two populations of CRISP1 have been detected in spermatozoa: one loosely associated which is released during capacitation and proposed to act as a decapacitation factor (Kohane et al. 1980b; Cohen et al. 2000a; Roberts et al. 2003), and one strongly bound which remains in spermatozoa after capacitation and migrates from the dorsal region of the acrosome to the ES concomitantly with the occurrence of the AR (Rochwerger and Cuasnicu 1992). The permanence of CRISP1 in the ES of acrosome-reacted spermatozoa opened the possibility of its involvement in gamete fusion. In vitro co-incubation of zona-free eggs and spermatozoa in the presence of anti-CRISP1 antibodies or purified CRISP1 protein supports a role for this protein in an event following sperm binding to the oolemma and leading to fusion through complementary sites localized in the fusogenic region of the egg (Rochwerger et al. 1992; Cohen et al. 2000b, 2001). Subsequent studies using *Crisp1*-deficient mice confirmed the involvement of CRISP1 in gamete fusion (Da Ros et al. 2008). Moreover, when zona-free eggs were simultaneously inseminated with $Crisp1^{+/+}$ and $Crisp1^{-/-}$ spermatozoa in a competition assay, the mutant cells exhibited an even lower fusion ability (Da Ros et al. 2008), confirming that null spermatozoa have a clear disadvantage in their ability to fuse with the egg. Thus, CRISP1 constitutes an example of a protein that relocalizes to the ES with the AR and plays a role during gamete fusion.

9.2.2.3 CRISP2

CRISP2 also belongs to the CRISP family of proteins (Foster and Gerton 1996; Kratzschmar et al. 1996), but, differently from CRISP1 which is located to the sperm surface, it has been described within the acrosome of guinea pig (Hardy et al. 1988), rat (O'Bryan et al. 2001), mouse (Busso et al. 2007), and human (Busso et al. 2005) spermatozoa, and as a component of the sperm tail in rats and mice (O'Bryan et al. 1998, 2001; Gibbs et al. 2007). Whereas in guinea pig results show that CRISP2 forms part of the soluble compartment of the acrosome (Hardy et al. 1991; Kim et al. 2001), in mouse CRISP2 has been associated with the acrosomal matrix (Busso et al. 2007). Evidence supports that CRISP2 is released

from the acrosome during the AR and binds to the surface of the ES (Busso et al. 2005, 2007; Munoz et al. 2012; Nimlamool et al. 2013) independently of actin polymerization (Nimlamool et al. 2013). Experiments using biotinylated CRISP2 revealed that the exogenous protein binds to acrosome-reacted but not intact or capacitated cells, indicating that CRISP2 associates with the ES only after this region is modified by the AR (Nimlamool et al. 2013). Consistent with the localization of CRISP2 in the ES after the AR, the presence of an anti-CRISP2 antibody during gamete co-incubation produces a significant decrease in the ability of spermatozoa to penetrate zona-free eggs (Busso et al. 2005, 2007; Munoz et al. 2012). These observations were confirmed by recent results revealing that CRISP2-deficient spermatozoa exhibit impaired fusion ability (Brukman et al. 2016). Moreover, CRISP2 binds to the same egg-binding sites than CRISP1 (Busso et al. 2007) and inhibits the already reduced ability of CRISP1-null spermatozoa to penetrate zona-free mouse eggs (Da Ros et al. 2008), supporting the existence of a cooperation between the two CRISP proteins during gamete fusion.

9.2.2.4 α-L-Fucosidase

α-L-fucosidase, a lysosomal enzyme ubiquitously expressed, has been found in spermatozoa from chimpanzees (Srivastava et al. 1981), bull (Jauhiainen and Vanha-Perttula 1986), rat (Hancock et al. 1993), human (Alhadeff et al. 1999), boar (Park et al. 2002), hamster (Venditti et al. 2010), and mouse (Phopin et al. 2012). Unlike the isoforms present in lysosomes and in seminal plasma, sperm α-L-fucosidase is not soluble, but rather it becomes associated with the plasma membrane of the cells presumably during spermatogenesis (Avilés et al. 1996; Alhadeff et al. 1999; Venditti et al. 2007; Phopin et al. 2012). In humans, α-L-fucosidase is represented in the general membrane system of ejaculated spermatozoa but becomes enriched within the ES during capacitation and AR (Venditti et al. 2007). In mice, enzyme activity assays and immunofluorescence studies support the original localization of α-L-fucosidase in the acrosomal area and its redistribution to the ES after the AR (Phopin et al. 2012), suggesting its role in recognition of the egg plasma membrane. Although some differences have been reported according to the species studied, different lines of evidence support roles for α-L-fucosidase in fertilization. In hamster, blocking α-L-fucosidase activity of capacitated spermatozoa does not inhibit ZP binding or penetration, but did inhibit sperm–oocyte membrane interactions and/or some early stages of embryogenesis (Venditti et al. 2010). Pretreatment of mouse spermatozoa with anti-fucosidase antibody or exposure of oocytes to purified human liver α-L-fucosidase inhibits sperm–oolemma binding and gamete fusion but not post-fusion events (Phopin et al. 2013). Accordingly, addition of L-fucose to the fertilization media results in a significant decrease in the penetration of zona-free eggs in mice (Boldt et al. 1989) supporting the involvement of α-L-fucosidase in gamete fusion. No information

from genetically modified animal models is available, however, to confirm the role of this protein in vivo.

9.2.2.5 SLLP1

SLLP1, sperm c lysozyme-like protein, is expressed in the testis and localizes mainly to the anterior acrosome of intact spermatozoa and to the ES of acrosome-reacted cells in both human and mouse (Mandal et al. 2003; Herrero et al. 2005). Treatment of capacitated human spermatozoa with an antiserum against the recombinant form of the protein produces a significant decrease in the percentage of penetrated zona-free hamster eggs, supporting the participation of SLLP1 in gamete interaction (Mandal et al. 2003). Similarly, SLLP1 antibody blocks in vitro fertilization and sperm–egg binding in mice, suggesting that SLLP1 may play a role in sperm–egg adhesion. The finding that recombinant SLLP1 binds to the microvillar region of the mouse egg surface supports the involvement of the protein in gamete fusion through complementary sites in the oolemma (Herrero et al. 2005). Moreover, the egg-specific metalloprotease SAS1B has been identified as the binding partner of SLLP1 on the egg surface (Sachdev et al. 2012). Whereas SAS1B-deficient females exhibit a subfertile phenotype that suggests that this protein is involved in female fertility (Sachdev et al. 2012), the SLLP1-deficient mouse model has not yet been developed. Thus, further experiments are required to elucidate the participation of SLLP1 in fertilization and its relevance to male fertility.

9.2.2.6 ADAMs

ADAMs (a disintegrin and metalloprotease) are a family of membrane-anchored glycoproteins of around 30 members, several of which are present in different regions of the sperm head (Evans 2002). Fertilin (Adam1b/Adam2 heterodimer), one of the best studied proteins of the family located in the post-acrosomal region of the head, has been depicted as a sperm–egg fusion protein (Blobel et al. 1992), and disruption of Adam2 resulted in infertile males (Cho et al. 1998). Although the infertility in ADAM2 knockout males was finally attributed to the inability of spermatozoa to pass through the utero-tubal junction (Nishimura et al. 2001; Shamsadin et al. 1999), in vitro studies showed that *Adam2*$^{-/-}$ spermatozoa exhibit defects in gamete fusion (Cho et al. 1998). However, in spite of its proposed role in gamete fusion, ADAM2 is neither modified nor relocalized to the ES by the occurrence of the AR. Another member of the family, ADAM15, is partially processed during sperm transit through the male and female reproductive tracts and further during the AR, exposing its disintegrin domain (Pasten-Hidalgo et al. 2008). Whereas in the mouse the protein can be detected diffusely distributed throughout the head and flagellum of acrosome-reacted spermatozoa (Pasten-Hidalgo et al. 2008), in guinea pig it remains associated with the ES and flagellum

after the AR (Pastén et al. 2014). Eto and coworkers (2002) showed that the disintegrin domain from human ADAM15 binds to murine oocytes and blocks the binding of spermatozoa to zona-free eggs in a dose-dependent manner. Moreover, a peptide of only 12 amino acids corresponding to the disintegrin loop from mouse ADAM15 inhibits sperm–egg binding and fusion (Pasten-Hidalgo et al. 2008). Although these results support a role for ADAM15 in gamete fusion, its role in vivo still remains to be examined.

9.2.2.7 Equatorin

Equatorin is a *N*,*O*-sialoglycoprotein identified as the antigen recognized by the monoclonal antibody MN9 (Toshimori et al. 1992; Yamatoya et al. 2009). Although originally described as localized within the anterior acrosome and ES in several mammalian species, including humans (Toshimori et al. 1992, 1998), subsequent studies revealed that it translocates to the plasma membrane of the ES during the AR (Yoshida et al. 2010). This more detailed observation of equatorin localization led the authors to postulate this protein as a model molecule to study the progression of the AR. Equatorin was proposed to be involved in mouse gamete fusion based on the accumulation of perivitelline spermatozoa when in vitro fertilization assays were carried out in the presence of the monoclonal antibody MN9 (Toshimori et al. 1998). The recent generation of *Eqtn*-deficient mice shows that $Eqtn^{-/-}$ male mice are subfertile and their spermatozoa exhibit reduced in vivo and in vitro fertilizing ability (Hao et al. 2014). The analysis of the mechanism underlying this reduction revealed a defect in the AR with normal acrosomal biogenesis. Moreover, co-immunoprecipitation studies indicated that equatorin might directly or indirectly interact with the SNARE complex (Hao et al. 2014) known to play an important role in membrane fusion during acrosome exocytosis (Tomes 2015). Further investigations are, therefore, required to more clearly establish the role of equatorin in the specific gamete fusion event.

9.3 Final Remarks

The AR is a critical event that renders the sperm cell capable of fertilizing the egg. In eutherian mammals, besides the release of the acrosomal content characteristic of this exocytotic event throughout the animal kingdom, there is an additional key consequence of the AR which is the acquisition of fusogenicity of the ES. Although still poorly understood, the mechanisms involved in this process seem to rely mainly on the ability of some proteins to modify cytoskeletal and/or membrane molecules as well as on a spatial reordering of acrosomal and membrane proteins (Fig. 9.1). Considering the numerous proteins proposed to be involved in the acquisition of ES fusogenicity, it is likely that a functional redundancy among them exists. The simultaneous deletion of more than one of these molecules will

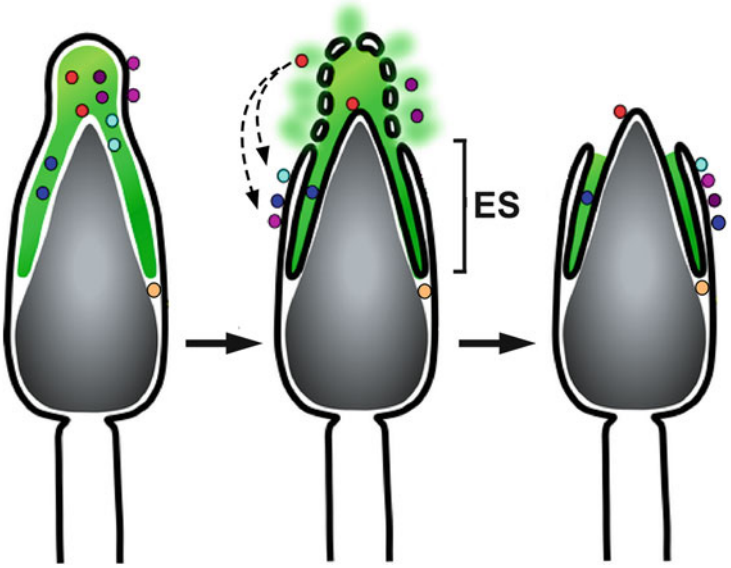

Fig. 9.1 Molecular changes during the AR leading to the acquisition of ES fusogenicity. Diagram showing different sperm proteins proposed to be involved in the acquisition of the ES fusogenicity either by modifying cytoskeletal proteins (i.e., *orange circle* TSSK6) and/or membrane proteins present in the ES (i.e., *red circle* acrosin, *dark blue circle* SPESP1) or by relocalizing to the ES to participate in gamete fusion (i.e., *light blue circle* IZUMO1, *pink circle* CRISP1, *magenta circle* CRISP2)

contribute to a better understanding of the mechanisms through which the AR prepares spermatozoa for subsequent fusion with the egg.

References

Alhadeff JA, Khunsook S, Choowongkomon K et al (1999) Characterization of human semen alpha-L-fucosidases. Mol Hum Reprod 5:809–815

Anderson DH, Michaelson JS, Johnson PM (1989) Trophoblast/leukocyte-common antigen is expressed by human testicular germ cells and appears on the surface of acrosome-reacted sperm. Biol Reprod 41:285–293

Anderson DJ, Abbott AF, Jack RM (1993) The role of complement component C3b and its receptors in sperm-oocyte interaction. Proc Natl Acad Sci USA 90:10051–10055

Aoyama T, Osaki Y, Suzumori K et al (2001) Involvement of μ-calpain in human sperm capacitation for fertilization. Am J Reprod Immunol 45:12–20

Avilés M, Abascal I, Martinez-Menarguez JA et al (1996) Immunocytochemical localization and biochemical characterization of a novel plasma membrane-associated, neutral ph optimum alpha-L-fucosidase from rat testis and epididymal spermatozoa. Biochem J 318:821–831

Baba T, Azuma S, Kashiwabara S et al (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. J Biol Chem 269:31845–31849

Bastián Y, Roa-Espitia AL, Mújica A et al (2010) Calpain modulates capacitation and acrosome reaction through cleavage of the spectrin cytoskeleton. Reproduction 140:673–684
Bedford JM (2004) Enigmas of mammalian gamete form and function. Biol Rev Camb Philos Soc 79:429–460
Bedford JM (2014) Singular features of fertilization and their impact on the male reproductive system in eutherian mammals. Reproduction 147:43–52
Bedford JM, Moore HD, Franklin LE (1979) Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. Exp Cell Res 119:119–126
Ben-Aharon I, Brown PR, Etkovitz N et al (2005) The expression of calpain 1 and calpain 2 in spermatogenic cells and spermatozoa of the mouse. Reproduction 129:435–442
Bianchi E, Doe B, Goulding D et al (2014) Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature 508:483–487
Blobel C, Wolsberg T, Turck C et al (1992) A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. Nature 356:248–252
Boldt J, Howe AM, Parkerson JB et al (1989) Carbohydrate involvement in sperm-egg fusion in mice. Biol Reprod 40:887–896
Brukman NG, Miyata H, Torres P et al (2016) Fertilization defects in sperm from Cysteine-rich secretory protein 2 (Crisp2) knockout mice: implications for fertility disorders. Mol Hum Reprod 22:240–251
Busso D, Cohen DJ, Hayashi M et al (2005) Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate alter capacitation and relevance for gamete interaction. Mol Hum Reprod 11:299–305
Busso D, Goldweic NM, Hayashi M et al (2007) Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. Biol Reprod 76:701–708
Cameo M, Blaquier J (1976) Androgen-controlled specific proteins in rat epididymis. J Endocrinol 69:317–324
Cervoni F, Oglesby TJ, Adams EM et al (1992) Identification and characterization of membrane cofactor protein of human spermatozoa. J Immunol 148:1431–1437
Chalbi M, Barraud-Lange V, Ravaux B et al (2014) Binding of sperm protein Izumo1 and its egg receptor Juno drives Cd9 accumulation in the intercellular contact area prior to fusion during mammalian fertilization. Development 141:3732–3739
Cho C, Bunch DO, Faure JE et al (1998) Fertilization defects in sperm from mice lacking fertilin β. Science 281:1857–1859
Cohen DJ, Rochwerger L, Ellerman DA et al (2000a) Relationship between the association of rat epididymal protein DE with spermatozoa and the behaviour and function of the protein. Mol Reprod Dev 56:180–188
Cohen DJ, Ellerman DA, Cuasnicú PS (2000b) Mammalian sperm-egg fusion: evidence that epididymal protein DE plays a role in mouse gamete fusion. Biol Reprod 63:462–468
Cohen DJ, Ellerman DA, Busso D et al (2001) Evidence that human epididymal protein ARP plays a role in gamete fusion through complementary sites on the surface of the human egg. Biol Reprod 65:1000–1005
Da Ros VG, Maldera JA, Willis WD et al (2008) Impaired sperm fertilizing ability in mice lacking Cysteine-RIch Secretory Protein 1 (CRISP1). Dev Biol 320:12–18
Ellerman DA, Cohen DJ, Weigel Muñoz M et al (2010) Immunologic behavior of human cysteine-rich secretory protein 1 (hCRISP1) in primates: prospects for immunocontraception. Fertil Steril 93:2551–2556
Eto K, Huet C, Tarui T et al (2002) Functional classification of ADAMs based on a conserved motif for binding to integrin $\alpha_9\beta_1$ implications for sperm–egg binding and other cell interactions. J Biol Chem 277:17804–17810
Evans JP (2002) The molecular basis of sperm-oocyte membrane interactions during mammalian fertilization. Hum Reprod Update 8:297–311
Fenichel P, Hsi BL, Farahifar D et al (1989) Evaluation of the human sperm acrosome reaction using a monoclonal antibody, GB24, and fluorescence-activated cell sorter. J Reprod Fertil 87:699–704

Flesch FM, Gadella BM (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 1469:197–235
Foster JA, Gerton GL (1996) Autoantigen 1 of the guinea pig sperm acrosome is the homologue of mouse Tpx-1 and human TPX1 and is a member of the cysteine-rich secretory protein (CRISP) family. Mol Reprod Dev 44:221–229
Franco SJ, Huttenlocher A (2005) Regulating cell migration: calpains make the cut. J Cell Sci 118:3829–3838
Fujihara Y, Murakami M, Inoue N et al (2010) Sperm equatorial segment protein 1, SPESP1, is required for fully fertile sperm in mouse. J Cell Sci 123:1531–1536
Gadella BM, Tsai PS, Boerke A et al (2008) Sperm head membrane reorganisation during capacitation. Int J Dev Biol 52:473–480
Garberi JC, Kohane AC, Cameo MS et al (1979) Isolation and characterization of specific rat epididymal proteins. Mol Cell Endocrinol 13:73–82
Garberi JC, Fontana JD, Blaquier JA (1982) Carbohydrate composition of specific rat epididymal protein. Int J Androl 5:619–626
Gibbs GM, Bianco DM, Jamsai D et al (2007) Cysteine-rich secretory protein 2 binds to mitogen-activated protein kinase kinase kinase 11 in mouse sperm. Biol Reprod 77:108–114
Goll DE, Thompson VF, Li H et al (2003) The calpain system. Physiol Rev 83:731–801
Haendler B, Kratzschmar J, Theuring F et al (1993) Transcripts for cysteine-rich secretory protein-1 (CRISP-1; DE/AEG) and the novel related CRISP-3 are expressed under androgen control in the mouse salivary gland. Endocrinology 133:192–198
Hancock LW, Raab LS, Aronson NN Jr (1993) Synthesis and processing of rat sperm-associated alpha-L-fucosidase. Biol Reprod 48:1228–1238
Hao J, Chen M, Ji S et al (2014) Equatorin is not essential for acrosome biogenesis but is required for the acrosome reaction. Biochem Biophys Res Commun 444:537–542
Hardy DM, Huang TTF, Driscoll WJ et al (1988) Purification and characterization of the primary acrosomal autoantigen of guinea pig epididymal spermatozoa. Biol Reprod 38:423–437
Hardy DM, Oda MN, Friend DS et al (1991) A mechanism for differential release of acrosomal enzymes during the acrosome reaction. Biochem J 275:759–766
Hayashi M, Fujimoto S, Takano H et al (1996) Characterization of a human glycoprotein with potential role in sperm-egg fusion: cDNA cloning, immunohistochemical localization, and chromosomal assignment of the gene (AEGL1). Genomics 32:367–374
Herrero MB, Mandal A, Digilio LC et al (2005) Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. Dev Biol 284:126–142
Inoue N, Ikawa M, Nakanishi T et al (2003) Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. Mol Cell Biol 23:2614–2622
Inoue N, Ikawa M, Isotani A et al (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434:234–238
Jauhiainen A, Vanha-Perttula T (1986) alpha-L-Fucosidase in the reproductive organs and seminal plasma of the bull. Biochim Biophys Acta 880:91–95
Johnson PM, Clift LE, Andrlíková P et al (2007) Rapid sperm acrosome reaction in the absence of acrosomal CD46 expression in promiscuous field mice (Apodemus). Reproduction 134:739–747
Kim KS, Foster JA, Gerton GL (2001) Differential release of guinea pig sperm acrosomal components during exocytosis. Biol Reprod 64:148–156
Kohane AC, Cameo MS, Piñeiro L et al (1980a) Distribution and site of production of specific proteins in the rat epididymis. Biol Reprod 23:181–187
Kohane AC, Gonzalez Echeverria F, Piñeiro L et al (1980b) Interaction of proteins of epididymal origin with spermatozoa. Biol Reprod 23:737–742
Kratzschmar J, Haendler B, Eberspaecher U et al (1996) The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. Eur J Biochem 236:827–836
Kwak KB, Kambayashi J, Kang MS et al (1993) Cell-penetrating inhibitors of calpain block both membrane fusion and filamin cleavage in chick embryonic myoblasts. FEBS Lett 323:151–154

Lebart MC, Benyamin Y (2006) Calpain involvement in the remodeling of cytoskeletal anchorage complexes. FEBS J 273:3415–3426
Lv ZM, Wang M, Xu C (2010) Antifertility characteristics of the N-terminal region of mouse equatorial segment protein. Anat Rec (Hoboken) 293:171–181
Mandal A, Klotz KL, Shetty J et al (2003) SLLP1, a unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. Biol Reprod 68:1525–1537
Mizuki N, Kasahara M (1992) Mouse submandibular glands express an androgen-regulated transcript encoding an acidic epididymal glycoprotein-like molecule. Mol Cell Endocrinol 89:25–32
Molinari M, Carafoli E (1997) Calpain: a cytosolic proteinase active at the membranes. J Membr Biol 156:1–8
Munoz MW, Ernesto JI, Bluguermann C et al (2012) Evaluation of testicular sperm CRISP2 as a potential target for contraception. J Androl 33:1360–1370
Nimlamool W, Bean BS, Lowe-Krentz LJ (2013) Human sperm CRISP2 is released from the acrosome during the acrosome reaction and re-associates at the equatorial segment. Mol Reprod Dev 80:488–502
Nishimura H, Cho C, Branciforte DR et al (2001) Analysis of loss of adhesive function in sperm lacking cyritestin or fertilin β. Dev Biol 233:204–213
O'Bryan MK, Loveland KL, Herszfeld D et al (1998) Identification of a rat testis-specific gene encoding a potential rat outer dense fibre protein. Mol Reprod Dev 50:313–322
O'Bryan MK, Sebire K, Meinhardt A et al (2001) Tpx-1 is a component of the outer dense fibers and acrosome of rat spermatozoa. Mol Reprod Dev 58:116–125
Okabe M (2015) Mechanisms of fertilization elucidated by gene-manipulated animals. Asian J Androl 17:646–652
Okabe M, Adachi T, Takada K et al (1987) Capacitation-related changes in antigen distribution on mouse sperm heads and its relation to fertilization rate in vitro. J Reprod Immunol 11:91–100
Okabe M, Nagira M, Kawai Y et al (1990) A human sperm antigen possibly involved in binding and/or fusion with zona-free hamster eggs. Fertil Steril 54:1121–1126
Okabe M, Ying X, Nagira M et al (1992) Homology of an acrosome-reacted sperm-specific antigen to CD46. J Pharmacobiodyn 15:455–459
Park CK, Hwang IS, Cheong HT et al (2002) Effect of a fertilization-promoting peptide on the fertilizing ability and glycosidase activity in vitro of frozen-thawed spermatozoa in the pig. Anim Reprod Sci 72:83–94
Pastén K, Bastián Y, Roa-Espitia AL et al (2014) ADAM15 participates in fertilization through a physical interaction with acrogranin. Reproduction 148:623–634
Pasten-Hidalgo K, Hernandez-Rivas R, Roa-Espitia AL et al (2008) Presence, processing, and localization of mouse ADAM15 during sperm maturation and the role of its disintegrin domain during sperm-egg binding. Reproduction 136:41–51
Phopin K, Nimlamool W, Bartlett MJ et al (2012) Distribution, crypticity, stability and localization of alpha-L-fucosidase of mouse cauda epididymal sperm. Mol Reprod Dev 3:208–217
Phopin K, Nimlamool W, Lowe-Krentz LJ et al (2013) Roles of mouse sperm-associated alpha-L-fucosidases in fertilization. Mol Reprod Dev 80:273–285
Roberts KP, Wamstad JA, Ensrud KM et al (2003) Inhibition of capacitation-associated tyrosine phosphorylation signaling in rat sperm by epididymal protein Crisp1. Biol Reprod 69:572–581
Rochwerger L, Cuasnicu PS (1992) Redistribution of a rat sperm epididymal glycoprotein after in vivo and in vitro capacitation. Mol Reprod Dev 31:34–41
Rochwerger L, Cohen DJ, Cuasnicú PS (1992) Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion. Dev Biol 153:83–90
Rojas FJ, Moretti-Rojas I (2000) Involvement of the calcium-specific protease, calpain, in the fertilizing capacity of human spermatozoa. Int J Androl 23:163–168
Rojas FJ, Brush M, Moretti-Rojas I (1999) Calpain-calpastatin: a novel, complete calcium-dependent protease system in human spermatozoa. Mol Hum Reprod 5:520–526

Sachdev M, Mandal A, Mulders S et al (2012) Oocyte specific oolemmal SAS1B involved in sperm binding through intra-acrosomal SLLP1 during fertilization. Dev Biol 363:40–51
Satouh Y, Inoue N, Ikawa M et al (2012) Visualization of the moment of mouse sperm-egg fusion and dynamic localization of IZUMO1. J Cell Sci 125:4985–4990
Schollmeyer JE (1986) Identification of calpain II in porcine sperm. Biol Reprod 34:721–731
Seya T, Turner JR, Atkinson JP (1986) Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. J Exp Med 163:837–855
Seya T, Hara T, Matsumoto M et al (1993) Membrane cofactor protein (MCP, CD46) in seminal plasma and on spermatozoa in normal and "sterile" subjects. Eur J Immunol 23:1322–1327
Shamsadin R, Adham IM, Nayernia K et al (1999) Male mice deficient for germ-cell cyritestin are infertile. Biol Reprod 61:1445–1451
Simpson KL, Holmes CH (1994) Differential expression of complement regulatory proteins decay-accelerating factor (CD55), membrane cofactor protein (CD46) and CD59 during human spermatogenesis. Immunology 81:452–461
Sivashanmugam P, Richardson RT, Hall S et al (1999) Cloning and characterization of an androgen-dependent acidic epididymal glycoprotein/CRISP1-like protein from the monkey. J Androl 20:384–393
Sosnik J, Miranda PV, Spiridonov NA et al (2009) Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. J Cell Sci 122:2741–2749
Spiridonov NA, Wong L, Zerfas PM et al (2005) Identification and characterization of SSTK, a serine/threonine protein kinase essential for male fertility. Mol Cell Biol 25:4250–4261
Srivastava PN, Farooqui AA, Gould KG (1981) Studies on hydrolytic enzymes of chimpanzee semen. Biol Reprod 25:363–369
Takano H, Yanagimachi R, Urch UA (1993) Evidence that acrosin activity is important for the development of fusibility of mammalian spermatozoa with the oolemma: inhibitor studies using the golden hamster. Zygote 1:79–91
Tomes CN (2015) The proteins of exocytosis: lessons from the sperm model. Biochem J 465:359–370
Topfer-Petersen E, Friess AE, Stoffel M et al (1990) Boar sperm membranes antigens. II. Reorganization of an integral membrane antigen during capacitation and acrosome reaction. Histochemistry 93:491–495
Toshimori K, Tanii I, Araki S et al (1992) Characterization of the antigen recognized by a monoclonal antibody MN9: unique transport pathway to the equatorial segment of sperm head during spermiogenesis. Cell Tissue Res 270:459–468
Toshimori K, Saxena DK, Tanii I et al (1998) An MN9 antigenic molecule, equatorin, is required for successful sperm-oocyte fusion in mice. Biol Reprod 59:22–29
Venditti JJ, Donigan KA, Bean BS (2007) Crypticity and functional distribution of the membrane associated alpha-L-fucosidase of human sperm. Mol Reprod Dev 74:758–766
Venditti JJ, Swann JM, Bean BS (2010) Hamster sperm-associated alpha-L-fucosidase functions during fertilization. Biol Reprod 82:572–579
Wolkowicz MJ, Shetty J, Westbrook A et al (2003) Equatorial segment protein defines a discrete acrosomal subcompartment persisting throughout acrosomal biogenesis. Biol Reprod 69:735–745
Wolkowicz MJ, Digilio L, Klotz K et al (2008) Equatorial segment protein (ESP) is a human alloantigen involved in sperm-egg binding and fusion. J Androl 29:272–282
Yamatoya K, Yoshida K, Ito C et al (2009) Equatorin: identification and characterization of the epitope of the MN9 antibody in the mouse. Biol Reprod 81:889–897
Yanagimachi R (1994) Mammalian fertilization. In: Knobil E, Neill JD (eds) The physiology of reproduction, 2nd edn. Raven, New York, NY, pp 189–317
Yoshida K, Ito C, Yamatoya K et al (2010) A model of the acrosome reaction progression via the acrosomal membrane-anchored protein equatorin. Reproduction 139:533–544
Yudin AI, Goldberg E, Robertson KR et al (2000) Calpain and calpastatin are located between the plasma membrane and outer acrosomal membrane of cynomolgus macaque spermatozoa. J Androl 21:721–729