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K^+ and Cl^- Channels and Transporters in Sperm Function

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Abstract

To succeed in fertilization, spermatozoa must decode environmental cues which require a set of ion channels. Recent findings have revealed that K^+ and Cl^- channels participate in some of the main sperm functions. This work reviews the evidence indicating the involvement of K^+ and Cl^- channels in motility, maturation, and the acrosome reaction, and the advancement in identifying their molecular identity and modes of regulation. Improving our insight on how these channels operate will strengthen our ability to surmount some infertility problems, improve animal breeding, preserve biodiversity, and develop selective and secure male contraceptives.

Keywords

Ionic currents; Chloride; Potassium; Capacitation; Hyperpolarization; Sperm

1. INTRODUCTION

Generating a unique individual requires that spermatozoa reach and fertilize the egg to deliver its genetic information. Cells build up and maintain ion concentration gradients across their membranes using ion pumps and transporters as a means of coding information related to their state which will then be used to respond to their changing environment. Ion channels are efficient transporters moving millions of ions per second which allow to rapidly modify the cell electric potential and the concentrations of internal second messengers within a wide time-range, depending on how they are regulated (Hille, 2001).

Spermatozoa are quite small, morphologically complex, and differentiated cells (Yanagimachi, 1994). Achieving their main goal of fertilizing the female gamete requires that sperm are equipped to decode multiple signals during their maturation and along their journey to reach the egg. Mammalian sperm travel through convoluted paths while maturing in the epididymis and then face many environmental changes while travel in female reproductive tract (Dacheux et al., 2012; Hung & Suarez, 2010; Visconti, Krapf, de la Vega-Beltran, Acevedo, & Darszon, 2011). The minimal set of ion channels and transporters needed for sperm to succeed in their fundamental task is still not fully known (Darszon, Nishigaki, Beltran, & Trevino, 2011; Lishko et al., 2012; Publicover & Barratt, 2012). In any case, ion channels play a major role in sperm maturation and in the regulation of

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motility and the acrosome reaction (AR) (Darszon et al., 2011; Lishko et al., 2012). Since mature sperm seem essentially unable to perform gene transcription or protein synthesis, the proteins needed for their function are generated during their differentiation (Baker, 2011).

Establishing the functional presence of an ion channel in a specific cell is not easy as these proteins are minor membrane components. Inklings of the characteristics of some sperm ion channels were initially derived from planar bilayers with incorporated sperm plasma membranes (reviewed in Darszon, Labarca, Nishigaki, & Espinosa, 1999). In fact, some of the first recordings of sperm K^+ and Cl^- single-channel activity were obtained in planar bilayers with incorporated sea urchin sperm plasma membranes (Labarca et al., 1996; Lievano, Sanchez, & Darszon, 1985; Morales, de la Torre, Moy, Vacquier, & Darszon, 1993) and of Ca^{2+} permeable channels from boar sperm plasma membranes (Cox & Peterson, 1989). For many years, achieving electrophysiological recordings in sperm to study their ion channels was exceedingly difficult (Darszon et al., 1999; Guerrero, Sanchez, & Darszon, 1987; Jimenez-Gonzalez, Michelangeli, Harper, Barratt, & Publicover, 2006; Kirichok & Lishko, 2011; Ren & Xia, 2010; Weyand et al., 1994). Fortunately, this experimental bottle neck was surmounted by obtaining whole-cell patch-clamp recordings sealing on the cytoplasmic droplet of mouse epididymal sperm (Kirichok, Navarro, & Clapham, 2006). This strategy was extended to human spermatozoa (Kirichok & Lishko, 2011) that have a cytoplasmic droplet in the head–flagellar junction (Cooper, 2011). This approach has allowed the characterization of CatSper (mouse and human) (Kirichok et al., 2006) and SLO3 (mouse) (Navarro, Kirichok, & Clapham, 2007; Santi et al., 2010; Schreiber et al., 1998; Zeng, Yang, Kim, Lingle, & Xia, 2011), so far, the only sperm-specific channels described whose elimination leads to infertility. CatSper is a tetrameric, Ca^{2+}-permeable cation channel potently regulated by intracellular pH (pHi) (Lishko et al., 2012), and SLO3 is a mildly K^+ selective and pHi-regulated channel which will be described in detail later. In addition, whole-cell recordings have revealed the presence of a voltage-sensitive H^+ channel involved in the pHi regulation in human sperm and much less in mouse sperm (Kirichok & Lishko, 2011), and of ATP-gated channels of the purinergic family, P2X2, in mouse epididymal sperm (Navarro, Miki, & Clapham, 2011). The presence of ion currents consistent with the properties of TRPM8 and absent in sperm from TRPM8 null mice have been recorded in testicular sperm (Gibbs et al., 2011; Martinez-Lopez et al., 2011). Functional evidence indicates that this channel is also present in human sperm (De Blas et al., 2009). An alternative to obtaining whole-cell recordings in sperm without using the cytoplasmic droplet has been recently described. This modified perforated patch-clamp strategy allows sealing directly onto the head of mature human spermatozoa where CatSper and the first human sperm Cl^- currents have been documented displaying characteristics associated with Ca^{2+}-dependent Cl^- channels (Orta et al., 2012).

Demonstrating the presence of a specific ion channel in a cell now requires controlled immunological or proteomic detection combined with electrophysiological, ion-sensitive fluorescent functional assays and pharmacology. In species that allow it, knocking out a specific ion channel from spermatozoa, might reveal its function and if good antibodies are available, establish its presence (Kirichok et al., 2006; Santi et al., 2010). This review will discuss the K^+ and Cl^- channels for which there is evidence indicating their presence in sperm and their participation in sperm's main functions: epididymal maturation, capacitation, motility, and/or the AR. Due to the particular relevance of these ions in the regulation of the membrane potential (Em), special emphasis will be given in Section 3 on the discussion of their role in the control of sperm-resting Em and the changes in Em that accompany the capacitation process.

2. EPIDIDYMAL MATURATION

After differentiation in the testis, sperm travel along the epididymis, a specialized duct of the male reproductive system that performs four important functions related to spermatozoa: transport, concentration, maturation, and storage (Turner, 2008). The function of various K^+ channels has been implicated at least during the transport and maturation processes. Transport of sperm through the epididymis is achieved by hydrostatic pressure and by smooth muscle contractions of the epididymis (Bellentani et al., 2011). It has been reported that voltage-gated K^+ channels play a role in the control of smooth muscle contraction. For example, Erg (ether-a-go-go related gene) K^+ channels participate in the contractibility of smooth muscle cells in addition to their contribution to membrane repolarization during the cardiac action potential. The former function is evident as pharmacological inhibition of Erg K^+ channels increases contraction activity of different smooth muscle cells. Mewe et al. (2008) demonstrated through RT-PCR and Western blot experiments the presence of *erg1a and erg1b* isoforms in the bovine epididymal duct. They also reported that Erg channel inhibitors increase the contractile activity of the duct, likely by depolarizing the resting potential and increasing its excitability. Additionally, Bellentani et al. (2011) and Nojimoto et al. (2009) showed that sibutramide (a K^+-channel blocker) increases the mechanical activity of the epididymis and of the vas deferens in rats, respectively. In both cases, the effect was attributed to the blockage of voltage-dependent K^+ channels implicated in smooth muscle contraction (Bellentani et al., 2011; Nojimoto et al., 2009).

The fact that sperm transport requires a relatively long time in many species—ranging 10–13 days (except for human sperm in which the transport time is between 2 and 6 days)—supports the notion that epididymal passage entails an indispensable maturation step rather than simply acting as a sperm conduit (Turner, 2008). Sperm from the caput epididymis are mostly immotile and are unable to undergo capacitation and fertilize the egg. In addition, such maturation process is evident by the greater fertilization ability of sperm obtained from cauda compared to that of sperm obtained from corpus epididymis.

The epididymal maturational process is complex and involves a series of modifications in the sperm, such as changes in the plasma membrane composition, modification, and/or remodeling which occur in the absence of transcription and *de novo* protein synthesis (Dun, Aitken, & Nixon, 2012). Although the complete process has not yet been fully elucidated, one important aspect is that cauda spermatozoa exhibit an increased volume regulation capacity. As spermatozoa leave the testis to transit into the epididymis, they encounter an increasing osmolarity ranging from 280 (rete testis fluid) to up to 400 mmol/kg (cauda epididymis fluid) (Yeung, Barfield, & Cooper, 2006). Upon ejaculation into the female reproductive tract, spermatozoa experience hypo-osmotic stress, which is counterbalanced through the process known as regulatory volume decrease (RVD) involving influx and efflux of water and osmolytes (Yeung et al., 2006).

2.1. Transporters involved in epididymal maturation

The role of K^+ channels during RVD is inferred by the observation that quinine, a general K^+-channel blocker, produces cell swelling upon a hypo-osmotic challenge; in other words, RVD is impaired when the channels are blocked. This notion is further supported by the fact that valinomycin (a K^+ ionophore) can reverse the quinine effect (Yeung et al., 2006). Cooper and Yeung (2007) summarized the pharmacological approaches that have been used by several laboratories to dissect the possible roles of various K^+, Cl^-, and K^+/Cl^- transporters in sperm RVD. Although an unequivocal identification is not possible due to a lack of specificity among blockers, the survey suggested the participation of the following K^+ channels in sperm RVD: $K_V1.5$ and $K_V7.1$, mink, and TASK2. The presence of $K_V1.5$ (human and mouse), mink (mouse), and TASK2 (human and mouse) has been confirmed by

Western blot analyses (Cooper & Yeung, 2007). Immunocytochemistry studies localized all these channels to the flagellum (Cooper & Yeung, 2007). Although sperm are believed by most researchers to be translationally and transcriptionally inactive after leaving the testis, transcripts for $K_V1.5$, mink, and TAKS2 were detected in human sperm (Cooper & Yeung, 2007) suggesting that their protein products are synthesized in spermatids and remain in posttesticular sperm. There is also evidence supporting the presence of a variety of K^+ channels in epididymis from several species using RT-PCR and immunodetection techniques. For example, evidence for the presence of K_{ATP} channels derived from RT-PCR and Western blot has been reported for rat and mouse epididymis, and in mature sperm of bovine, feline, canine, mouse, and human origin (Acevedo et al., 2006; Lybaert et al., 2008).

As in somatic cells, the aforementioned evidence for a role of K^+ channels in sperm volume regulation during epididymal maturation suggests a parallel involvement of Cl^- channels in compensating the positive charges and maintaining electroneutrality. The identity of Cl^- channels involved in volume regulation is not well understood. It has been proposed that ClC-2 (CLCN2) and ClC-3 (CLCN3) play a role in somatic cells (Furst et al., 2002; Nilius & Droogmans, 2003); however, their function is still controversial (Sardini et al., 2003). In sperm, CLCN3 was detected by Western blot and localized to the sperm tail by immunofluorescence (Yeung, Barfield, & Cooper, 2005). While the function of K^+ and Cl^- channels in the regulation of sperm volume is still under study, their presence in sperm from several species suggests that they may play an important role during epididymal maturation and warrants further research.

3. CAPACITATION

Mammalian sperm acquire fertilization capacity only after residing in the female genital tract for a finite period of time (Austin, 1952; Chang, 1951). This maturation process is called capacitation and results in two major changes in sperm physiology: (1) they develop a distinctive motility pattern known as hyperactivation and (2) they become competent to undergo the AR, an exocytotic event that allows the sperm to fertilize the egg. Among physiological changes which take place during capacitation are: (a) activation of PKA (Harrison, 2004); (b) intracellular alkalinization (Zeng, Clark, & Florman, 1995); (c) increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) (Baldi et al., 1991; Breitbart, 2003; DasGupta, Mills, & Fraser, 1993; Suarez, Varosi, & Dai, 1993; Xia & Ren, 2009); (d) changes in the plasma membrane composition (Cross, 1998; Davis, 1981; Gadella & Harrison, 2000; Go & Wolf, 1983; Travis & Kopf, 2002; Visconti et al., 1999); (e) activation of PKA, upstream of an increase in tyrosine phosphorylation (Visconti, Bailey, et al., 1995; Visconti, Moore, et al., 1995); and (f) hyperpolarization of the sperm plasma membrane (Arnoult et al., 1999; Demarco et al., 2003; Munoz-Garay et al., 2001; Zeng et al., 1995).

3.1. Changes in Em during sperm capacitation

The role of Em in regulating any of the multiple processes that take place during capacitation at the molecular level is an important avenue of research that has many questions yet to be answered. Sperm membrane hyperpolarization is a key event in mouse and bovine sperm capacitation (Arnoult, Cardullo, Lemos, & Florman, 1996; Arnoult et al., 1999; Demarco et al., 2003; Munoz-Garay et al., 2001; Zeng et al., 1995), but as yet has not been demonstrated in human sperm. This may be due to the fact that a much smaller fraction of human spermatozoa is capacitated at any given time compared to mouse sperm (Cohen-Dayag, Tur-Kaspa, Dor, Mashiach,& Eisenbach, 1995). Thus, capturing voltage changes occurring in a small fraction of sperm may be difficult. Sperm Em was described in mouse and bovine sperm populations using the voltage-sensitive dye DisC3 by several groups showing that the sperm-resting potential is relatively depolarized (between −30 and −40

mV) but hyperpolarizes during capacitation (Demarco et al., 2003; Espinosa & Darszon, 1995; Hernandez-Gonzalez et al., 2006; Munoz-Garay et al., 2001; Santi et al., 2010; Zeng et al., 1995). However, the absolute level of membrane hyperpolarization has not been well established. Since this type of experiment uses a nonhomogeneous population of sperm where only a portion of the population may be capacitated *in vitro*, the Em absolute values have to be taken cautiously. Therefore, the ~−60 mV value obtained in sperm populations in capacitated conditions could be an average value in a mixed population of sperm(and thus an underestimation of the real Em of a subfraction of capacitated sperm).

In an effort to circumvent this problem, Arnoult et al. (1999) measured Em in individual spermatozoa using the voltage-sensitive dye di8-ANEPPS. Their results corroborate earlier experiments showing the Em of noncapacitated sperm to be relatively depolarized as reported earlier, and that two populations of sperm are present under capacitated conditions: one that are hyperpolarized (~−80 mV) and may represent capacitated sperm able to undergo the AR and another population that appear to be noncapacitated with resting potentials averaging ~−43 mV. Regarding the role of Em changes in capacitation, some experiments suggest that hyperpolarization is essential for the sperm to acquire the ability to undergo a physiological AR. For example, if capacitation is inhibited by the application of high external KCl during this process, the AR induced by zona pellucida (ZP) is significantly reduced (Arnoult et al., 1999; Zeng et al., 1995). The authors of those experiments reached the conclusion that a significant hyperpolarization is an essential part of the capacitation process and is required for the AR to take place.

3.2. What is the functional role of hyperpolarization during capacitation?

3.2.1 Hyperpolarization and sperm motility—As spermatozoa must find and deliver their genetic material to the homologous female gamete, highly sophisticated strategies to regulate their motility and succeed in their search for the egg or oocyte have evolved. Motility is therefore a fundamental function of the mammalian male gamete. It is not surprising that a multitude of gene-targeted transgenic mice, without obvious sperm morphological defects, nevertheless have motility alterations and are sterile (e.g., sAC, PKA, sNHE, GAPDHs, CatSper, PMCA4, SLO3) (Esposito et al., 2004; Miki et al., 2004; Nolan et al., 2004; Okunade et al., 2004; Quill, Ren, Clapham, & Garbers, 2001; Ren et al., 2001; Santi et al., 2010; Wang et al., 2007; Zeng et al., 2011). The flagellum generates the driving force for motility in most male gametes, including some plant kingdom species. The propelling machinery of cilia and flagella is the axoneme (Lindemann & Goltz, 1988) which consists of nine microtubule doublets around a central pair of microtubules (commonly denoted the 9+2 structure). The axonemal motor proteins that generate the force that slides the microtubules producing flagellar beating are the dynein ATPases whose activity is modulated by pH, ATP, ADP, Ca^{2+}, and phosphorylation (Christen, Schackmann, & Shapiro, 1983; Lindemann & Goltz, 1988). Ion transport which supports and controls flagellar beating plays key roles in sperm motility regulation (Guerrero et al., 2011; Kaupp, Kashikar, & Weyand, 2008).

Mammalian spermatozoa display two main modes of motility, activated and hyperactivated. Mature sperm stored in the caudal epididymis and vas deferens before ejaculation are immotile or weakly motile. Sperm activate upon release by ejaculation into media containing bicarbonate and Ca^{2+} and swim propelled by a relatively low-amplitude flagellar beat corresponding to the activated mode (Wennemuth, Carlson, Harper, & Babcock, 2003). The activation of the sperm AdCyc10 by HCO_3^- and the consequent cAMP/PKA activation is the main factor responsible for the activated motility (Carlson, Hille, & Babcock, 2007; Esposito et al., 2004; Hess et al., 2005; Nolan et al., 2004; Xie et al., 2006). Subsequently, the initiation of hyperactivated sperm motility, characterized by high amplitude and

asymmetrical flagellar beating, allows the sperm to detach from temporary binding sites along the female genital tract and penetrate the extracellular matrix of cumulus cells and the ZP surrounding the oocyte (Suarez, 2008). Though it is believed that hyperactivation is encompassed by the capacitation process, there is a relative independence between them. Exactly how hyperactivation is triggered remains not well understood; however, it has been shown to involve a rise in $[Ca^{2+}]i$ carried mainly by CatSper channels in the flagellar plasma membrane. This Ca^{2+} channel, only present in the sperm flagella, is weakly voltage dependent and activated by an increase in pHi (Kirichok et al., 2006; Ren et al., 2001). CatSper null male mice are infertile mainly because of failure to hyperactivate (Carlson et al., 2005, 2003; Ren et al., 2001; Quill et al., 2001). Hyperpolarization of the sperm plasma could play an important role in hyperactivation by facilitating Ca^{2+} influx through CatSper channels during cytosolic alkalinization due to an increase in the driving force on Ca^{2+} (Navarro et al., 2007). Since CatSper is only weakly voltage dependent, it is likely to retain a significant conductance during capacitation-induced hyperpolarization (Kirichok et al., 2006).

3.2.2 Hyperpolarization and the AR—Originally it was proposed that hyperpolarization occurring during capacitation might be necessary for the AR to occur by enabling sperm to generate transient $[Ca^{2+}]i$ elevations; the proposed mechanism involved removing the inactivation of T-type voltage-dependent Ca^{2+} channels (Ca_V3), which could then be subject to induction by physiological agonists (e.g., ZP) (Arnoult et al., 1996, 1999; Santi, Darszon, & Hernandez-Cruz, 1996; Zeng et al., 1995). However, $Ca_V3.1$ and 3.2 knockout mice are fertile (Stamboulian et al., 2004) and Ca_V channels, although recorded in testicular sperm, were not found in epididymal sperm (Martinez-Lopez et al., 2009; Ren & Xia, 2010). These findings bring into question the participation of Ca_V3 channels in the mouse sperm AR, although they do not completely rule it out. Alternatively, hyperpolarization of the sperm plasma membrane could play an important role in sustaining Ca^{2+} influx, essential for the AR to take place, through other Ca^{2+} entry pathways like TRP or SOCs channels that have been described in sperm plasma membrane (Darszon et al., 2011). Several lines of evidence suggest that hyperpolarization of the sperm plasma membrane is necessary for the AR to take place, although the actual role of hyperpolarization is not yet understood.

3.3. What is the molecular mechanism that underlies the sperm hyperpolarization associated with capacitation?

The mechanism of hyperpolarization is not very well understood yet, but evidence about the molecular players is beginning to emerge. In the absence of changes in the bulk ionic concentration, hyperpolarization could, for instance, be due to (1) an increase in K^+ permeability caused by the activation of K^+ selective channels and (2) a reduction of Na^+ permeability, by decreasing the activity of Na^+ channels. Although not directly affecting membrane voltage, the regulation and activity of Cl^- permeability through Cl^- channels and transporters could also play a direct or indirect role in the regulation of the sperm plasma Em (see below).

3.4. Role of K^+ channels in sperm capacitation

The resting potential of sperm plasma membrane prior to capacitation is around −40 mV (Arnoult et al., 1999; Demarco et al., 2003; Espinosa & Darszon, 1995; Munoz-Garay et al., 2001; Zeng et al., 1995), significantly less negative than the equilibrium potential for K^+ ions (E_K~−90 mV). This indicates that K^+ permeability is not the sole determinant of sperm-resting Em at least in noncapacitated sperm. The opening of K^+ channels in these conditions will displace the Em toward E_K resulting in membrane hyperpolarization, while the closure of these channels will cause depolarization. Since the hyperpolarization that accompanies mouse sperm capacitation is influenced by the external K^+ concentration and K^+-channel

blockers, it is reasonable to assume that this hyperpolarization could be due to the opening of these channels (Arnoult et al., 1999; Zeng et al., 1995). The types of K^+ channels present in spermatogenic cells and sperm and their possible roles in sperm capacitation and other sperm functions will be discussed in the following sections.

K^+ channels can be classified in four major classes: (1) voltage-gated K^+ channels (K_V channels) that open or close in response to changes in Em; (2) ion-activated K^+ channels that open in response to the presence of $[Ca^{2+}]i$ or other intracellular signaling molecules; (3) inwardly rectifying K^+ channels that conduct K^+ more easily in the inward direction (into the cell) than outward; (4) tandem pore domain K^+ channels which may be constitutively open or possess high basal activity. The first two above mentioned classes of K^+ channels, voltage gated and ion activated, may have overlapping properties as in the case of SLO3, a unique sperm-specific K^+ channel which is modulated by intracellular H^+ but is also voltage gated. More will be said about SLO3 later in this review. Biochemical, molecular biology, and electrophysiological data support the presence of several of the above mentioned K^+ channels in spermatogenic cells and sperm (Acevedo et al., 2006; Chan et al., 1998; Felix et al., 2002; Hagiwara & Kawa, 1984; Jacob, Hurley, Goodwin, Cooper, & Benoff, 2000; Martinez-Lopez et al., 2009; Munoz-Garay et al., 2001; Navarro et al., 2007; Salvatore, D'Adamo, Polishchuk, Salmona, & Pessia, 1999; Santi et al., 2010; Schreiber et al., 1998; Wu et al., 1998).

3.4.1 Delayed outward voltage-dependent K+ currents—Initial whole-cell patch-clamp studies in rodent spermatogenic cells showed the presence of only one functional type of K^+ channel, a delayed non-inactivating tetraethyl ammonium (TEA)-sensitive K^+ channel (Hagiwara & Kawa, 1984). The molecular identity of these currents was not known at that time. Subsequent studies done by Felix et al. (2002) revealed the presence of at least two types of delayed rectifier currents in spermatogenic cells, one sensitive and one resistant to external TEA. It was speculated that the TEA-sensitive channel was probably $K_V3.1$ that was identified by RT-PCR in spermatogenic cells and by immunocytochemistry in mature sperm. On the other hand, the delayed rectifier component less sensitive to TEA was hypothesized to correspond to the SLO3 K^+ channels (see below). Other channels that were identified by immunocytochemistry and RT-PCR in mouse sperm and spermatogenic cells, respectively, were $K_V1.1$, $K_V1.2$, and GIRK1 channels (Felix et al., 2002). Also, Jacob et al. (2000), using RT-PCR and Northern blot analysis showed the presence of $K_V1.3$ mRNA in rat spermatogenic cells. Ca^{2+}-dependent K^+ currents resembling delayed rectifier currents have also been recorded in *Xenopus* oocytes injected with RNAs from rat spermatogenic cells. These currents showed the typical properties of the SLO1 family of Ca^{2+}-activated K^+ channels such as block by charybdotoxin and low concentrations of TEA (Chan et al., 1998). Of all the delayed rectifier currents mentioned above, only currents having the properties of SLO3 have been observed in mature corpus epididymal sperm (see Section 3.4.3).

3.4.2 Inward rectifier K^+ currents—Inward rectifiers are a class of K^+ channels that conduct larger inward currents at membrane voltages negative to the K^+ equilibrium potential than outward currents at positive voltages. This property, called inward rectification, enables these channels to function at negative voltages. Inward rectifier K^+ channels differ in their degree of rectification. Rectification is not an inherent property of the channel protein itself, but reflects strong voltage dependence of channel block by intracellular cations such as Mg^{2+} and polyamines (Hibino et al., 2010). Among the various classes of inward rectifiers are K_{ATP} channels which are heteromeric complexes of two types of protein subunits, the Kir 6 subfamily and the sulfonylurea receptors (SURs). SUR is a member of the ATP-binding cassette (ABC) family. These channels are blocked by elevated levels of intracellular ATP, and several therapeutic agents including sulfonylureas, and respond to K^+ channel openers like pinacil and diasoxide (Aguilar-Bryan & Bryan,

1999; Aguilar-Bryan et al., 1998; Mannhold, 2004; Seino, 1999). Different types of SUR subunits endow the channels with differential sensitivity to sulfonylureas: SUR1 possesses a high-affinity binding site for tolbutamide and glibenclamide, whereas SUR2A binds glibenclamide but not tolbutamide with high affinity (Dorschner, Brekardin, Uhde, Schwanstecher, & Schwanstecher, 1999).

Two types of inward rectifier K^+ channels were electrophysiologically characterized in spermatogenic cells using the whole-cell patch-clamp technique: (1) K^+ channels with strong inward rectification were reported in mouse spermatids and primary spermatocytes by Munoz-Garay et al. (2001). These currents were highly K^+ selective, showed strong inward rectification, and were inhibited by intracellular acidification. Additionally, they were blocked by 0.3–1 mM external Ba^{2+}, which also inhibits the hyperpolarization associated with capacitation and partially inhibits the AR induced by ZP (Munoz-Garay et al., 2001). (2) Weakly rectifying K^+ channels sensitive to ATP (K_{ATP} channels) were also reported by Darszon's group and others in spermatogenic cells (Acevedo et al., 2006; Zhou et al., 2011). These currents were sensitive to micromolar concentrations of the K_{ATP}-channel blockers tolbutamide and glibeclamide, and channel activity also increased when glucose was removed from the external media, a maneuver to reduce internal ATP (Acevedo et al., 2006). It has also been shown that tolbutamide produces both an inhibition of the hyperpolarization at micromolar concentrations and an inhibition of the AR induced by ZP in a dose-dependent manner, suggesting a plausible participation of K_{ATP} channels in the hyperpolarization associated with capacitation (Acevedo et al., 2006).

The molecular identities of the K_{ATP} channels were determined by isolating Kir 6.1, Kir 6.2, and SUR1 and SUR2 transcripts in mouse and rat spermatogenic cells. In addition, immunocytochemistry experiments showed that Kir 6.1 and Kir 6.2 as well as SUR1 and SUR2B subunits are expressed in both spermatogenic cells and mature sperm (Acevedo et al., 2006; Lybaert, Leleux, Meuris, & Lebrun, 2010; Lybaert et al., 2008). In mouse, while Kir 6.1 was detected in the flagellum midpiece, the Kir 6.2 was localized both in the midpiece and in the postacrosomal region of the sperm head. The SUR1 subunit displays the same distribution pattern as Kir 6.2, suggesting that Kir 6.2 and SUR1 subunits probably are colocalized and form K_{ATP} channels similar to those seen in pancreatic beta cells. The SUR 2 subunit that was detected in the flagellum principal piece and to lesser extent in the midpiece (Acevedo et al., 2006; Zhou et al., 2011) showed that in rat, Kir6.2 colocalized with SUR2B in acrosome of spermatids.

3.4.3 SLO3 K^+ channels—Although several types of K^+ currents have been detected in spermatogenic cells by electrophysiological methods, and voltage-dependent K^+ channels were identified by immunocytochemistry in spermatogenic cells and sperm, these types of currents have not been identified by patch-clamp experiments in mature spermatozoa. The only K^+ current detected to date in corpus epididymal sperm is a pH-sensitive K^+ current, originally named IK_{Sper} (Navarro et al., 2007) that was later identified as the SLO3 current (Santi et al., 2010; Zeng et al., 2011). IK_{Sper} was first recorded in 2007 by Navarro et al. using the whole-cell patch-clamp technique (Navarro et al., 2007). The current activates at ~ −70 mV (in 5 mM external K^+, 130 mM internal K^+, and in the absence of external Na^+) and shows weak voltage and time-dependent activation. Consistent with the properties of SLO3, the current is potentiated by intracellular alkalinization increasing approximately eightfold when pHi changed from 6.0 to 8.0. Pharmacological studies on IK_{Sper} revealed that this current is sensitive to quinine, clofilium, EIPA, mibefradil, and Ba^{2+} and is insensitive to 10 mM external TEA and $CdCl_2$. These authors also showed that intracellular alkalinization under current clamp conditions produced a rapid hyperpolarization of the sperm plasma membrane due to the activation of a K^+ current which has the same pharmacology of IK_{Sper}. They concluded that the hyperpolarization of the sperm plasma membrane produced by

intracellular alkalinization is due to the activation of IK_{Sper} channels. In 2009, Martínez-López et al. described a pH-sensitive K^+ current present in testicular sperm (Martinez-Lopez et al., 2009). Like IK_{Sper} recorded in more mature sperm, this current is also activated by membrane depolarization and intracellular alkalinization; it is strongly blocked by 1 mM Ba^{2+} and weakly blocked by external TEA. The current was also activated by intracellular cAMP. Although the molecular identity of IK_{Sper} and the pH-sensitive current present in testicular sperm was not proven at the time, some of the electrophysiological characteristics of these currents, such as outward rectification, pH modulation, and modulation by PIP_2 (Tang, Zhang, Xia, Ren, & Logothetis, 2010), suggested that the channel carrying those currents could be the high-conductance K^+ channel SLO3. However, validation of this hypothesis had to wait until 2010–2011 when Santi et al. (2010) and Zeng et al. (2011) using SLO3 knockout mice strains corroborated that SLO3 channels were responsible for these currents.

Slo3 is one of four mammalian genes encoding high-conductance potassium (K^+) channels of the SLO K^+ channel family. SLO3 channels from mouse were first cloned in the Salkoff lab in 1998, from a testis cDNA library based on its homology to the BK (mSLO1 channel). Expression of these channels in *Xenopus* oocytes showed that they are high-conductance K^+ channels activated by both voltage and intracellular alkalinization (Schreiber et al., 1998). These channels are highly homologous to SLO1 (BK), channels especially in the transmembrane regions with most of the differences located in the C-terminus. Unlike SLO1 which is conserved in *Drosophila* (Atkinson, Robertson, & Ganetzky, 1991), *C. elegans*, and mammals, SLO3 channels are only present in mammals and are only expressed in testis (Martinez-Lopez et al., 2009; Santi et al., 2010; Schreiber et al., 1998; Zeng et al., 2011). An interesting property of SLO3 channels is their low sequence conservation among different mammalian species. While SLO1 channels are extremely conserved in evolution, we found that SLO3 is highly divergent. This is not surprising for genes that mediate sexual reproduction (Swanson & Vacquier, 2002; Torgerson, Kulathinal, & Singh, 2002; Wyckoff, Wang, & Wu, 2000). Positive Darwinian selection has been reported in many genes involved in the immunity of reproduction and also in ion channels like CatSper (Podlaha, Webb, Tucker, & Zhang, 2005). The mouse and bovine SLO3 channels (mSLO3, bSLO3) are the only species of SLO3 channels that have been cloned and expressed in *Xenopus* oocytes (Santi, Butler, Kuhn, Wei, & Salkoff, 2009; Schreiber et al., 1998).

The first evidence about the molecular identity of the pH-sensitive K^+ current in sperm and its physiological role was obtained by Santi et al. (2010). Using a SLO3 knockout mouse in which the first two coding exons of the *Kcnu1* gene (*slo3* gene) were deleted, they showed that mutant testicular sperm lack the pH-sensitive K^+ current. Male SLO3 knockout mice are infertile; homozygous mutant males mated to wild-type females do not produce offspring in extended mating periods. On the other hand, homozygous mutant females and heterozygous males have normal fertility consistent with the male-specific expression of SLO3. This result was corroborated in 2011 by Zeng et al. (2011), who showed the absence of IK_{Sper} current in corpus epididymal sperm in another SLO3 knockout mouse which lacks the last coding exon (exon 27) of the *Kcnu1* gene (Zeng et al., 2011).

Experiments measuring Em before and after capacitation with voltage-sensitive dyes in SLO3 knockout sperm populations confirmed that SLO3 channels, directly or indirectly, are the main channels responsible for the hyperpolarization that occurs during *in vitro* capacitation; apparently no other channels can compensate for the loss of SLO3. Mutant sperm show a small but significant depolarization after capacitation (Santi et al., 2010). Consistent with these results, current clamp experiments demonstrated that the application of NH_4Cl failed to hyperpolarize mutant sperm, resulting in a small depolarization instead (Zeng et al., 2011).

The SLO3 knockout mouse has helped to unravel the physiological role of this channel in mouse sperm. Since hyperpolarization is removed, the SLO3 knockout mouse would be very helpful in understanding the impact of membrane voltage in different sperm functions. Although SLO3 mutant spermcan, to some degree, undergo the spontaneous AR, they fail to undergo this exocytotic event when exposed to solubilized ZP. This phenotype is rescued by incubation of the mutant sperm with valinomycin, a K^+ ionophore that hyperpolarizes the sperm Em bringing it to the E_K. This result supports the hypothesis that membrane hyperpolarization during capacitation is a key factor required for the induction of the AR (Zeng et al., 1995). Interestingly, although A23187 is capable of inducing a significant AR in the mutant sperm, the efficiency is significantly lower than in wild-type sperm (Santi et al., 2010). This intriguing result suggests that, in addition to Ca^{2+} entry, other voltage-sensitive processes might be required for the AR to take place, and are thus deficient in SLO3 mutant sperm.

In addition to impaired Ca^{2+} entry, fertility depends in part, on the ability of sperm to respond to osmotic challenges encountered in their journey to meet the egg. Therefore, volume regulation may rely on the movement of K^+ (Barfield, Yeung, & Cooper, 2005; Yeung et al., 2006). Perhaps not coincidentally, volume regulation has a distinctive pharmacology that overlaps with the pharmacology of SLO3 channels, an area that remains to be explored (Yeung et al., 2006). Thus, SLO3 channels could well participate in volume control. When volume regulation fails, sperm swell and undergo characteristic morphological changes. Angulated sperm fail to migrate from the uterus to the oviduct, a deficiency resulting in infertility (Yeung & Cooper, 2001). Both Santi et al. (2010) and Zeng et al. (2011) reported that 60–70% of SLO3 mutant sperm are angulated when they are isolated in 330 mOsm/kg medium. Zeng et al. (2011) also found that this SLO3 mutant phenotype is rescued by isolating sperm in a higher osmolarity medium of 430 mOsm/kg.

3.5. Role of Cl^- in sperm capacitation

Work from our group has recently shown that when sperm are incubated in media lacking Cl^- anions, most of the capacitation-associated processes are blocked (Hernandez-Gonzalez et al., 2007; Wertheimer et al., 2008). In particular, Cl^--free media support neither the increase in tyrosine phosphorylation nor the hyperpolarization of the sperm Em. Not surprisingly, sperm incubated in these conditions do not hyperactivate, do not acquire the ability to undergo the AR, and do not fertilize a metaphase II-arrested egg *in vitro*. Interestingly, although in the absence of Cl^-, cAMP agonists induced phosphorylation events, the activation of the cAMP pathway is not sufficient to allow the sperm to fertilize *in vitro*. Also, using a Cl^- indicator MQAE, we have shown that intracellular Cl^- concentration ($[Cl^-]i$) increased during capacitation (Hernandez-Gonzalez et al., 2007; Wertheimer et al., 2008). These results indicate that the regulation of Cl^- homeostasis in sperm is needed for capacitation and suggest that one or more Cl^- transport systems are present in sperm. Despite these findings, little is known regarding specific sperm Cl^- transporters and how Cl^- affects capacitation. Below, we summarize some of the Cl^- translocation systems that have been proposed to be expressed in sperm with special emphasis on those that might play a role in the capacitation process.

In all cells, $[Cl^-]i$ levels are established by the relative contributions of all Cl^- transporters present in their plasma membrane. Those Cl^- transporters can be divided in two categories: Cl^- channels and specialized Cl^- carriers (Jentsch, Neagoe, & Scheel, 2005; Nilius & Droogmans, 2003). Among the Cl^- channels, four structural families have been identified: (1) CFTR channels; (2) the γ-aminobutyric (GABA)-gated and related glycine-gated neurotransmitter receptors; (3) Ca^{2+}-activated Cl^- channels (CaCCs); and (4) CLC channels. On the other hand, Cl^- can also cross the plasma membrane through secondary active transporters. In these molecules, Cl^- translocation is coupled to the movement of another ion

in either opposite direction (anti-porter) or in the same direction (cotransporter or symporter). Therefore, the energy to transport one of the ions against its electrochemical gradient is obtained by coupling it to the translocation of a second ion down its electrochemical gradient. Therefore, the process does not require the direct use of ATP. The Cl^- carrier proteins include (1) the electroneutral cation-Cl^- cotransporter family. This later family is composed of seven members, one Na^+/Cl^- cotransporter (NCC), two $Na^+/K^+/2Cl^-$ cotransporters (NKCC), and four Na^+-independent K^+/Cl^- cotransporters (KCC); and (2) the electroneutral Cl^-/HCO_3^- exchanger family.

3.5.1 CFTR channels—The cystic fibrosis transmembrane conductance regulator (CFTR) is a unique member of the ABC transporter family that forms an anion channel modulated by cAMP/PKA and ATP. The anion selectivity sequence of cAMP-regulated channels in cells containing either endogenous or recombinant CFTR is $Br^->Cl^->I^->F^-$ (Anderson et al., 1991). CFTR is composed of five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory (R) domain. While the MSDs form the channel pore, phosphorylation of the R domain determines channel activity; and ATP hydrolysis by the NBDs controls the channel-gating properties (Sheppard & Welsh, 1999). It is well established that mutations in CFTR cause cystic fibrosis (CF), a disease characterized by defective Cl^- and HCO_3^- transport. About the role of CFTR in reproduction, although more than 95% of all CF male patients are infertile because of congenital bilateral absence of the vas deferens, it is still unknown if CFTR mutations are involved in other forms of male infertility. Using specific antibodies, our group and others have shown that CFTR is present in both human and mouse sperm (Chan et al., 2006; Hernandez-Gonzalez et al., 2007; Li et al., 2010; Xu et al., 2007). It has also been shown that the fertilizing capacity of sperm obtained from heterozygous CFTR mutant mice is also significantly lower than that of wild type (Xu et al., 2007). More recently, whole-cell patchclamp recordings from testicular and epididymal mouse sperm revealed membrane currents containing a Cl^- selective component that is ATP dependent, stimulated by cAMP, cGMP, and genistein, and inhibited by DPC and $CFTR_{inh}$-172 (Fierro et al., 2012). Furthermore, the Cl^- current component activated by cAMP and inhibited by $CFTR_{inh}$-172 is absent in recordings on testicular sperm from mice in which CFTR was replaced by a loss-of-function mutation of the *CFTR* gene (ΔF508). Altogether these findings indicate that CFTR is present in mature mouse sperm and support the hypothesis that this Cl^- channel is involved in the regulation of capacitation.

The mechanism by which Cl^- and other anions are involved in the regulation of the sperm Em is not well understood. When Cl^- is replaced by nonpermeable anions (e.g., gluconate or methanesulfonate), there is no change in the sperm-resting membrane potential (Hernandez-Gonzalez et al., 2007). However, as mentioned above, in conditions that support capacitation, the associated hyperpolarization is inhibited in Cl^- free medium. Because CFTR is mainly a Cl^- transporter, one possibility is that this channel mediates the role of Cl^- in the regulation of both the resting sperm Em and the capacitation-associated hyperpolarization. Three lines of evidence support this hypothesis in mouse sperm: (1) the CFTR inhibitor diphenylamine-2-carboxylic acid (DPC 250 μM) inhibits the capacitation-associated hyperpolarization and decreases the ZP-induced AR without affecting the increase in tyrosine phosphorylation; (2) a CFTR agonist (genistein; 5–10 μM) promotes hyperpolarization in noncapacitated mouse sperm; and (3) addition of permeable analogs of cAMP to noncapacitated mouse sperm elevates $[Cl^-]i$ (Hernandez-Gonzalez et al., 2007). In addition to its role as a Cl^- channel, CFTR is also known to interact with and regulate other ion channels including epithelial Na^+ channels (ENaC) (Berdiev, Qadri, & Benos, 2009; Konig, Schreiber, Voelcker, Mall, & Kunzelmann, 2001; Kunzelmann & Schreiber, 1999; Perez-Cornejo & Arreola, 2004). As mentioned in the previous section, the sperm-resting Em is relatively depolarized and cannot be explained only by active K^+ channels. An

approximate 10% contribution of Na^+ permeability would predict an Em of −35 mV which is close to experimental observations. Consistent with a Na^+ contribution: (1) when sperm are incubated in media in which Na^+ is replaced by choline$^+$ or glutamine$^+$, the sperm Em is hyperpolarized to an Em approaching the K^+ equilibrium (Hernandez- Gonzalez et al., 2006); (2) addition of pulses of Na^+ to sperm incubated in Na^+-free media induces sperm depolarization, suggesting the presence of an open Na^+ channel in these conditions; and (3) using the Na^+ indicator, CoroNa Red in combination with flow cytometry analysis, we have recently shown that the intracellular Na^+ ($[Na^+]i$) decreases when the sperm are incubated under capacitating conditions (Escoffier, Krapf, Navarrete, Darszon, & Visconti, 2012). All these results are consistent with ENaC channels being present in the membrane. In this regard, ENaC subunits have been detected in mature mouse sperm by Western blot analysis and immunofluorescence (Hernandez-Gonzalez et al., 2006). Furthermore, amiloride, a compound known to block these channels, induces sperm hyperpolarization in noncapacitated sperm to levels similar to those observed in capacitated conditions and blocks the capacitation-induced decrease in $[Na^+]i$. Altogether these results indicate that Na^+ influx is involved in establishing the sperm-resting Em and support the hypothesis that ENaC downregulation plays a role in the capacitation-associated sperm hyperpolarization. Although it has been demonstrated in other systems (Konig et al., 2001) that CFTR inhibits ENaC through an increase in the $[Cl^-]i$ (Stutts et al., 1995), there is no direct evidence of CFTR and ENaC interaction in sperm. However, the hypothesis that activation of CFTR downregulates ENaC is supported by the findings that activation of CFTR by genistein hyperpolarizes the sperm Em (Hernandez-Gonzalez et al., 2007) and decreases $[Na^+]i$ (Escoffier et al., 2012). Interestingly, immunofluorescence experiments show midpiece localization for both ENaC alpha subunit and CFTR (Hernandez-Gonzalez et al., 2006, 2007).

3.5.2 GABA and glycine channels—$GABA_A$ receptors are Cl^- channels that mediate inhibitory neurotransmission in the central nervous system (CNS). They were first identified pharmacologically as being activated by GABA and the selective agonist muscimol, blocked by bicuculline and picrotoxin, and modulated by benzodiazepines, barbiturates, and certain other CNS depressants (Macdonald & Olsen, 1994; Sieghart, 1995). In sperm, the presence of $GABA_A$ receptors has been studied by Meizel's group (Meizel, 1997; Wistrom & Meizel, 1993). It has been shown that GABA can induce the AR (Shi, Yuan, & Roldan, 1997) and also that $GABA_A$ receptors can modulate the response to progesterone in these cells (Hu, He, Wu, Yan, & Koide, 2002; Ritta, Calamera, & Bas, 1998; Shi & Roldan, 1995; Turner, Garcia, & Meizel, 1994). The role of GABA in the regulation of the AR will be discussed in Section 4. Regarding sperm capacitation, it is not clear whether GABA has an effect on this process. Ritta et al. have studied the role of this neurotransmitter in both human and bovine sperm capacitation, and their experiments suggested that GABA has a role in the regulation of sperm motility (Ritta, Bas, & Tartaglione, 2004; Ritta et al., 1998). Also in rat sperm, GABA and progesterone have been proposed to accelerate capacitation and hyperactivated motility, followed by an increase in the AR. Bicuculline and picrotoxin, antagonists of $GABA_A$ receptor/Cl^- channels, inhibit the effects of both GABA and progesterone (Jin et al., 2009). This evidence suggests that activation of $GABA_A$ receptor/Cl^- channels may contribute to sperm capacitation and hyperactivated motility.

3.5.3 Ca^{2+}-activated Cl^- channels—CaCCs are activated by increases in cytosolic free Ca^{2+} concentrations due to release from intracellular stores or influx through plasma membrane channels. The molecular identity of some types of Cl^- channels is still unknown. Recently, three research groups have arrived independently at the identification of TMEM16A (also known as anoctamin-1) as a membrane protein strongly related to the activity of CaCCs (Caputo et al., 2008; Ferrera, Caputo, & Galietta, 2010; Schroeder,

Cheng, Jan, & Jan, 2008; Yang et al., 2008). TMEM16A is part of a family of proteins that includes nine other members named as TMEM16B-K (Galindo & Vacquier, 2005). TMEM16B coexpression with CaCCs, exhibits biophysical characteristics (voltage dependence, unitary conductance) different from those associated with TMEM16A coexpression (Pifferi, Dibattista, & Menini, 2009; Scudieri, Sondo, Ferrera, & Galietta, 2012). All TMEM16 proteins have a similar putative topology, consisting of eight transmembrane segments and cytosolic N- and C-termini (Galietta, 2009).

Patch-clamp studies in cell-attached mode "mapping" ion channel activity in human sperm head reveals a Cl^--permeable channel showing long stable openings. Different parts of the sperm head possess different channels, and there is remarkable clustering which may have a functional significance (Jimenez-Gonzalez, Gu, Kirkman-Brown, Barratt, & Publicover, 2007). Recently, a CaCC in human sperm was characterized (Orta et al., 2012). Using the perforated patch-clamp technique to obtain whole-cell recordings from the head of mature human spermatozoa revealed the presence of CaCC currents which could be carried by TMEM16A proteins. CaCCs play an important role in the physiology of human spermatozoa and are likely to participate in the solubilized ZP-induced AR, but their role in sperm motility is not yet determined.

3.5.4 Secondary active Cl^-- transporters—As mentioned in the previous section, there is evidence supporting a role of CFTR in the changes in sperm Em that occur during capacitation. However, CFTR inhibitors do not inhibit other aspects of capacitation, such as the increase in tyrosine phosphorylation, which may suggest that, in addition to CFTR, other Cl^- transport systems are present in sperm. Cl^- can also be translocated into sperm through electroneutral carriers (e.g., NCC, NKCC, and KCC). Although these three cation/Cl^- cotransporters can work in both ways (e.g., Cl^- influx or efflux), under physiological conditions, the NCC and the NKCC carriers normally transport Cl^- into the cell, while the KCC family transport Cl^- out of the cell (Russell, 2000). During capacitation, it has been shown that $[Cl^-]i$ is increased (Hernandez-Gonzalez et al., 2007; Meizel & Turner, 1996); therefore, if present, NCC and NKCC might play a role in the regulation of Cl^- homeostasis during this process. This hypothesis was tested by observing the effect of Cl^- transport inhibitors on capacitation (Wertheimer et al., 2008). General Cl^- transport blockers such as stilbenes (e.g., DIDS and SITS) reduced sperm capacitation parameters to similar levels as those observed in the absence of Cl^-. However, most of the other Cl^- transport inhibitors tested, including the NCC inhibitor thiazide, failed to block capacitation-associated processes. Exceptions were bumetanide and furosemide, two NKCC inhibitors, which blocked the increase in tyrosine phosphorylation, hyperactivation, and the ability of the sperm to fertilize *in vitro*. However, the concentration necessary for these inhibitory effects is higher than that reported to be effective in inhibiting NKCC (Garg et al., 2007; Russell, 2000). Moreover, although the presence of Cl^- and Na^+ is essential for the increase in tyrosine phosphorylation, K^+, another ion needed for the function of NKCCs is not required. These results suggest that high concentrations of bumetanide act on a mechanism different from the inhibition of NKCC. Conversely, the ZP-induced AR was inhibited at a much lower concentration of bumetanide and was dependent on the presence of Cl^-, K^+, and Na^+ suggesting that NKCC might have a role in the preparation of the sperm for the physiologically induced AR. Interestingly, NKCC1 transcripts are present in spermatids, and null mutants of this protein have defects in spermatogenesis and are infertile (Pace et al., 2000). Although likely to be present in sperm, more research will be needed to understand the role of these cation/Cl^- exchangers in capacitation.

3.5.5 Cl^-/$HCO3^-$ exchangers—Cl^- carriers also include those molecules that exchange Cl^- for HCO_3^- in either direction. In sperm, HCO_3^- has been shown to activate cAMP synthesis through the atypical soluble adenylyl cyclase (AdCyc10 a.k.a. SACY) (Hess et al.,

2005; Okamura, Tajima, Soejima, Masuda, & Sugita, 1985). The specific carriers responsible for HCO_3^- transport have not yet been fully defined. Our group has provided evidence that Na^+/HCO_3^- cotransporters are present in mouse sperm and are responsible for initial HCO_3^- influxes (Demarco et al., 2003). However, it is not clear whether other HCO_3^- transport systems can also play a role in the control of HCO_3^- levels in sperm. In this respect, Cl^-/HCO_3^- exchangers have been proposed to play a role in the regulation ofHCO_3^- homeostasis. These exchangers are expressed in multiple cell types and are relevant to the regulation of pHi, cell volume, and the regulation of Em through their contribution to the Cl^- gradient. The molecules responsible for the exchange of HCO_3^- and Cl^- belong to two evolutionary independent gene superfamilies, SLC4 and SLC26, which exhibit unique patterns of anion selectivity and tissue distribution. The SLC4 superfamily is composed by three genes (*AE1*, *AE2*, and *AE3*), each of them, represented by more than one alternative spliced sequences. The *SLC26* gene superfamily, comprising the SLC26 transporter superfamily, is represented by 11 genes. Of those, only *SLC26A3*, *SLC26A4*, and *SLC26A6* have Cl^-/HCO_3^- exchange activity.

Although little is known about the contribution of the *SLC4* and *SLC26* genes to sperm function, their presence has been proposed for many years (Ruknudin & Silver, 1990; Visconti, Muschietti, Flawia, & Tezon, 1990). From the SLC4 superfamily, only AE2 is prominent in testicular germ cells. Its expression pattern suggests a role for this gene product in either spermatogenesis or later on in sperm function. The *AE2* gene is represented by five splice variants (AE2a, AE2b1, AE2b2, AE2c1, and AE2c2). Mice lacking expression of all of them die before weaning due to severely retarded development (Gawenis et al., 2004); however, those mice retaining AE2c but lacking AE2a, AE2b1, and AE2b2 have a milder phenotype. Consistent with the observation that AE2 is highly expressed in the testis, these mice are infertile and exhibit testicular dysplasia (Medina et al., 2003).

Regarding the SLC26 superfamily, recent work from our group and others using Western blot and immunofluorescence approaches show the presence of SLC26A3 and SLC26A6 in the sperm midpiece (Chan et al., 2009; Chavez et al., 2011; Chen et al., 2009). In this last chapter, we provided evidence that these transporters coprecipitate with CFTR and that tenidap, a SLC26A3-specific inhibitor, blocked the capacitation-associated hyperpolarization and the ZP-induced AR. However, tenidap did not block the activation of a cAMP pathway and the increase in tyrosine phosphorylation, suggesting that these transporters are not directly involved in the regulation of AdCyc10.

4. ACROSOME REACTION

The acrosome is a large secretory vesicle located at the posterior end of the sperm head (Yanagimachi, 1998). The AR is a unique, single-vesicle exocytotic event required for sperm of many species to achieve fusion with the female gamete. During the AR, now considered as a multistep process, many fusion points occur between the sperm head plasma membrane and the outer acrosomal membrane. Plasma membrane–outer acrosome hybrid vesicles are liberated as a result of the multiple fenestrations that occur in this irreversible reaction, which leads to acrosomal content release of hydrolytic enzymes. Not surprisingly, the fusion machinery conserved in many neuroendocrinal secretory cells and regulated by Ca^{2+} is present in sperm and involved in the AR (Bello, Zanetti, Mayorga, & Michaut, 2012; Castillo Bennett, Roggero, Mancifesta, & Mayorga, 2010). At present, where the AR takes place in the female tract and the identity of the molecules inducing this exocytotic reaction is controversial (Jin et al., 2011; Visconti & Florman, 2010; Yanagimachi, 1998). However, among the many inducers of the AR described, the ZP and progesterone are the ones considered more physiologically relevant (Litscher, Williams, & Wassarman, 2009; Mayorga, Tomes, & Belmonte, 2007). In addition to its participation in sperm–egg

signaling, ZP is also a protective layer (Litscher et al., 2009). The ZP-induced AR requires the convergence of several transduction pathways (for review, see Mayorga et al., 2007) which result in a complex cascade of $[Ca^{2+}]i$ changes. The physiologically relevant AR requires changes in $[Ca^{2+}]i$; external and internal sources contribute to its modulation (Breitbart, Rotman, Rubinstein, & Etkovitz, 2010; Costello et al., 2009; Darszon et al., 2011; Florman, Jungnickel, & Sutton, 2008).

4.1. Ca^{2+} channels and the AR

Three distinct Ca^{2+} channels have been proposed to contribute to $[Ca^{2+}]i$ responses associated with the AR. Though not fully understood, these channels are functionally linked (reviewed in Darszon et al., 2011; Florman et al., 2008; Publicover, Harper, & Barratt, 2007). The first is a voltage-dependent Ca^{2+} (Ca_V) channel whose involvement in the AR was deduced from functional and pharmacological observations. Supporting this possibility, sperm from many mammalian species undergo $[Ca^{2+}]i$ increases in response to K^+ depolarization and ZP that are sensitive to Ca_V-channel blockers (reviewed in Darszon et al., 2011; Florman et al., 2008; Lishko et al., 2012; Ren & Xia, 2010). Ca_V channels have been proposed to participate in the transient elevation induced by ZP which last ~1 s and has been best characterized in mouse sperm (Florman et al., 2008). However, even though $Ca_V3.2$ (T-type Ca^{2+} channel) has been considered the most likely Ca_V candidate to participate in the mouse AR (Arnoult et al., 1996; Escoffier et al., 2007; Lievano et al., 1996; Trevino et al., 2004), knockout mice lacking this channel are fertile and undergo the ZP-induced AR. In addition, recent patch-clamp whole-cell recordings on the cytoplasmic droplet of epididymal mouse sperm have failed to detect Ca_Vs (Xia, Reigada, Mitchell, & Ren, 2007). These findings have questioned the presence of functional Ca_Vs in mature sperm and their involvement in the AR, in spite of solid immunological data demonstrating their presence (Escoffier et al., 2007; Trevino et al., 2004). Alternatively, the fertile phenotype of $Ca_V3.2$ null male mice can be explained by compensation from other Ca_V channels, by the possible participation of high-voltage-activated Ca^{2+} channels (HVA) in the AR (Escoffier et al., 2007), or by activation of CatSper channels as recently proposed (Xia et al., 2007).

Following the fast transient $[Ca^{2+}]i$ elevation induced by ZP, a sustained $[Ca^{2+}]i$ increase lasting up to minutes occurs. This $[Ca^{2+}]i$ change results from the release of Ca^{2+} from internal stores (i.e., the acrosome). As the IP3 receptor, the second type of Ca^{2+} channel involved in the AR, is activated due to IP3 production (reviewed in Publicover et al., 2007; Florman et al., 2008; Darszon et al., 2011). Ca^{2+} store emptying leads to the opening of plasma membrane Ca^{2+} channels (SOCs) which in turn, as in somatic cells, cause a sustained $[Ca^{2+}]i$ increase. Since the SOC machinery is more complex than originally thought (Moreno & Vaca, 2011), several components such as STIM, ORAI, and TRPCs may participate in the sustained Ca^{2+} uptake which is the third type of Ca^{2+} channels involved in the AR. Preliminary reports of the presence of STIM and ORAI in human and mouse sperm are consistent with this proposal (Costello et al., 2009; Darszon et al., 2012).

4.2. Cl^- channels and the AR

While there is no doubt that Ca^{2+} fluxes are key to the AR, little is known about the participation of Cl^- movement during this event. As mentioned in Section 3, using different approaches, several Cl^- channels and exchangers have been detected in sperm from various species, and it has been previously established that Cl^- is essential for capacitation. Regarding the participation of Cl^- channels in the mammalian sperm AR, niflumic acid (NFA) has been shown to inhibit Ca^{2+}-induced hyperpolarization partially driven by Cl^- (Espinosa et al., 1998). Furthermore, this stilbene inhibited the first Cl^- single-channel activity recorded in mammalian sperm as well as the AR induced by solubilized ZP, progesterone, and GABA in mouse sperm (Espinosa et al., 1998). Recently, our group has

provided evidence that anion-channel blockers like NFA, DIDS, and others inhibit the mouse and human sperm AR as well as Cl^- channels detected in these cells (Espinosa & Darszon, 1995; Espinosa et al., 1998; Orta et al., 2012).

In addition, neurotransmitter receptors have been identified and implicated during the AR of sperm from several species, based primarily on pharmacological and genetic evidence. $GABA_A$ and glycine receptors are of particular interest in this review, since they are also associated with Cl^- fluxes. Although the best studied inductor of the AR is ZP3, GABA and glycine are present in the female reproductive tract and have been shown to also induce the AR, possibly through Cl^- fluxes. For example, Burrello et al. (2004) reported AR induction by GABA in human sperm. They also observed that the AR induced by progesterone is blocked in the presence of picrotoxin (a GABA Cl^- channel inhibitor) and that GABA and Pg together exert a stronger AR induction compared to each component alone. These researchers postulated that GABA present in the follicular fluid, together with Pg, activates the same receptor (probably a $GABA_A$-like receptor). However, when they used specific inhibitors of $GABA_A$ and $GABA_B$ receptors (bicuculline and saclofen, respectively), the AR induced by follicular fluid was unaffected, unless these drugs where used simultaneously. Burrello et al. (2004) concluded that both $GABA_A$ and $GABA_B$ receptors participate in the human sperm AR. However, it has been shown that the Ca^{2+} channel CatSper can be activated by progesterone in human sperm (Lishko, Botchkina, & Kirichok, 2011; Strunker et al., 2011), and due to the promiscuous activation of this channel (Brenker et al., 2012), it is now important to establish whether GABA is acting via CatSper or via a GABA receptor. In any case, the induction of the AR by GABA has been corroborated by several researchers in different species such as mouse, rat, human, porcine, guinea pig, and bull (Espinosa et al., 1998; Hu et al., 2002; Melendrez & Meizel, 1995; Roldan, Murase, & Shi, 1994; Shi & Roldan, 1995; Shi et al., 1997). The participation of CatSper in this induction is yet to be established.

Pharmacological evidence has also implicated the glycine receptor in the ZP- or glycine-induced AR in human, mouse, and hamster sperm (Bray, Son, Kumar, Harris, & Meizel, 2002; Llanos, Ronco, Aguirre, & Meizel, 2001; Sato, Son, & Meizel, 2000). Sperm from glycine receptor null mice are unable to respond to ZP, although fertilization *in vitro* can still proceed, albeit at a lower rate and attributable to a spontaneous AR (Meizel & Son, 2005). It has been proposed that a Cl^- efflux takes place via a glycine receptor causing a depolarization, which in turn may trigger the voltage-dependent Ca^{2+} channel opening required for the ZP-induced AR (Llanos et al., 2001). This hypothesis is also under scrutiny, as both the functional presence of voltage-dependent Ca^{2+} channels and the relevance of ZP-induced AR is questioned in the light of new evidence regarding this process (reviewed in Darszon et al., 2011).

As discussed earlier, there is also electrophysiological evidence for the presence of Cl^- channels in sperm. The first recordings were performed by directly patching mouse sperm. Using this technique, Espinosa et al. (1998) recorded an anion channel in epididymal sperm with biophysical properties and sensitivity to NFA, similar to the Ca^{2+}-dependent Cl^- channels (Hogg, Wang, & Large, 1994). Recently, Orta et al. (2012) reported the presence of CaCCs, possibly TMEM16A, in human sperm. Interestingly, the pharmacology profile of the recorded currents was consistent with inhibition of the ZP3-induced AR. $TMEM16A_{inh}$ (20 μM), so far the most specific antagonist of TMEM16A Cl^- channels, inhibited nearly 80% of the AR, supporting the participation of TMEM channels during this process. In this regard, the AR involves a Ca^{2+}-dependent swelling of the acrosome (Zanetti & Mayorga, 2009), a process in which Cl^- currents may participate.

Interestingly, evidence gathered in recent years has uncovered an unexpected pharmacological overlap between CaCCs and the large conductance, Ca^{2+}-gated K^+ channels (BK_{Ca} or K_{Ca} 1.1) (Greenwood & Leblanc, 2007; Sones, Leblanc, & Greenwood, 2009). Various compounds with structurally different characteristics considered to be Cl^--channel blockers, such as NFA, anthracene-9-carboxylate, and ethacrynic acid, enhance $K_{Ca}1.1$ currents (Greenwood & Large, 1995; Ottolia & Toro, 1994; Toma, Greenwood, Helliwell, & Large, 1996). Since there are evidences of the presence of $K_{Ca}1.1$ channels in mammalian sperm (Rossato, Di Virgilio, Rizzuto, Galeazzi, & Foresta, 2001; Wu et al., 1998), the combined effects of anion-channel blockers such as NFA on CaCCs and K_{Ca} 1.1s could account for their potent ability to inhibit the AR. The large $[Ca^{2+}]i$ changes that occur during the AR result in significant morphological sperm head alterations which seem to involve acrosome swelling and an RVD in which CaCCs may participate. By blocking CaCCs, NFA, DIDS, and $TMEM16A_{inh}$ could alter the regulatory volume decrease that appears to be important to regulate the distance between the outer acrosomal membrane and the plasma membrane, which is critical for acrosome exocytosis (Zanetti & Mayorga, 2009).

5. FINAL REMARKS

As mentioned in the introduction, the role of ionic fluxes in sperm has been hindered due to many factors which include: (1) the small sperm size which has made the use of electrophysiological techniques very difficult; and (2) the lack of transcription and translation, making it very challenging to perform knock down or to express exogenous proteins. In the past years, the use of genetically modified mice has accelerated the process of identifying molecules which are essential for different aspects of sperm function. Among them, knockout mice for two sperm-specific ion channels, CatSper and SLO3, were key to reveal that these channels are necessary for fertilization. Also, the increasing sensitivity of mass spectrometry has helped to identify some of the proteins associated with these ion channel-associated proteins. Finally, new patch-clamp techniques have allowed the study of ion movements in sperm. Overall, the chapters discussed in this review support the idea that the activity of sperm ion channels is involved in the regulation of sperm maturation, capacitation, and the AR. However, currently little is known about how these sperm ion channels are regulated during these processes. Several lines of experimentation now show promise in uncovering these mechanisms including identifying posttranslational modifications in those proteins that modulate ion fluxes and making direct electrophysiological observations in sperm which has only recently been possible. These as well as other new techniques will almost certainly accelerate the pace of our understanding of the physiology of sperm. Finally, from the applied point of view, a better understanding of sperm ion channels exposed to the extracellular milieu offers opportunities for intervention in fertility treatments as well as for their use as possible contraceptive targets.

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