Chapter 6

Cl⁻ Channels and Transporters in Sperm Physiology

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Abstract Spermatozoa must decode environmental and cellular cues to succeed in fertilization, and this process relies heavily on ion channels. New observations bring to light the relevant participation of Cl⁻ channels and anion transporters in some of the main sperm functions. Here we review the evidence that indicates the participation of Cl⁻ channels in motility, maturation, and the acrosome reaction (AR), and what is known about their molecular identity and regulation. Our better understanding of sperm anion transport will yield tools to handle some infertility problems, improve animal breeding and preserve biodiversity, and develop selective and secure male contraceptives.

Keywords Capacitation • Chloride • Hyperpolarization • Ionic currents • Sperm

6.1 Introduction

Spermatozoa must find and fertilize the egg to deliver their genetic information and generate a unique individual in organisms of sexual reproduction. Ion pumps and transporters are used by cells to build up and maintain ion concentration gradients across their membranes. These ionic gradients allow cells to respond to their changing environment, to signals from other cells and to perform secondary transport. Ion

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channels can transport millions of ions per second, which enables them to rapidly modify the cell electric potential and the concentrations of internal second messengers within a wide time range, depending on their mode of regulation (Hille 2001).

Spermatozoa are small, differentiated, and morphologically complex cells (Yanagimachi 1994). They must be endowed with decoding systems for multiple signals along their journey to reach the egg to succeed in fertilizing it. During their maturational convoluted journey through the epididymis and the female reproductive tract, mammalian sperm encounter many environmental changes (Dacheux et al. 2012; Hung and Suarez 2010; Visconti et al. 2011). Although significant advances have been made in recent years, the set of ion channels and transporters needed for sperm to achieve fertilization is still not fully known (Darszon et al. 2011; Lishko et al. 2012; Publicover and Barratt 2012). Ion channel inhibition and knockout experiments have clearly revealed the major role these transporters play in sperm maturation, the regulation of motility, and the acrosome reaction (AR) (Darszon et al. 2011; Lishko et al. 2012). As gene transcription and protein synthesis seem not to occur in mature sperm, their proteins are generated during spermatogenesis (Baker 2011).

Considering that ion channels are minor membrane protein components, demonstrating the functional presence of a particular ion channel in spermatozoa needs controlled immunological or proteomic detection combined with electrophysiological, ion-sensitive fluorescent functional assays and pharmacology. When possible, eliminating the specific ion channel from spermatozoa might unravel its function (Kirichok et al. 2006; Santi et al. 2010). Initial glimpses of the properties of some sperm ion channels were derived from planar bilayers with incorporated sperm plasma membranes (reviewed by Darszon et al. 1999). Notably, some of the first sperm single-channel recordings were of K^+ and Cl^- channels obtained in planar bilayers with incorporated sea urchin sperm plasma membranes (Labarca et al. 1996; Lievano et al. 1985; Morales et al. 1993) and of Ca^{2+} channels from boar sperm plasma membranes (Cox and Peterson 1989).

Obtaining electrophysiological recordings directly in sperm to study their ion channels was exceedingly difficult for a long time (Darszon et al. 1999; Guerrero et al. 1987; Jimenez-Gonzalez et al. 2006; Kirichok and Lishko 2011; Ren and Xia 2010; Weyand et al. 1994). Achieving whole-cell patch-clamp recordings became more feasible when Kirichok et al. (2006) were able to seal the cytoplasmic droplet of mouse epididymal spermatozoa and then mature human spermatozoa (Kirichok and Lishko 2011). This novel strategy is allowing the characterization of sperm-specific channels such as CatSper (Kirichok et al. 2006) and SLO3 (Navarro et al. 2007; Santi et al. 2010; Schreiber et al. 1998; Zeng et al. 2011), and of sperm anion channels that are present in other cell types (Orta et al. 2012; Ferrera et al. 2010). Additionally, a voltage-sensitive H^+ channel involved in the intracellular pH (pH_i) regulation in human sperm and less importantly in mouse sperm (Kirichok and Lishko 2011), and ATP-gated channels of the purinergic family, P2X2, in mouse epididymal sperm have been recorded (Navarro et al. 2011). Ionic currents with properties consistent with TRPM8 channels were recorded in testicular sperm (Gibbs et al. 2011; Martinez-Lopez et al. 2011). Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$

imaging experiments at different temperatures and membrane potential (E_m) measurements suggested TRPM8-like channels are present in more mature mouse and human sperm (De Blas et al. 2009; Martinez-Lopez et al. 2011).

Alternatively, whole-cell recordings have now been obtained directly patching the head of human spermatozoa using a modification of the perforated patch-clamp strategy. Employing this approach, Cl⁻ currents displaying characteristics associated with Ca²⁺-dependent Cl⁻ channels were documented in human spermatozoa (Orta et al. 2012).

Research in past years has established that in most cells Cl⁻ is actively transported and not at electrochemical equilibrium. As a result, Cl⁻ can participate in signaling and perform work, an important matter when considering the functional roles of anion channels and transporters, in addition to their more established participation in fluid secretion and volume regulation (Duran et al. 2012). In spermatozoa, as in other cells, Cl⁻ is the main anion and is involved in volume regulation and osmotic stress protection (Cooper and Yeung 2007; Furst et al. 2002; Yeung et al. 2005). Capacitation, a maturational process and the AR, a unique exocytotic event essential for fertilization, both discussed next, are significantly affected in mouse and human spermatozoa when external Cl⁻ concentrations are lowered (Chen et al. 2009; Figueiras-Fierro et al. 2013; Orta et al. 2012; Wertheimer et al. 2008; Yeung and Cooper 2008). Although these findings suggest that Cl⁻ plays a relevant role in sperm physiology, not much is known about its transport across the membrane of this fundamental cell. This review summarizes information about Cl⁻ channels and transporters for which evidence suggests their presence in sperm and their involvement in important sperm functions such as epididymal maturation, capacitation, motility, and the AR.

6.2 Maturation During Epididymal Transit

The epididymis is a specialized duct of the male reproductive system that fulfills four important functions for spermatozoa: transport, concentration, maturation, and storage (Turner 2008). The movement of sperm through the epididymis involves hydrostatic pressure and smooth muscle contractions. Depending on the species, sperm transit through the epididymis ranges from 2 to 13 days (Turner 2008). The epididymis is divided into three main sections: caput, corpus, and cauda, proximal to distal to the exit from the testis. Sperm from the caput section are immotile and lack important characteristics required for fertilization. In contrast, sperm obtained from the cauda have the highest fertilizing capacity. The osmolarity encountered by sperm during transit into the epididymis increases from 280 (in the rete testis fluid) to up to 400 mmol/kg (in the cauda epididymis fluid) (Yeung et al. 2006). On 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). RVD capability is acquired during epididymal transit, and sperm from the

cauda exhibit the greatest RVD capacity. Results indicating the role of K^+ channels during sperm RVD at the time of epididymal maturation suggest a parallel involvement of Cl^- channels to compensate the positive charges and maintain electroneutrality (Cooper and Yeung 2007). The molecular identity of the Cl^- channels involved in volume regulation is not established. Candidates such as CIC-2 (CLCN2) and CIC-3 (CLCN3) have been proposed to play a role in somatic cells (Furst et al. 2002; Nilius and Droogmans 2003); however, their function is still controversial (Sardini et al. 2003). Interestingly, CLCN3 was detected by Western blot and localized to the sperm tail by immunofluorescence (Yeung et al. 2005). Although the function of K^+ and Cl^- channels in RVD is still under study, the expression of such channels in sperm from several species suggests they may play an important role during epididymal maturation, a matter that awaits further research.

6.3 Motility

Sperm motility is activated when spermatozoa enter the female tract. Motility is one of the most important functions carried out by the sperm because it is essential to achieve fertilization. Indeed, several sperm motility defects can cause male sterility (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 et al. 2001; Ren et al. 2001; Santi et al. 2010; Wang et al. 2007; Zeng et al. 2011). Sperm motility is driven by the flagellum, an appendage with an ultrastructure very similar to that of cilia. The axoneme is the principal structure that propels a flagellum (Lindemann and Goltz 1988); it is composed of a particular arrangement of microtubules, usually in the configuration of nine doublets surrounding a central pair. Movement results by repetitive cycles of flagellar bending, arising from microtubule sliding using the force generated by dynein ATPases whose activity is modulated by pH, ATP, ADP, Ca^{2+} , and phosphorylation (Christen et al. 1983; Lindemann and Goltz 1988). Ion transport that supports and controls flagellar beating plays key roles in sperm motility regulation (Guerrero et al. 2011; Kaupp et al. 2008).

Upon ejaculation, sperm initiate motility with a relatively low-amplitude flagellar beat known as activated motility (Wennemuth et al. 2003). The stimulation of the sperm soluble adenylate cyclase by HCO_3^- and the consequent cAMP/PKA stimulation is required for the activated motility (Carlson et al. 2007; Esposito et al. 2004; Hess et al. 2005; Nolan et al. 2004; Xie et al. 2006). After some time (varying according to species) in the female tract, spermatozoa become hyperactivated, displaying vigorous asymmetrical flagellar beating with large amplitude and high curvature. Hyperactivation helps sperm to detach from temporary binding sites along the female genital tract and to penetrate the extracellular matrix of cumulus cells and the zona pellucida (ZP) surrounding the oocyte (Suarez 2008). The mechanisms involved in hyperactivation are not well understood; however, it is known that a $[Ca^{2+}]_i$ rise mediated by CatSper channels is required for hyperactivation. This Ca^{2+} channel, only present in the sperm flagella, is weakly voltage dependent and

activated by an increase in pH_i (Kirichok et al. 2006; Ren et al. 2001). CatSper null male mice are infertile mainly because of a failure to hyperactivate (Carlson et al. 2005; Carlson et al. 2003; Quill et al. 2001; Ren et al. 2001). It has been proposed that the hyperpolarization of the sperm plasma associated with capacitation increases the driving force for Ca^{2+} , facilitating Ca^{2+} influx through CatSper channels during cytosolic alkalinization (Navarro et al. 2007).

6.4 Capacitation

A defined period of time in the female genital tract is necessary for mammalian spermatozoa to acquire their ability to fertilize (Austin 1952; Chang 1951). Altogether the set of changes required for this maturation process is called capacitation and involves the development of a distinctive sperm motility pattern known as hyperactivation, and the sperm capacity to undergo the AR, an exocytotic event that allows the sperm to fertilize the egg. During sperm capacitation PKA is activated (Harrison 2004), leading to tyrosine phosphorylation increases (Visconti et al. 1995a, b); pH_i (Zeng et al. 1995) and $[Ca^{2+}]_i$ elevate (Baldi et al. 1991; Breitbart 2003; DasGupta et al. 1993; Suarez et al. 1993; Xia and Ren 2009); plasma membrane composition and organization are modified (Cross 1998; Davis 1981; Gadella and Harrison 2000; Go and Wolf 1983; Travis and Kopf 2002; Visconti et al. 1999); and the cell E_m is hyperpolarized in the mouse and other species (Arnoult et al. 1999; Demarco et al. 2003; Munoz-Garay et al. 2001; Zeng et al. 1995).

6.4.1 Membrane Potential Changes During Sperm Capacitation

It is yet not fully understood how and why E_m is hyperpolarized in some mammalian sperm species. Indeed, hyperpolarization is important in mouse, bovine, and equine sperm capacitation (Arnoult et al. 1996; Arnoult et al. 1999; De La Vega-Beltran et al. 2012; Demarco et al. 2003; McPartlin et al. 2011; Munoz-Garay et al. 2001; Zeng et al. 1995), although it has not been demonstrated in human sperm. The sperm resting E_m is relatively depolarized in most mammalian sperm (between -30 and -40 mV) (De La Vega-Beltran et al. 2012; Demarco et al. 2003; Espinosa and Darszon 1995; Hernandez-Gonzalez et al. 2006; McPartlin et al. 2011; Munoz-Garay et al. 2001; Santi et al. 2010; Zeng et al. 1995). Sperm populations are very heterogeneous and only a fraction of the cells capacitate in vitro ($\sim 30\%$); therefore, the average E_m values must be cautiously considered. Capacitated mouse spermatozoa display an average E_m approximately -60 mV. Indeed, when Arnoult et al. (Arnoult et al. 1999) measured E_m in individual spermatozoa using di-8-AN-EPPS, a voltage-sensitive dye, they documented that capacitated sperm populations consisted of at least two groups: one hyperpolarized (~ -80 mV), possibly

representing capacitated sperm, and another of noncapacitated sperm with a resting E_m approximately -43 mV. Various findings suggest that hyperpolarization is essential for sperm to acquire the ability to undergo a physiological AR. For instance, carrying out capacitation in the presence of high external KCl significantly reduces the ZP-induced mouse sperm AR (Arnoult et al. 1999; De La Vega-Beltran et al. 2012; Zeng et al. 1995). These observations lead to the proposal that an E_m hyperpolarization is important for capacitation and thus is required for the AR.

Initially the role of the capacitation-associated hyperpolarization was thought to be needed to remove inactivation from T-type voltage-dependent Ca^{2+} channels (Ca_v3), which could then be activated by physiological agonists (e.g., ZP) to culminate with the induction of the AR (Arnoult et al. 1996; Arnoult et al. 1999; Santi et al. 1996; Zeng et al. 1995). Recent evidence suggests that hyperpolarization of the sperm plasma membrane is necessary and sufficient to prepare sperm for the AR (De la Vega et al. 2012). Even though the molecular entities and the mechanisms responsible for the hyperpolarization are not yet established, it could be the result of (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. In this context, 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 E_m .

6.5 The Acrosome Reaction

The sperm head contains a large secretory vesicle located at its posterior end called the acrosome (Yanagimachi 1998). Spermatozoa of many species need to undergo the fusion of their single acrosome to the plasma membrane to be able to fertilize the female gamete. This process, called the AR, is now believed to occur in multiple steps. As multiple fusion points between the acrosomal and plasma membrane are involved in the AR, plasma membrane–outer acrosome hybrid vesicles are liberated, resulting in the release of the acrosomal content (Buffone et al. 2012). The fusion machinery involved in this reaction is regulated by Ca^{2+} and similar to that found in many neuroendocrinal secretory cells (Bello et al. 2012; Castillo Bennett et al. 2010). Notably, where and what triggers the physiologically relevant AR is being reevaluated (Inoue et al. 2011; Jin et al. 2011; Visconti and Florman 2010; Yanagimachi 1998).

In this context ZP and progesterone, as well as other AR inducers, require reexamination to establish their physiological relevance.

Various transduction pathways are required to converge for the ZP-induced AR to occur, and complex $[\text{Ca}^{2+}]_i$ changes are involved (for review, see Mayorga et al. 2007). The physiologically relevant AR changes in $[\text{Ca}^{2+}]_i$ include external and internal Ca^{2+} sources (Breitbart et al. 2010; Costello et al. 2009; Darszon et al. 2011; Florman et al. 2008). At the present time three different Ca^{2+} channels are thought to mediate the $[\text{Ca}^{2+}]_i$ responses associated to the AR. They appear to be functionally

linked in a manner that is not fully understood (Darszon et al. 2011; Florman et al. 2008; Publicover et al. 2007). Early on it was thought that voltage-dependent Ca^{2+} (Ca_v) channels were involved in the initial $[\text{Ca}^{2+}]_i$ increase detected during the AR induced by ZP in mouse sperm, taking into account their pharmacology and that of this reaction (Darszon et al. 2011). $\text{Ca}_v3.2$ was 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). However, $\text{Ca}_v3.1$ and 3.2 knockout mice are fertile (Stambouljian et al. 2004), and Ca_v currents, although recorded in testicular sperm, were not detected in epididymal sperm (Martinez-Lopez et al. 2009; Ren and Xia 2010). These results raised doubts about the participation of Ca_v3 channels in the mouse sperm AR, although solid immunological data demonstrate their presence (Escoffier et al. 2007; Trevino et al. 2004).

A sustained $[\text{Ca}^{2+}]_i$ increase lasting up to minutes is associated with the AR. Evidence indicates internal Ca^{2+} stores (i.e., the acrosome) participate releasing Ca^{2+} through the IP3 receptor, the second type of Ca^{2+} channel involved in the AR, which is activated as a consequence of IP3 production during the AR (Darszon et al. 2011; Florman et al. 2008; Mayorga et al. 2007; Publicover et al. 2007). Plasma membrane Ca^{2+} channels (SOCS) activate as Ca^{2+} store emptying occurs, causing the sustained $[\text{Ca}^{2+}]_i$ increase. Several components such as STIM, ORAI, and TRPCs may constitute SOCS (Moreno and Vaca 2011). STIM and ORAI have been suggested to be present in human and mouse sperm and contribute to the sustained $[\text{Ca}^{2+}]_i$ elevation leading to the AR (Costello et al. 2009; Darszon et al. 2012).

As discussed, Ca^{2+} transport plays a fundamental role in the AR. Much less is known about how Cl^- movements influence this event. Interestingly, niflumic acid (NFA), best known as a Ca^{2+} -activated Cl^- channel inhibitor, was reported a long time ago to block 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). NFA also partially inhibited a Ca^{2+} -induced hyperpolarization partially driven by Cl^- in mouse spermatozoa (Espinosa et al. 1998). Anion channel blockers such as NFA, for example, DIDS, also inhibit the mouse and human sperm AR, as well as Cl^- channels detected in these cells (Espinosa and Darszon 1995; Espinosa et al. 1998; Figueiras-Fierro et al. 2013; Orta et al. 2012).

Considering the reevaluation of the site(s) and mechanisms that trigger the AR requires also a reexamination of the evidence involving neurotransmitter receptors in this process. These matters are discussed in the section on the GABA_A and glycine receptors, which are particularly relevant to this review as they mediate Cl^- fluxes.

6.6 Cl^- Channels and Transporters Linked to Sperm Physiology

In a recent paper our group reported that when sperm are incubated in Cl^- -free media, most of the capacitation-associated processes are impaired (Hernandez-Gonzalez et al. 2007; Wertheimer et al. 2008). In this condition, increase in tyrosine

phosphorylation and hyperpolarization of the sperm E_m are not observed. Consistently, sperm did not hyperactivate (discussed below), were unable to undergo the AR, and failed to fertilize in an in vitro assay. Although in the absence of Cl^- cAMP agonists rescued phosphorylation events, this condition was not sufficient to allow the sperm to fertilize in vitro. These findings highlight the importance of Cl^- homeostasis in sperm during capacitation, and suggest that one or more Cl^- transport systems are present in sperm. The identity of the specific sperm Cl^- transporters involved in capacitation is still unclear.

The activity of all Cl^- transporters present in a particular cell type defines its $[\text{Cl}^-]_i$ levels. These transporters can be divided in two categories: Cl^- channels and specialized Cl^- carriers (Jentsch et al. 2005; Nilius and Droogmans 2003). Cl^- channels are distributed in four groups: (1) CFTR channels; (2) the γ -aminobutyric (GABA)-gated and related glycine-gated neurotransmitter receptors; (3) Ca^{2+} -activated Cl^- channels; and (4) CLC channels. Cl^- carriers couple the transport of Cl^- to the movement of another ion in either opposite direction (antiporter) or in the same direction (cotransporter or symporter). Cl^- carriers are classified in two main families: (1) the electro-neutral cation Cl^- cotransporter family and (2) the electro-neutral $\text{Cl}^-/\text{HCO}_3^-$ exchanger family.

6.6.1 CFTR Channels

The ABC transporter family has a unique member, the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel modulated by cAMP/PKA and ATP. The CFTR consists of two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory (R) domain. The MSDs have six transmembrane helices each, which are linked by the regulatory domain. In cells possessing either endogenous or recombinant CFTR, the channel displays the following anionic selectivity sequence: $\text{Br}^- > \text{Cl}^- > \text{I}^- > \text{F}^-$ (Anderson et al. 1991). The channel pore is formed by the MSDs, whereas the gating activity is related to ATP hydrolysis by the NBDs and phosphorylation of the R domain (Sheppard and Welsh 1999). Mutations in CFTR cause cystic fibrosis (CF), an autosomic recessive genetic disease characterized by abnormal transport of Cl^- and HCO_3^- leading to viscous secretions in many epithelial cells, but especially the lungs, pancreas, liver, and intestine.

CFTR mutations affect male fertility; as on average 95 % of male CF patients have congenital bilateral absence of the vas deferens, making them infertile. In addition, other mechanisms related to sperm physiology may be affected by CFTR mutations, leading to infertility in CF (Popli and Stewart 2007). Supporting this notion, it has been found that fertility in uremic patients may be reduced through alterations in CFTR expression (Xu et al. 2012). CFTR has been shown to be present in both human and mouse sperm by our group and others, using specific antibodies (Chan et al. 2006; Hernandez-Gonzalez et al. 2007; Li et al. 2010; Xu et al. 2007).

Xu et al. (2007) showed, using CFTR inhibitors and specific antibodies, as well as heterozygous CFTR mutants, that sperm capacitation and the associated HCO_3^- transport are significantly reduced when compared to mice in normal fertilizing conditions. CFTR function has also been identified electrophysiologically as ATP-dependent Cl^- currents that are stimulated by cAMP, cGMP, and genistein, and inhibited by DPC and CFTR_{inh}-172 in whole-cell clamp recordings from testicular and epididymal mouse sperm (Figueiras-Fierro et al. 2013). The biophysical and pharmacological properties of CFTR recorded from epididymal mice spermatozoa, as well as its role in the AR, are shown in Fig. 6.1. Moreover, this particular Cl^- current is absent in testicular sperm from mice displaying the most common variant of the CF mutation, the loss-of-function mutation of the CFTR gene known as ΔF508 . All these findings support the idea that CFTR is present in mature spermatozoa and that it is involved in the capacitation events.

How E_m is regulated by Cl^- and other anions is still poorly understood. As CFTR is mainly a Cl^- channel, it possibly participates in regulation of the resting E_m ; however, Cl^- substitution by nonpermeable anions (e.g., gluconate or methanesulfonate) does not modify it. On the other hand, this procedure inhibits hyperpolarization development during capacitation (Hernandez-Gonzalez et al. 2007). Findings supporting this idea in mouse sperm are (1) adding 250 μM of the CFTR antagonist DPC (diphenylamine-2-carboxylic acid) inhibits hyperpolarization associated with capacitation and also the AR induced by sZP, without modifying tyrosine phosphorylation levels; (2) the CFTR agonist genistein (5–10 μM) hyperpolarizes noncapacitated spermatozoa; and (3) addition of permeable analogues of cAMP to noncapacitated sperm elevates $[\text{Cl}^-]_i$ (Hernandez-Gonzalez et al. 2007). CFTR channels are also known to interact with and regulate other ion channels (i.e., epithelial Na^+ channels, ENaCs) and transporters (i.e., the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, SCL family) (Berdiev et al. 2009; König et al. 2001; Kunzelmann and Schreiber 1999; Perez-Cornejo and Arreola 2004). The interaction of CFTR with the $\text{Cl}^-/\text{HCO}_3^-$ exchangers is important to explain its role in pH_i regulation (discussed in Sect. 6.6.6). Of particular relevance to understand how CFTR influences the resting E_m is its interaction with ENaC. As the sperm resting E_m is mildly depolarized (~ -35 mV) and thus shifted from the K^+ equilibrium potential, it cannot be explained mainly by a high K^+ permeability through K^+ channels, as it usually happens in many cells. Just a 10 % contribution of Na^+ permeability would explain the observed resting E_m in noncapacitated sperm, and their inhibition during capacitation would contribute to the observed hyperpolarization (see Hernandez-Gonzalez et al. 2006; Hernandez-Gonzalez et al. 2007). CFTR can downregulate ENaCs through mechanisms still not fully understood (König et al. 2001), and the CFTR agonist genistein hyperpolarizes sperm E_m (Hernandez-Gonzalez et al. 2007) and diminishes $[\text{Na}^+]_i$ (Escoffier et al. 2012). In summary, CFTR is important in sperm physiology regulating pH_i and E_m in the resting and capacitating conditions, directly through its Cl^- and anion permeability or indirectly through its interaction with ENaC and $\text{Cl}^-/\text{HCO}_3^-$ exchangers.

Fig. 6.1 Cystic fibrosis transmembrane conductance regulator (CFTR) channels are functional in epididymal mouse spermatozoa. **(a)** Representative whole-cell patch-clamp currents recorded by applying voltage pulses from a holding potential of -40 mV to test potentials ranging from 100 to -80 mV in 20 -mV steps. The protocol used for eliciting CFTR currents is shown between **a** and **c** traces. Sperm were exposed to recording solutions where the primary ion is Cl^- . Control Cl^- currents (filled squares) were significantly stimulated by extracellular db-cAMP (100 μM ; filled circles) and inhibited following the addition to the external media of the specific inhibitor, CFTR_{inh}-172 (5 μM ; filled triangles db-cAMP+CFTR_{inh}-172). CFTR_{inh}-172 inhibition was partially reversible (hollow squares). The effects of agonist and CFTR_{inh}-172 were recorded in the presence of niflumic acid (NFA) (50 μM), to eliminate the contribution of other Cl^- channels. **(b)** I - V relationship of the currents in **a**. CFTR currents similar to those detected in epididymal sperm were also present in testicular sperm. **(c)** CFTR inhibition during capacitation significantly inhibited acrosome reaction (AR). Mouse sperm were capacitated in medium supplemented with bovine serum albumin and NaHCO_3 , and AR was induced with ZP or A23187 (Ca^{2+} ionophore). The presence of DPC, a classic CFTR inhibitor, during sperm capacitation decreased the ZP-induced AR if added during capacitation but not if added during AR induction. For **a** and **b**, symbols represent the mean $\pm$ SEM of four experiments; some SE bars were smaller than the symbols. The currents were normalized with respect to the stimulated Cl^- current at 100 mV. For **c**, data are normalized with respect to spontaneous AR and presented as the mean $\pm$ SEM with $n \geq 3$

6.6.2 GABA and Glycine Receptors

GABA_A receptors are ligand-gated anion channels selective for Cl⁻ ions, which mediate inhibitory neurotransmission in the central nervous system (CNS). These receptors are pentameric and composed of different subunits. 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 central nervous system (CNS) depressants (Macdonald and Olsen 1994; Sieghart 1995). GABA_A receptors are also found outside the CNS, in tissues such as liver, lung, and immune cells (Sigel and Steinmann 2012). Polymerase chain reaction (PCR) and immunocytochemistry studies revealed the presence of GABA_A and GABA_B receptors in spermatogenic cells, and patch-clamp studies showed that GABA application to round spermatogenic cells induced an inward Cl⁻ current (Kanbara et al. 2011). The presence and function of GABA_A receptors in sperm from different species have been explored by several laboratories (Jin et al. 2009; Meizel 1997; Puente et al. 2011; Wistrom and Meizel 1993). For example, Ritta et al. established that this neurotransmitter plays a role in the regulation of motility in bovine and human sperm (Ritta et al. 2004; Ritta et al. 1998). In rat sperm, GABA and progesterone accelerated the process of capacitation and hyperactivated motility, effects that were inhibited by bicuculline and picrotoxin (Jin et al. 2009). Additionally, it has been shown that GABA can induce the AR (Puente et al. 2011; Shi et al. 1997) and also that GABA_A receptors can modulate the response to progesterone in these cells (Hu et al. 2002; Ritta et al. 1998; Shi and Roldan 1995; Turner et al. 1994).

Glycine receptors are also heteropentameric Cl⁻ channels that mediate inhibitory transmission in the CNS, although they are also found in retina and macrophages (Webb and Lynch 2007). The alkaloid strychnine has been used as a very specific antagonist for these receptors. The presence and functionality of glycine receptors in sperm have been studied especially in Meizel's laboratory. They reported the expression of different glycine receptor isoforms in sperm from human, porcine, mouse, and hamster using both immunocytochemistry and Western blot analysis (Bray et al. 2002; Kumar and Meizel 2008; Llanos et al. 2001; Meizel and Son 2005; Melendrez and Meizel 1995; Sato et al. 2000). These receptors were detected in the head and flagellum, suggesting distinct roles at different cell locations. For example, antibodies against glycine receptors A1 and A2 inhibited the ZP3-induced AR in human sperm (Bray et al. 2002).

Compounds such as glycine, GABA, and acetylcholine were reported many years ago to induce the AR; however, the physiological significance of these observations was unclear, considering that the accepted physiological inductor for this reaction was ZP3. Recent findings have challenged this paradigm, and where, when, and what induces the AR are open questions again (Inoue et al. 2011; Jin et al. 2011; Kunzelmann et al. 2011).

6.6.3 Ca^{2+} -Activated Cl^- Channels (CaCCs)

In many cell types, volume control and secretion are critical (i.e., reproductive tract smooth muscle cells, oviduct and ductus epididymis cells, spermatids, epithelial cells in exocrine glands and trachea, airway, and vascular smooth muscle cells), and spermatozoa are not an exception. Usually these cells possess Ca^{2+} -activated Cl^- channels (CaCCs) that display similar biophysical and molecular features (Hartzell et al. 2005; Huang et al. 2009). Such currents were initially documented in *Xenopus* oocytes (Miledi 1982) but have now been recorded in many cell types.

An elevation of $[\text{Ca}^{2+}]_i$ resulting from release from intracellular stores or influx through plasma membrane channels activates CaCCs. In spite of the frequent presence of CaCCs in cells, their molecular identity is not fully known. Candidates considered for CaCCs are bestrophins, tweety, and CLCA. However, all of them failed to reproduce the native behavior of CaCCs when expressed in heterologous expression systems (Hartzell et al. 2009). Recently, using bioinformatics approaches, different research teams succeeded isolating and expressing genes in heterologous expression systems that elicit almost identical CaCCs currents to those reported in native cells. The identity of these molecules that seem to be the molecular basis of CaCCs are anoctamins (TMEM16) (Caputo et al. 2008; Ferrera et al. 2010; Schroeder et al. 2008; Yang et al. 2008). The term anoctamin refers to the property of being anion selective and having subunits with a putative topology consisting of eight transmembrane segments and cytosolic N- and C-termini (Galiotta 2009). One of the main TMEM16 proteins whose activity strongly resembles that of CaCCs is TMEM16A, corresponding to anoctamin-1. The TMEM16/anoctamin family has nine more members named TMEM16B to -K, or anoctamin 2–10 (Galindo and Vacquier 2005). The biophysical characteristics among the members of this family of ion channels such as voltage dependence, selectivity, and conductance differ (Pifferi et al. 2009; Scudieri et al. 2012). Almost all show enzymatic properties in addition to the transport function, which has led to the speculation that they are multifunctional proteins, one of them being permeable to Cl^- (Tian et al. 2012).

As mentioned, earlier electrophysiological evidence for the presence of Cl^- channels in sperm had been gathered. The initial patch-clamp recordings directly on epididymal mouse sperm revealed an anion channel displaying biophysical properties and sensitivity to NFA, resembling to the Ca^{2+} -dependent Cl^- channels (Espinosa et al. 1998; Hogg et al. 1994). Thereafter, a Cl^- -permeable channel showing long stable openings was documented in patch-clamp studies in the cell-attached mode in the human sperm. Distinct channel clustering and activity was detected in different sperm head regions whose functional significance awaits determination (Jimenez-Gonzalez et al. 2007).

More recently, using a modified perforated patch-clamp technique, whole-cell recordings of the human mature spermatozoa head revealed that an important component of their Cl^- currents is in fact caused by CaCCs, possibly of the TMEM16A type (Orta et al. 2012). Supporting evidence was based on the biophysical properties and in the pharmacology profile of the recorded currents. The typical results of such experiments characterizing biophysically and pharmacologically CaCCs in epididymal human spermatozoa are shown in Fig. 6.2. One of the most specific

Fig. 6.2 Ca^{2+} -activated Cl^- channel (CaCC) currents in epididymal human spermatozoa. (a) Whole-cell CaCC currents recorded from a human spermatozoa. Currents were obtained at a holding potential of 0 mV with the indicated voltage step protocol (*top panel*). The control currents (Control) were recorded before and after exposure to 10 μM TMEM16A_{inh} (*bottom panel*). (b) I - V plot of the blockade of the CaCC currents ($[\text{Ca}^{2+}]_i = 250 \text{ nM}$) by 10 μM NFA and 10 μM TMEM16A_{inh}. Currents were normalized with respect to maximal current in control measurements at the end of each voltage pulse. (c) Dose-dependent blockade of CaCC currents by TMEM16A_{inh}. Current amplitudes were measured at +100 mV by averaging seven to nine original current traces and normalized with respect to the maximal blocked fraction. (d) Concentration-response curve at different $[\text{Ca}^{2+}]_i$ on the macroscopic inward and outward Cl^- currents obtained from seals on the cytoplasmic droplet against the current obtained at 1,000 nM $[\text{Ca}^{2+}]_i$ recorded at +100 mV. The continuous lines represent the data fitted to the Hill equation with the following parameters: $K_d = 163 \text{ nM}$ and $n_H = 1.9$ at +100 mV. For **b** and **c**, data represent the mean $\pm$ SEM with $n = 6$; for **d**, data represent mean $\pm$ SEM with $n = 7$.

antagonists of TMEM16A channels, the drug TMEM16A_{inh} (20 μM), inhibited these currents and reduced $\sim 80\%$ of the AR. These results suggest the critical participation of these channels during the AR. Results supporting this hypothesis are shown in Fig. 6.3. It is known that during the AR there are large $[\text{Ca}^{2+}]_i$ changes leading to profound modifications in the sperm head morphology involving acrosome swelling and a decrease in regulatory volume; therefore, CaCCs channels may

Fig. 6.3 The rhZP3-induced AR in human spermatozoa is inhibited by CaCC/TMEM16A_{inh}. Motile human spermatozoa were obtained by the swim-up technique and capacitated during 5 h. Sperm populations were preincubated for 15 min with different NFA, DIDS, and TMEM16A_{inh} concentrations. The AR was induced with rhZP3 (10 ng μl^{-1}). Cells were fixed with cold methanol and the acrosomal status evaluated after staining sperm with FITC-PSA. Acrosomal reaction was expressed as an index (ARI=percentage of AR normalized against the maximum AR with ionomycin) and was used to estimate the percentage of AR inhibition. NFA (a) and DIDS (b), two CaCCs blockers, inhibited 90 % of the AR. TMEM16A_{inh} (c) blocked approximately 80 %, indicating that TMEM16A channels may have an essential contribution in the AR. For all data, error bars represent mean $\pm$ SEM with $n=4-6$. Statistical comparisons according to Student's unpaired t test indicated * $P<0.05$; ** $P<0.01$; *** $P<0.001$ versus spermatozoa incubated with 0.1 % DMSO + 10 ng μl^{-1} of rhZP3

participate in this event (Zanetti and Mayorga 2009). Blockage of CaCCs by NFA, DIDS, and TMEM16A_{inh} may affect the reduction in regulatory volume, which in turn seems to be important to regulate acrosomal-plasma membrane distance, essential for acrosomal exocytosis (Zanetti and Mayorga 2009).

Evidence gathered recently has shown that CaCCs and voltage and Ca^{2+} -gated K^+ channels (BK_{Ca}, Slo1, or K_{Ca}1.1) have some unexpected similarities regarding their pharmacological properties (Greenwood and Leblanc 2007; Sones et al. 2009). Classical Cl^- channel inhibitors with different structures such as anthracene-9-carboxylate, NFA, and ethacrynic acid also behave as BK_{Ca} agonists (Greenwood and Large 1995; Ottolia and Toro 1994; Toma et al. 1996). The potent ability to inhibit the AR by classical anion channel blockers such as NFA may be explained by a combination of the effects of this drug on BK_{Ca} and CaCCs, as BK_{Ca} channels are also present in mammalian sperm (Rossato et al. 2001; Wu et al. 1998).

Notably, it has also been shown that TMEM16A channels may increase their permeability to HCO_3^- on raising $[\text{Ca}^{2+}]_i$ through Ca^{2+} /calmodulin modulation (Jung et al. 2013). This finding is relevant to sperm physiology because HCO_3^- plays a key role in cAMP production and pH_i regulation (Visconti et al. 2011). It has also been shown that CaCCa can be modulated through the activity of other ion channels also present in spermatozoa (i.e., purinergic receptors) (Wang et al. 2013). Taking these facts into account, it is likely that new evidence of their critical relevance will be gathered in the future.

In summary, CaCCs significantly influence sperm physiology and are likely players in the solubilized ZP-induced AR. It will be interesting to further investigate their role in sperm motility, as inklings of their involvement in sea urchin sperm

chemotaxis have been reported (Alvarez et al. 2012; Guerrero et al. 2013; Ingermann et al. 2008; Wood et al. 2003; Wood et al. 2007).

6.6.4 Voltage-Dependent Anion Channels (VDACs)

VDACs are porins, usually located on the outer mitochondrial membrane (Liu et al. 2010). They seem to be critical for mitochondria metabolism and regulation of apoptosis as they are permeant to small hydrophilic molecules. In this sense, they have been described as molecules able to “regulate cell life and death” (Shoshan-Barmatz et al. 2010). They seem to be involved in the pathogenesis of a variety of processes such as cancer, neurodegenerative diseases, and ischemic-reperfusion injuries in the heart (Peixoto et al. 2012). VDAC2 is the main variant present in the male germline, although VDAC3 has also been found (Liu et al. 2010). VDACs have been localized not only in the mitochondrial sheath but also in the plasma membrane and outer dense fiber (Hinsch et al. 2004; Liu et al. 2009; Triphan et al. 2008). It has been reported recently that they might play a role in human fertility (Kwon et al. 2013). Consistent with this proposal, VDAC2 was found to be one of the putative sperm head membrane proteins that bind ZP (Petit et al. 2013).

6.6.5 Secondary Active Cl^- Transporters

A $[\text{Cl}^-]_i$ increase has been reported to occur during capacitation (Hernandez-Gonzalez et al. 2007; Meizel and Turner 1996), and it is possible that several Cl^- transport systems participate in this process. Under physiological conditions electro-neutral carriers such as NCC, NKCC support transport of Cl^- into the cell and KCC outside the cell (Russell 2000). Pharmacological approaches have been used to test the participation of these carriers during sperm capacitation. Stilbenes such as DIDS and SITS, which are general Cl^- transport blockers, reduced sperm capacitation parameters to similar levels as those observed in the absence of Cl^- . Two specific inhibitors (bumetanide and furosemide) for NKCC blocked the increase in tyrosine phosphorylation, hyperactivation, and the ability of the sperm to fertilize in vitro, but the concentration required to observe these effects was higher than that specifically reported to inhibit NKCC (Garg et al. 2007; Russell 2000). NKCC function requires the presence of Na^+ , K^+ , and Cl^- , but tyrosine phosphorylation is affected in the absence of Na^+ and Cl^- but not K^+ , suggesting that bumetanide and furosemide may be acting through a target other than NKCC. It is worth noting that the ZP-induced AR depended on the presence of the three ions and was inhibited at much lower concentrations of bumetanide, suggesting that NKCC might have a role in the preparation of the sperm for the physiologically induced AR. NKCC1 transcripts were detected in spermatids, and male mouse null mutants of this protein have defects in spermatogenesis and are infertile (Pace et al. 2000). Thiazide, a specific NCC inhibitor, did not interfere with capacitation-associated processes.

6.6.6 $\text{Cl}^-/\text{HCO}_3^-$ Exchangers

$\text{Cl}^-/\text{HCO}_3^-$ transporters are proteins that exchange Cl^- for HCO_3^- in either direction. HCO_3^- regulation is particularly important in spermatozoa because it activates cAMP synthesis by the atypical soluble adenylyl cyclase present in these cells (Hess et al. 2005; Okamura et al. 1985). The specific carriers responsible for HCO_3^- transport have not yet been fully defined. Experiments from our group suggest that $\text{Na}^+/\text{HCO}_3^-$ cotransporters allow HCO_3^- influx into mouse sperm (Demarco et al. 2003). However, other HCO_3^- transport systems can also play a role in the control of HCO_3^- levels in sperm. In particular, $\text{Cl}^-/\text{HCO}_3^-$ exchangers have been proposed to be involved in HCO_3^- homeostasis. These exchangers also influence pH_i , cell volume, and E_m through their contribution to determine the Cl^- gradient. Two superfamilies group the $\text{Cl}^-/\text{HCO}_3^-$ exchangers, SLC4 and SLC26; they have different anion selectivity and unique tissue distribution. The SLC4 superfamily is composed of 3 genes (AE1, AE2, and AE3), each of them represented by more than one alternative spliced sequence. The SLC26 gene superfamily consists of 11 genes but only SLC26A3, SLC26A4, and SLC26A6 have $\text{Cl}^-/\text{HCO}_3^-$ exchange activity.

The AE2 gene is the only member from the SLC4 superfamily reported in spermatogenic cells. This gene has five splice isoforms (AE2a, AE2b1, AE2b2, AE2c1, AE2c2), and mice lacking their expression die before weaning of severely retarded development (Gawenis et al. 2004). Mice expressing only the AE2c isoform survive but they are infertile and exhibit testicular dysplasia, consistent with the observation that AE2 is highly expressed in the testis (Medina et al. 2003).

Recent Western blot and immunofluorescence results from several laboratories, including ours, indicate the presence of SLC26A3 and SLC26A6 in the sperm mid-piece (Chan et al. 2009; Chavez et al. 2011; Chen et al. 2009). Chavez et al. (2011), also provided evidence that these transporters co-precipitate with CFTR and that tenidap, a specific SLC26A3 inhibitor, blocked the capacitation-associated hyperpolarization and the ZP-induced AR. However, tenidap did not affect the activation of a cAMP pathway or the increase in tyrosine phosphorylation, suggesting that these transporters are not directly involved in the regulation of the soluble adenylate cyclase.

6.7 Final Remarks

Work reported in recent years corroborates the significant repercussions of ion channels in sperm maturation, capacitation, and the AR. However, our knowledge regarding the molecular mechanisms regulating these processes is still limited. The study of ion transport in spermatozoa, specially the use of electrophysiological techniques, has been a difficult enterprise because of their small size and the stiffness of their membrane. Fortunately, new patch-clamp strategies have improved our ability to study sperm ion channels. Although the inability of these cells to perform transcription and translation significantly complicates knocking down or expressing

exogenous proteins, advances in the production of genetically modified mice have enhanced the identification of key proteins, processes, and mechanisms essential to sperm function. The increasing sensitivity and speed of single-cell ion-imaging strategies is helping to unravel sperm signaling networks and how ion channels participate in them. We hope the readers have become aware that the study of sperm anion transport requires our full attention, as it is deeply implicated in the physiology of this important cell. Enhancing our understanding of sperm ion transport will impact our capacity to preserve animal species and improve our control of fertility.

Acknowledgments This work was supported by DGAPA: IN202312 (to A.D.) and IN202212-3 (to C.T.), CONACyT: 128566 (to A.D.) and 99333 (to C.T.), CSIC-UdelaR international collaboration grants to G.F. with A.D. and C.T. NIH: R01 HD038082 (to Pablo Visconti) and CSIC p944 (to Gonzalo Ferreira).

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