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Review

Regulation mechanisms and implications of sperm membrane hyperpolarization

Carla Ritagliati[a,*], Carolina Baro Graf[a], Cintia Stival[a], Dario Krapf[a,b,*]

[a] Laboratory of Cell Signal Transduction Networks, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET-UNR), Rosario 2000, Argentina
[b] Laboratorio de Especialidades Reproductivas, Facultad de Ciencias Bioquimicas y Farmacéuticas, UNR, Rosario 2000, Argentina

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ABSTRACT

Mammalian sperm are unable to fertilize the egg immediately after ejaculation. In order to gain fertilization competence, they need to undergo a series of biochemical and physiological modifications inside the female reproductive tract, known as capacitation. Capacitation correlates with two essential events for fertilization: hyperactivation, an asymmetric and vigorous flagellar motility, and the ability to undergo the acrosome reaction. At a molecular level, capacitation is associated to: phosphorylation cascades, modification of membrane lipids, alkalinization of the intracellular pH, increase in the intracellular Ca^{2+} concentration and hyperpolarization of the sperm plasma membrane potential. Hyperpolarization is a crucial event in capacitation since it primes the sperm to undergo the exocytosis of the acrosome content, essential to achieve fertilization of the oocyte.

1. Introduction

Fertilization involves the fusion of male and female gametes, which is the first step in creating a new organism. However, mammalian sperm, unlike spermatozoa of many other animals, are unable to fertilize the egg immediately after ejaculation. In order to gain fertilization competence, they need to undergo a series of physiological modifications inside the female reproductive tract, collectively known as capacitation (Austin, 1952; Chang, 1951). This process can be mimicked in vitro by sperm incubation in chemically defined media containing Ca^{2+}, HCO_3^-, energy sources, and a cholesterol acceptor that is usually BSA, as first demonstrated by Toyoda et al. (1971) in the mouse. At a molecular level, capacitation is associated to: activation of a cAMP/PKA pathway (Harrison, 2004; Krapf et al., 2010), loss of membrane cholesterol (Cross, 1996; Davis et al., 1980) and modification of other membrane lipids (Gadella and Harrison, 2000), increase in protein tyrosine phosphorylation, intracellular pH (pHi) and intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Ruknudin and Silver, 1990), and hyperpolarization of the sperm plasma membrane potential (*Em*) (De La Vega-Beltran et al., 2012; Escoffier et al., 2012; Gervasi and Visconti, 2016). The sperm is a highly differentiated and compartmentalized cell, composed of a flagellum, in charge of the cell propulsion, and a head, which contains the highly compacted DNA surrounded by a vesicle named acrosome. These compartments exhibit differential distribution of membrane channels restricted to specific localizations (Fig. 1). Capacitation correlates with flagellar vigorous motility called hyperactivation (Yanagimachi, 1994a) and the ability to undergo the acrosome reaction in response to a physiological agonist, both of which are believed to be essential for successful sperm penetration into oocytes (Yanagimachi, 1994b). Therefore, the two essential physiological events, hyperactivation and acrosome reaction, take place in different compartments. However, it is also now known that activation of signaling pathways in the sperm tail plays a role in the regulation of events happening in the head. Navarro et al. showed ten years ago, by injecting fluorescent dyes, that diffusion among compartments is possible (Navarro et al., 2007). It should be noted that trans-signaling events by second messengers as cAMP, produced in micro-domains by the action of transmembrane (only in the head) and/or soluble adenylyl cyclase (presumably in the whole sperm) is improbable, since cAMP diffusion is restricted by hydrolyzing enzymes and extrusion pumps (Alonso et al., 2017; Wertheimer et al., 2013). However, signaling events can also be orchestrated by diffusion of ions across compartments, influencing pH, Ca^{2+} concentration, and membrane potential.

As already mentioned, capacitation correlates with changes in the sperm plasma membrane potential (*Em*) (Zeng et al., 1995). When cauda epididymal mouse sperm are incubated in culture media, their *Em* is relatively depolarized. However, this membrane potential hyperpolarizes as capacitation proceeds, whereas no change is observed when sperm are incubated in conditions that do not support capacitation (Demarco et al., 2003; Zeng et al., 1995; Espinosa and Darszon,

* Corresponding authors at: Instituto de Biología Molecular y Celular de Rosario (CONICET-UNR), Ocampo y Esmeralda, Rosario 2000, SF, Argentina.
E-mail addresses: ritagliati@ibr-conicet.gov.ar (C. Ritagliati), krapf@ibr-conicet.gov.ar (D. Krapf).

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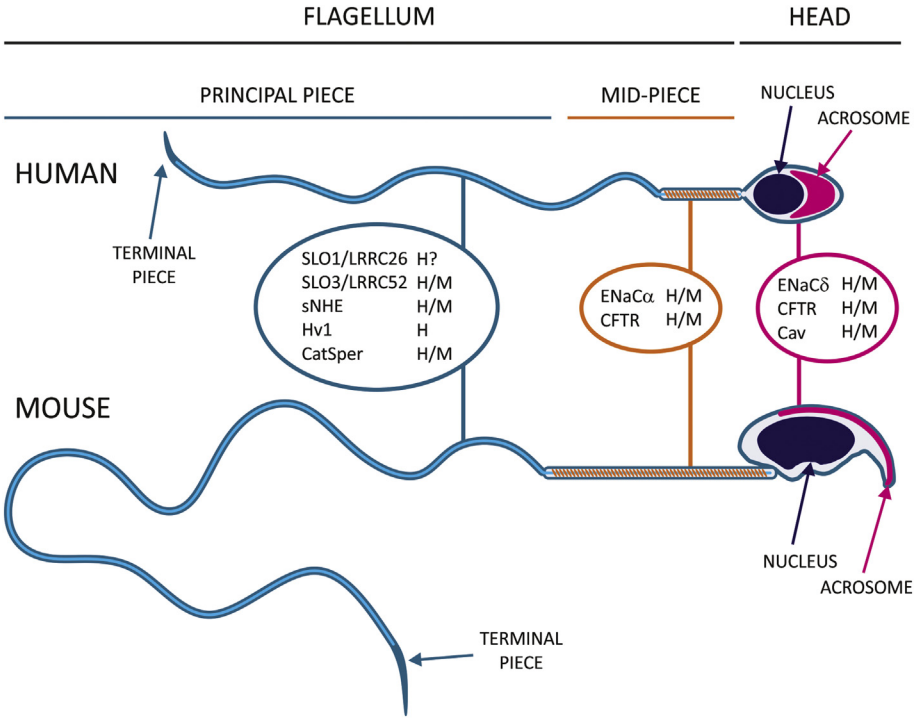

Fig. 1. Schematic representation of human and mouse sperm. Cellular compartments and localization of species-specific ion channels, found within each section, which are important for (or directly involved in) *Em* determination as described in this review. H: human; M: mouse. Slowpoke potassium channel (SLO1/3), leucine-rich repeat-containing 26/52 (LRRC26/52), sperm-specific Na^+/H^+ exchanger (sNHE), voltage-gated H^+ channel (Hv1), cation channel of sperm (CatSper), epithelial sodium channel (ENaC), cystic fibrosis transmembrane conductance regulator (CFTR), and voltage-activated calcium channel (Cav).

1995). This *Em* hyperpolarization has been shown to be both necessary and sufficient to prepare mice sperm to undergo an induced acrosome reaction. Non-capacitated sperm pharmacologically hyperpolarized, acquire acrosomal responsiveness even when phosphorylation pathways associated to sperm capacitation are turned off. Moreover, when sperm are capacitated in depolarized *Em* clamped conditions, therefore unable to hyperpolarize, both PKA activation and tyr phosphorylation activate normally, but the agonist-induced acrosome reaction is inhibited. This points towards the key role of hyperpolarization on enabling mouse sperm to undergo the acrosome reaction (De La Vega-Beltran et al., 2012). Despite its importance, the mechanisms that underlie hyperpolarization during capacitation are poorly understood. This review will focus on the channels and events involved in the regulation of membrane potential that takes place in capacitation, with special emphasis on differences and similarities between mouse and human sperm, and its implications on other capacitation-associated events. The involvement of other channels, which have not been thoroughly studied and related to capacitation-associated hyperpolarization, cannot be completely ruled out, but were therefore not included in this review.

2. Membrane potential

In sperm, as in most cells, the internal ion concentrations are markedly different from those in the extracellular medium. These differences result from the relative permeability of the plasma membrane to each of the ions found in the inner and outer media given by the specific ion channels and transporters present in the cell, to the gradients they establish and the metabolic state of the cell. At rest, the balance of these fluxes, gradients and permeabilities results in an electric potential, known as the resting *Em* (Stival et al., 2016; Visconti et al., 2011). Mammalian sperm encounter environments with very different ionic composition on their journey to meet the egg. For example, external K^+ concentration ($[K^+]_e$) may change from ~39 to 5–8 mM, external Cl^- concentration ($[Cl^-]_e$) from ~27 to 130 mM, and external Na^+ concentration ($[Na^+]_e$) from 38 to 140 mM in the cauda epididymus and oviduct respectively (Neill, 2006). Sperm must regulate their *Em* and adapt to the changes in external ion concentration, while also achieving membrane hyperpolarization at the appropriate time (Chávez et al., 2013). Before capacitation mouse sperm are relatively depolarized (*Em* around −35 mV) and become hyperpolarized (*Em* around −70 mV) during capacitation (Arnoult et al., 1999; Zeng et al., 1995), as determined by a fluorometric population assay (Fig. 2A, described below). This change results from a combination of electrogenic ion permeability alterations that shift the *Em* towards the K^+ equilibrium potential (around −90 mV) (Demarco et al., 2003). However, using flow cytometry (Fig. 2B, described below), it has been reported that in mouse sperm samples subjected to capacitating medium, only 40% or less actually achieve the capacitated state, resulting in a heterogeneous sperm population with resting membrane potentials that fall into two subpopulations; those that appear not to have achieved a capacitated state with a membrane potential less than −50 mV, while those that appear to have achieved capacitation and hyperpolarization with membrane potentials approaching −80 mV (Arnoult et al., 1999). This is also the case for human sperm, as shown by López-González et al. (2014) using flow cytometry, only a subpopulation hyperpolarizes under capacitating conditions. However, there is a large heterogeneity in the percentage of hyperpolarized cells among samples from different individuals. Therefore, it is likely that the membrane potential measured in population assays results from the average of these two subpopulations (Escoffier et al., 2015).

2.1. Methodology used to study Em

2.1.1. Fluorometric population assay

The fluorometric population assay allows the determination of absolute *Em* values, which are relatively easy to measure (Fig. 2A). The equipment needed consists of a spectrofluorometer with a thermostatized holder at 37 °C, and a potentiometric probe. Among many, the most widely used is $DiSC_3(5)$, a cationic carbocyanine dye with a short (C_3) alkyl tail that readily partitions across the membrane depending on *Em* and aggregates within the membrane, causing self-quenching. Therefore, upon *Em* hyperpolarization more dye enters the cell, resulting in a decreased fluorescence from the extracellular millieu. When constant concentrations of sperm and probe are used, this method provides a highly reproducible value of plasma membrane potential

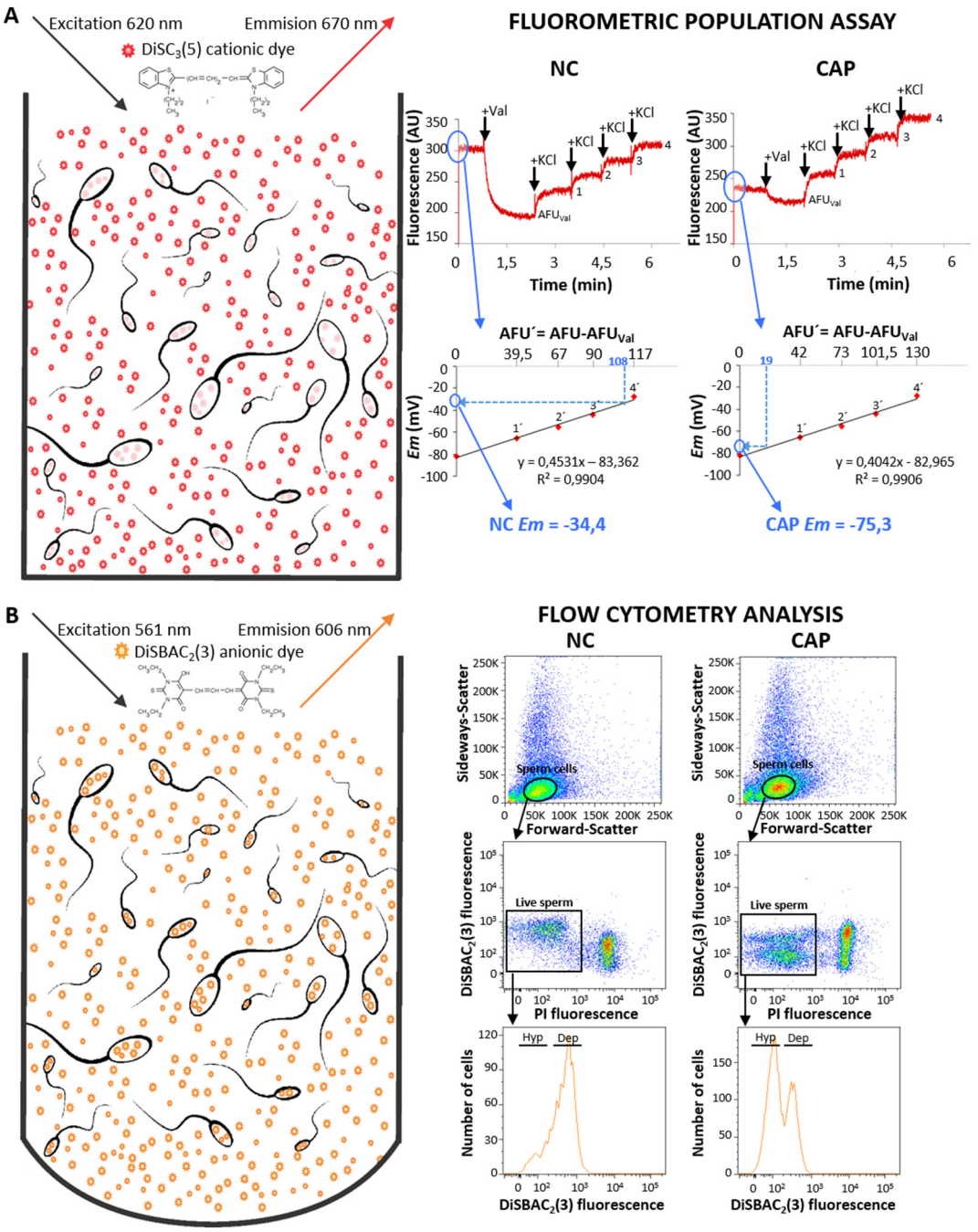

Fig. 2. Methodology used to study *Em*. A, Representative image of a fluorometric population assay. Non-capacitated (NC) and capacitated (CAP) sperm are loaded with $DiSC_3(5)$ (1 μM) for 2–3 min, transferred to a gently stirred quartz cuvette at 37 °C, and the fluorescence is monitored with a spectrofluorometer at 620/670 nm excitation/emission wavelength. Recordings are initiated when steady-state fluorescence is reached (approximately 1 min). Calibration is performed by adding 1 μM valinomycin (Val) and sequential additions of KCl. The Nernst equation determines a theoretical *Em* value for every arbitrary fluorescence unit (AFU) obtained for each KCl concentration (points 1–4). In order to construct the calibration curve, the difference between each point and the fluorescence obtained after the addition of valinomycin (AFU_{Val}) is calculated: AFU′(1′-4′). Finally, the sperm *Em* is obtained by linearly interpolating the theoretical *Em* values against the initial AFU registered. The dye is depicted in red, while the intracellular quenched fluorophore is in pink. B, Representative image of the flow cytometry analysis. Non-capacitated (NC) and capacitated (CAP) sperm are loaded with $DiSBAC_2(3)$ (15 μM) at 37 °C for 30 min. Alternatively, cells can be loaded with $DiSC_3(5)$ (50 nM). Then, propidium iodide (PI) is added (at a final concentration of 2 μM for mouse sperm and 50 nM for human sperm) and the cells are analyzed in a flow cytometer. Non-viable cells become PI positive, and their red fluorescent signal detected as fluorescence of wavelength ≥ 670 nm. Orange fluorescence from $DiSBAC_2(3)$-positive cells was detected at 561–606 nm. The first two dot plots correspond to Sideways-Scatter versus Forward-Scatter analysis of NC and CAP sperm in the absence of 0.1% Triton X-100. When Triton X-100 is added, since it solubilizes non-sperm particles, it makes it possible to distinguish between non-sperm particles and sperm cells (maintained in the presence of detergent, circled in black), which are further analyzed in the $DiSBAC_2(3)$ versus PI two-dimensional fluorescence dot plot. Live sperm populations (low PI, squared in black) were used for histogram analysis depicting number of cells versus $DiSBAC_2(3)$ fluorescence. Sperm incubated in capacitating conditions show two subpopulations: one with high dye fluorescence, which are depolarized (Dep) and also present in the non-capacitating condition, and another with low dye fluorescence corresponding to the capacitated hyperpolarized (Hyp) cells. The dye is depicted in orange.

after calibration using valinomycin (a K^+ ionophore) and sequential additions of KCl, increasing extracellular K^+ concentration to different known values. The final *Em* is obtained following the Nernst equation, assuming an intracellular K^+ concentration of 120 mM and that the membrane is a K^+ electrode under the influence of valinomycin, by linearly interpolating the theoretical *Em* values (for each KCl concentration) against arbitrary fluorescence units of each trace (Espinosa and Darszon, 1995). The fluorescence emission of $DiSC_3(5)$ is affected by sperm number, dye concentration (which needs to be adjusted depending on the species), sperm viability and dimensions of the quartz cuvette, as well as by the presence of compounds that chemically interact with $DiSC_3(5)$. However, the internal calibration compensates for these variables, resulting in a robust determination. For mouse sperm, no toxic effects on sperm function have been observed at the concentrations used (< 5 μM) (Zeng et al., 1995). Moreover, it has been shown that mitochondrion *Em* contribution is negligible (Chávez et al., 2013). In this regard, it should be noted that keeping dye loading times to the minimum possible aids to avoid intracellular compartmentalization.

2.1.2. Flow cytometry analysis

Cells are usually loaded with $DiSC_3(5)$ or with the fluorescent anionic dye $DiSBAC_2(3)$, which contrary to the $DiSC_3(5)$ dye, increases its signal inside the cell and exhibits enhanced fluorescence and a red spectral shift (Fig. 2B). Considering the $DiSBAC_2(3)$ properties, hyperpolarization results in a decrease in fluorescence, while with $DiSC_3(5)$ hyperpolarization results in an increase in fluorescence, in a condition where only cell-originated fluorescence is recorded. DiSBAC dyes are excluded from mitochondria because of their overall negative charge (Escoffier et al., 2015). This method allows the study of *Em* of individual cells in a population, but does not provide absolute *Em* values.

3. Channels involved in *Em* hyperpolarization

There are two mechanisms that mainly contribute to sperm *Em* hyperpolarization: 1) Decreasing activity of Na^+ channels reduces Na^+ permeability (P_{Na}) since both, the decrease in $[Na^+]_e$, or the addition of the Na^+ channel blocker amiloride, produce membrane hyperpolarization in non-capacitated sperm (Hernández-González et al., 2006). 2) Activation of K^+ selective channels increases K^+ permeability. The involvement of K^+ channels in this change was proposed because hyperpolarization was affected by external K^+ and reduced by K^+ channel blockers (Espinosa and Darszon, 1995; Zeng et al., 1995).

3.1. Epithelial sodium channels (ENaCs)

ENaCs are made up of four subunits: α, β, γ and δ, where α and δ can replace each other and are directly involved in forming the channel pore (Canessa et al., 1994). These channels may be regulated by pH, Ca^{2+}, Na^+, Cl^- and phosphorylation (Kellenberger and Schild, 2002), parameters that change during capacitation. ENaCs are expressed in many invertebrate and vertebrate cell types. The presence of ENaC α and δ subunits in mouse sperm was confirmed by Western blot analysis using polyclonal antibodies, which detected the proteins in testis and sperm. As seen by immunolocalization, although ENaC α was observed in the flagellum mid-piece, ENaC δ antibodies stained the anterior acrosome. Neither subunit changed its localization following sperm capacitation (Hernández-González et al., 2006). Furthermore, ENaC type currents were detected using electrophysiological techniques in spermatogenic cells, the precursors of sperm (Hernández-González et al., 2006) and in testicular sperm (Martínez-López et al., 2009). In human sperm, Kong et al. (2009) demonstrated by immunoblotting the presence of ENaC α protein in the flagellar mid-piece. Na^+ permeability is involved in the establishment of the sperm resting *Em* and this permeability is thought to be reduced during sperm capacitation (Demarco et al., 2003; Hernández-González et al., 2006), since $[Na^+]_i$ decreases (Escoffier et al., 2012) and replacement of external Na^+ by non-permeable cations results in sperm *Em* hyperpolarization. The addition of external Na^+ produces a depolarization that is potently inhibited by amiloride and its analog EIPA (5-(N-ethyl-N-isopropyl)-amiloride), two ENaC inhibitors. Altogether, these results suggest that epithelial Na^+ channels (ENaCs) are present in mouse (Hernández-González et al., 2006) and human (Kong et al., 2009) sperm and that they may contribute to the capacitation-associated hyperpolarization.

3.1.1. ENaC regulation by Cystic Fibrosis Transmembrane conductance Regulator (CFTR)

Capacitation is associated with an elevation of intracellular cAMP levels. It is noteworthy that elevated cAMP levels inhibit ENaCs in other cell types. This inhibition can be caused by a cAMP-dependent activation of the cystic fibrosis transmembrane regulator (CFTR), either through direct stimulation involving a nucleotide binding domain of CFTR (Schreiber et al., 1999) or through increase of intracellular Cl^- concentration (König et al., 2001).

CFTR is an ATP-gated cAMP-modulated Cl^- and HCO_3^- channel with a modest permeability ratio ($P_{HCO_3^-}/P_{Cl^-} \sim 0.25$) (Tang et al., 2009), and a regulator of several transporters and proteins, including K^+ channels, such as ROMK1 and ROMK2 (Kunzelmann and Schreiber, 1999), anion exchangers, aquaporins, and ENaCs. Cystic fibrosis, the most prevalent human genetic disease, is caused by CFTR mutations (Guggino and Stanton, 2006). Additionally, the involvement of CFTR in male and female infertility has long been recognized (Jarzabek et al., 2004). Nearly all men with cystic fibrosis are infertile due to a congenital bilateral absence of the vas deference. In addition, the higher incidence of CFTR mutations in a male infertile subpopulation may indicate its participation in other fertilization related events like sperm capacitation (Schulz et al., 2006).

CFTR mRNA was detected in rat testis and in spermatogenic cells (Gong et al., 2001). In 2007, Hernandez-Gonzalez et al. (2007) documented the presence of CFTR in both mouse and human sperm and demonstrated that diphenylamine-2-carboxylic acid (DPC), an inhibitor of CFTR, blocks the capacitation-associated hyperpolarization. Moreover, genistein, known to activate CFTR channels, hyperpolarized sperm under conditions that do not support capacitation. These genistein-induced changes in *Em* were inhibited by DPC (Hernandez-Gonzalez et al., 2007). Electrophysiological evidence indicates that functional CFTR channels are present in mouse sperm (Figueiras-Fierro et al., 2013). Moreover, sperm from CFTR-mutated heterozygous mice have reduced fertilizing capacity in vitro and in vivo, implying an important role of CFTR in determining sperm-fertilizing ability (Xu et al., 2007) and consistent with the fact that inhibition of CFTR impairs the ability of sperm to undergo increases in intracellular pH (see below, Section 5.2) (Xu et al., 2007) and membrane hyperpolarization (Hernandez-Gonzalez et al., 2007). It was reported that human sperm treated with a specific CFTR inhibitor reduces the percentage of sperm undergoing progesterone-induced acrosome reaction, hyperactivation and penetration of zona-free hamster eggs (Li et al., 2010).

Genistein, the CFTR activator, diminished the amiloride-induced hyperpolarization and the amiloride-sensitive Na^+ permeability in non-capacitated sperm suggesting that CFTR regulates the capacitation-associated hyperpolarization in mouse sperm through the inhibition of ENaCs. Both CFTR and ENaC are found in the mid-piece and head of the sperm. Although there is no data implying their physical interaction, their localization in the same sperm region is consistent with the hypothesis that ENaC may be regulated by CFTR (Hernandez-Gonzalez et al., 2007; Xu et al., 2007).

Although all the aforementioned results indicate that the closure of Na^+ channels after capacitation might have a larger influence on hyperpolarization, Chavez et al. measured no significant decrease in the Na^+ permeability of mouse sperm in capacitating conditions (0.13 in non-cap and 0.13 in cap) and only a slight decrease when

hyperpolarization is elicited by pH 8 external media (from 0.15 to 0.12) (Chávez et al., 2013). A small decline in P_{Na} after alkalization might be expected since amiloride-sensitive Na^+ channels have been reported to be sensitive to external pH (Chen et al., 2009). On the other hand, the P_K exhibits a significant increase when incubated in capacitating conditions (1.6 in non-cap and 4.38 in cap) or in a pH 8 external media (from 1.6 to 8.2). These results show the primacy of K^+ channels in hyperpolarization, since the permeability changes reported account for approximately 95% of membrane potential changes, where P_K increases ~5 times (Chávez et al., 2013).

3.2. SLO potassium channel

Spermatogenic cells and mature spermatozoa have been reported to possess Ca^{2+}-activated K^+ channels (Chan et al., 1998), voltage-gated K^+ channels (Felix et al., 2002; Salvatore et al., 1999), two-pore domain K^+ channels (Hur et al., 2009), inwardly rectifying K^+ (Kir) channels (Muñoz-Garay et al., 2001) and SLO K^+ channels (Navarro et al., 2007; Santi et al., 2010; Schreiber et al., 1998).

The SLO or BK (Big Potassium) channels, originally cloned from *Drosophila* (Atkinson et al., 1991; Elkins et al., 1986), are characterized by their large conductance for potassium ions (K^+) through cell membranes. Opening of BK channels allows K^+ to passively flow through the channel, down the electrochemical gradient. Under typical physiological conditions, this results in an efflux of K^+ from the cell, which leads to cell membrane hyperpolarization. The Slo gene family is represented by Slo1, Slo2, and Slo3 (Gutman et al., 2005). These channels possess seven transmembrane helices S0–S6, with the S1–S6 helices exhibiting homology to classic voltage-gated K^+ channels; the K^+-selective pore is formed by S5 and S6 (Adelman et al., 1992; Butler et al., 1993). The pore-forming α subunits of SLO channels are associated with auxiliary β and γ subunits (Behrens et al., 2000; Brenner et al., 2000; Uebele et al., 2000), which interact with the S0 segment of the α subunit. Several studies demonstrated that the association with different subunits impacts channel pharmacological and gating properties. There are four α subunits, where each type can form a functional tetramer (Salkoff et al., 2006) and four different β subunits that regulate the function and expression of SLO channels (Yang et al., 2009).

The family of SLO channels evolved from voltage-dependent potassium channels, but acquired a large conserved carboxyl extension, called the gating ring, which allows channel gating to be altered in response to direct sensing of different intracellular ions, and by other second-messenger systems. The α subunit has the large cytosolic C-terminal proposed to be responsible for ligand regulation. A specific cytosolic ion regulates each of the SLO members: Ca^{2+} for SLO1, Na^+ for both SLO2.1 (Slick) and SLO2.2 (Slack), and H^+ for SLO3 (reviewed in Salkoff et al., 2006).

SLO1 is conserved in *Drosophila*, *C. elegans* and mammals. Slo1 channels cloned from mouse and human show strong conservation of sequence and functional properties (Butler et al., 1993; Dworetzky et al., 1994). SLO1 channels are expressed in many tissues where voltage-gated calcium channels are present, such as in hippocampus (Hicks and Marrion, 1998), smooth muscle cells (Knaus et al., 1994) and adrenal chromaffin cells (Solaro and Lingle, 1992).

In contrast to other multigene voltage-gated K^+ channel families, SLO1 was for many years the sole functionally characterized representative of its family (Wei et al., 1996), until Schreiber et al. (1998) described mSLO3, a pH- and voltage-dependent SLO family member, which was thought to be exclusively expressed in mammalian sperm until Vicens et al. (2017) challenged this notion. Using a phylogenomic approach that involves comparative genomics and transcriptomic analyses, they found that the emergence of the Slo3 gene dates to the radiation of ancestral vertebrates and that it is conserved in birds and reptiles. However, to date, the role of SLO3 channels in non-mammalian sperm is not known (Vicens et al., 2017). SLO1 and SLO3 are homologues closely related in amino acid sequence, and, in fact, sequence analysis suggests that SLO3, which exists only in mammals, evolved from a duplication of the Slo1 gene, which is found across metazoans (Salkoff et al., 2006). The mSlo3 cDNA was isolated from a testis cDNA library based on its homology to mSlo1. A detailed comparison of mSLO3 and mSLO1 sequences implied two functional properties of mSLO3: 1) The absence of the "calcium bowl" (Schreiber and Salkoff, 1997) suggested that mSLO3 is activated by factors other than Ca^{2+}; and 2) A GFG motif in the K^+-selective pore, rather than the typical GYG, suggested differences in ionic selectivity between the two channels, rendering SLO3 only mildly sensitive to voltage-activation (Hartmann et al., 1991; Yool and Schwarz, 1991). When expressed in *Xenopus oocyte*, mSlo3 cRNA produced currents that were sensitive to both pH and voltage. This was demonstrated in observations of single channel behavior, macroscopic currents in the patch configuration, and whole-cell oocyte currents recorded in the two-electrode voltage clamp mode. Despite similarity in sequence of mSLO3 to mSLO1, mSLO3 is insensitive to calcium over a wide concentration range. Furthermore, they display a low selectivity for K^+ over Na^+ ($P_K/P_{Na} \sim 5$) in comparison with SLO1 channels ($P_K/P_{Na} > 50$) (Schreiber et al., 1998). The expression of mouse and human Slo3 transcripts is largely restricted to the testis, where it is expressed in the seminiferous tubules with the signal directly over developing spermatocytes (Schreiber et al., 1998).

By using whole-sperm and whole-flagellum current clamp, Navarro et al. (2007) found that a pHi-sensitive K^+ current (KSper) was responsible for changes of epididymal spermatozoan *Em*. Both putative SLO3 currents observed in sperm, and SLO3 currents recorded in heterologous oocyte expression are similarly activated by cAMP, blocked by 1 mM Ba^{2+} and high tetraethylamonium (TEA) concentration (60 mM), and activated by alkaline pHi (Martínez-López et al., 2009). The identity and importance of the SLO3 channel for murine sperm physiology was confirmed by recordings from Slo3-deficient mice that also display severely reduced male fertility where these currents are absent (Santi et al., 2010; Zeng et al., 2011). Sperm from Slo3 null mice are unable to swim progressively, to hyperpolarize and to undergo the acrosome reaction. Remarkably, these sperm cannot acrosome react even when exposed to Ca^{2+} ionophore A23187. These results show the importance of SLO3 channel activation in the capacitation-associated processes necessary for fertilization (Santi et al., 2010). In murine sperm, SLO3 mediates a voltage- and alkalization-activated K^+ current essential for fertility. However, while KSper is activated by alkalization in the range of pH 6.4–7.2 at membrane potentials between −50 and 0 mV, the heterologously expressed SLO3 channels are largely closed at potentials negative to 0 mV at physiological pH. The auxiliary subunit LRRC52 (leucine-rich repeat-containing 52), shifts SLO3 gating into a range of voltages and pH values similar to that producing KSper current activation. LRRC52 protein is detected only in testis. It is markedly diminished from $Slo3^{-/-}$ testis and completely absent from $Slo3^{-/-}$ sperm, indicating that LRRC52 expression is critically dependent on the presence of SLO3 (Yang et al., 2011). LRRC52 is a homolog of the SLO1-modifying LRRC26, which has been shown to markedly shift gating of SLO1 in prostate tumor cells, even in the absence of Ca^{2+} (Yan and Aldrich, 2010). These accessory proteins belong to an extracellular leucine-rich-repeat-only (Elron) cluster, which includes other LRRC proteins: LRRC38, LRRC55, LRTM1 and LRTM2. Elron cluster members are all predicted to contain a single transmembrane segment with an N-terminal signal peptide resulting in extracellular localization of the LRR domain and a short cytoplasmic C-terminal tail containing a short stretch of acidic residues. Despite shared organization of leucine-rich-repeats among Elron family LRRC proteins, additional amino acid homology is modest. However, their common structural organization suggests that they may share structurally similar interaction partners (Yang et al., 2011).

Human SLO3 (hSLO3) also functions as a pH-gated channel when expressed in *Xenopus* oocytes, as determined by inside-out patch-clamp electrophysiology experiments, in which the intracellular solution can

be precisely controlled (Leonetti et al., 2012). hSLO3 currents open in the same range of pH as mSLO3. However, upon co-expression with the associated subunit LRRC52, the pH dependences of mSLO3 and hSLO3 differ markedly: hSLO3 + LRRC52 currents show significant activation at pH < 6.7, a pH at which mSLO3 + LRRC52 activity is essentially suppressed (Leonetti et al., 2012). However, the K^+ current in human sperm is much less sensitive to changes in intracellular pH than the K^+ current of mouse sperm (Lishko et al., 2012). This led to an uncertainty about hSLO3 being responsible for K^+ currents in human sperm. In this regard, Mannowetz et al. (2013) by applying the patch-clamp technique to ejaculated and epididymal human sperm, found that human K^+ currents, originating from the flagellum, are insensitive to intracellular alkalinization, and dependent on intracellular [Ca^{2+}]. Correspondingly, *Em* is strongly regulated by Ca^{2+} and less so by pHi. On the other hand, the hKSper is inhibited by three known SLO1 channel inhibitors, as well as by micromolar concentrations of progesterone. These results indicated that the molecular identity of human KSper was distinct from that of murine KSper, which is represented by the SLO3 protein, and that SLO1 was the principal potassium channel in human sperm (Mannowetz et al., 2013). However, Brenker et al. (2014) refuted this idea, identifying the SLO3 and LRRC52 proteins in the flagellum of human sperm and showing that SLO3 inhibitors, but not SLO1 inhibitors, suppressed human KSper. López-González et al. (2014), also found that human sperm hyperpolarization is partially sensitive to diverse K^+ channels blockers, suggesting that two or more K^+ channels, including SLO3, are involved in it. The pharmacological profile comparison of capacitation-associated hyperpolarization and heterologously expressed hSLO3 in CHO cells indicates the involvement of other K^+ channels, possibly hSLO1, in addition to hSLO3 (Sánchez-Carranza et al., 2015). Moreover, human KSper and heterologously expressed hSLO3 currents shared similar biophysical properties, pharmacology, and ligand dependence, showing that activation of human SLO3 is regulated by $[Ca^{2+}]_i$ and also, more weakly, by cytosolic alkalization (Brenker et al., 2014). This is unconventional because, as stated above, SLO channels are defined based on their gating properties: SLO3 is pH-gated, SLO2 is Na^+-gated and SLO1 is gated by Ca^{2+}. Besides, the crystal structure of human SLO3 indicated the channel lacks a calcium-sensitive bowl (Leonetti et al., 2012). A conceivable explanation could be that hKSper consists of a heteromer of SLO1 and SLO3 subunits (Mansell et al., 2014; Sánchez-Carranza et al., 2015). Another possibility could be that hSLO3 is rapidly evolving, and that it acquired Ca^{2+} sensitivity. Geng et al. (2017) analyzed Slo3 sequence conservation within the Exome Aggregation Consortium (a database of > 64,000 individuals), and identified a conspicuous non-synonymous single nucleotide polymorphism that maps to the site of interaction between the gating ring and the pore gate domain. The biophysical characterization of this variant showed that it confers heightened sensitivity to both Ca^{2+} and pHi relative to the wild-type hSLO3 channel (Geng et al., 2017).

4. The importance of K^+ channels in fertility

Since Slo3 or Lrrc52 null mice have markedly reduced fertility and sperm from these animals show functional impairments (Santi et al., 2010; Zeng et al., 2015, 2011), it is inferred that the malfunction of K^+ channels in human spermatozoa might also contribute significantly to the occurrence of subfertility in men. In order to determine the importance of K^+ channels in human fertility, Brown et al. (2016) used whole-cell patch clamp electrophysiology to assess the biophysical characteristics of spermatozoa from semen samples provided by men undergoing fertility treatments and healthy donors. In ~10% of patients there was either a negligible outward conductance or an enhanced inward current, both of which caused depolarization of *Em*. Analysis of clinical data from the IVF patients showed significant association of depolarized *Em* with low fertilization rate (Brown et al., 2016).

5. Capacitation-associated events related to *Em* hyperpolarization

5.1. Protein phosphorylation

One of the first events of the capacitation-signaling pathway is the HCO_3^--dependent stimulation of cAMP synthesis, which activates PKA (Buffone et al., 2014). In mice sperm, PKA activation appears to be involved in *Em* hyperpolarization since the percentage of sperm undergoing membrane hyperpolarization increased as a function of HCO_3^- concentration and sperm incubation with increasing concentrations of the PKA inhibitor H89 significantly reduced the percentage of sperm membrane hyperpolarization (Escoffier et al., 2015). These results are consistent with the hypothesis that sperm hyperpolarization is downstream of a cAMP/PKA signaling pathway. However, the activation kinetics of PKA (~1 min) and hyperpolarization (~30 min) suggest that the role of PKA in the regulation of hyperpolarization is indirect. In this regard, Stival et al. (2015) showed that Src kinase (Krapf et al., 2012) is a connecting player between PKA activation and hyperpolarization in mouse sperm. Inhibition of Src activity blocks the capacitation-induced hyperpolarization of the sperm plasma membrane, as well as the ability of progesterone to induce the acrosome reaction, without blocking the increase in tyrosine phosphorylation. However, the acrosome reaction responsiveness was rescued by pharmacologically inducing hyperpolarization with the K^+ ionophore Valinomycin. In addition, Src inhibition decreases mSLO3 currents when heterologously expressed in *Xenopus* oocytes (Stival et al., 2015). Consistently, in human (Varano et al., 2008), bovine (Etkovitz et al., 2009) and porcine sperm (Bragado et al., 2012), Src was shown to be involved in the acquisition of acrosomal responsiveness, its inhibition completely blocked the progesterone-induced acrosome reaction. Furthermore, the SLO3 homolog SLO1 is known to be regulated by Src activation in other cell types, through phosphorylation of a tyrosine located in the highly conserved carboxyl-terminus of the channel (Ling et al., 2000). Altogether, these results support the hypothesis that Src is involved in the regulation of *Em* downstream of PKA activation.

5.2. Intracellular pH

Regulation of sperm pHi is fundamental for capacitation, being so for both hyperactivation and the acrosome reaction. Even the basal sperm motility is pH-sensitive since dynein's ability to hydrolyze ATP and provide axonemal bending greatly increases with the rise of pHi. The motile sperm flagellum constantly generates intracellular protons via glycolysis, ATP hydrolysis and proton/calcium exchange (Lishko et al., 2012). The faster a flagellum moves, the more acidic it becomes. Babcock et al. (1983) suggested that the mechanism for proton efflux from bovine sperm was via a voltage-gated proton channel, based on the fact that the sperm cytosol becomes alkaline upon membrane depolarization (Babcock et al., 1983). On the basis of experiments with pHi-sensitive fluorescent probes that detect changes in pHi, the NHE (Garcia and Meizel, 1999) and a Na^+-dependent Cl^-/HCO_3^- exchanger (Zeng et al., 1996) were proposed to participate in sperm alkalinization. The Na^+/H^+ exchangers (NHE), involved in pH homeostasis of the cytosol and intracellular organelles, are encoded by the SLC9 gene family that comprises nine members in mammals (Donowitz et al., 2013; Orlowski and Grinstein, 2011). These can be divided in two subfamilies: a plasma membrane subfamily including NHE1–5 and an organellar subfamily comprising NHE6–9. Sperm express a specific member of the mammalian NHE superfamily of Na^+/H^+ exchangers, sNHE (Wang et al., 2003), which is localized in the principal piece of the sperm flagellum and predicted to have 14 membrane-spanning helices. sNHE contains a nucleotide-binding domain close to its intracellular C-terminus and four putative transmembrane helices analogous to the voltage sensor of voltage-dependent channels. These characteristics suggest that sNHE may be regulated by cyclic nucleotides and *Em* (Wang et al., 2007). $sNHE^{-/-}$ mice were completely

infertile due to impaired motility; however, they also had unexpectedly low expression of soluble adenylate cyclase (sACY) (Wang et al., 2003). Since the motility could be rescued by application of membrane permeable cAMP analogues, it is likely that the absence of sACY was the cause of the infertile sNHE$^{-/-}$ phenotype (Wang et al., 2007). Recently, Oberheide et al. (2017) reported that mice lacking the Golgi-localized NHE8 globally or only in germ cells produce round-headed spermatozoa with no acrosomal cap, abnormal mitochondrial distribution and decreased motility, resulting in male infertility. This resembles human globozoospermia, which likewise is associated with infertility (Oberheide et al., 2017).

The proton-selective, voltage-gated ion channel Hv1 (HVCN1) was cloned in 2006. This channel is composed of a voltage sensor domain homologous to that of voltage-gated cation channels, and is activated by the combination of the pH gradient and membrane depolarization (Ramsey et al., 2010, 2006). In addition, it is proposed that Hv1 may also be activated by the removal of zinc during sperm passage through the female genital tract and by encountering anandamide during sperm penetration through the cumulus oophorus (Lishko et al., 2010; Lishko and Kirichok, 2010). In 2010, direct electrophysiological recordings of human sperm revealed a large voltage-activated outwardly rectifying H^+ current (HSper) highly H^+ selective. Hv1 is abundantly expressed in human sperm cells within the principal piece of the sperm flagellum, making it ideally positioned to activate pH-dependent proteins of the axoneme and thus to control sperm motility (Lishko et al., 2010). In contrast to human sperm, mouse spermatozoa do not possess Hv1, and, not surprisingly, Hv1$^{-/-}$ mice are fertile. Furthermore, electrophysiological recordings assessing rat and bovine spermatozoa have indicated that these animals do not possess an Hv1 current. It would appear that humans are unique in this respect (Miller et al., 2015). In species that lack sperm Hv1, it is still unclear how intracellular pH is regulated, though sNHE remains an attractive mechanism. Unfortunately, as sNHE is suggested to be electro-neutral (no overall net charge), traditional electrophysiological techniques cannot be implemented to investigate if this is in fact the alkalizing mechanism (Miller et al., 2015).

5.3. Calcium influx and hyperactivation

Calcium signaling is essential for all cell types. In spermatozoa, swimming behavior is controlled by a propagation of a Ca^{2+}-induced wave that changes flagellar beat pattern. This asymmetrical, whip-like bending of the flagellum, commonly referred to as hyperactivation, is essential for mammalian sperm to overcome the protective vestments of the oocyte (Suarez et al., 1993; White and Aitken, 1989). Apart from its fundamental role in motility and phosphorylation signaling pathways (Navarrete et al., 2015), Ca^{2+} is also required for initiation of the acrosome reaction (Lucchesi et al., 2016).

5.3.1. Cav channels

Before 2001, the channel responsible for sperm Ca^{2+} elevation was believed to be a voltage-gated Ca^{2+} channel (Cav). These channels convert *Em* changes into Ca^{2+} signals and are present in several cell types. Electrophysiological evidence has revealed the functional presence of Cav3 channels in mouse and human spermatogenic cells (Lievano et al., 1996; Publicover and Barratt, 1999) and in mouse testicular sperm (Arnoult et al., 1996). Weak depolarizations can open Cav3 channels, while high depolarizations are needed to open Cav1 and Cav2 isoforms (Catterall and Few, 2008). However, data from several experiments challenge the Cav-based models in mammalian sperm. First, genetic ablation studies of Cav2.2, Cav3.1, and Cav3.2 suggested that these proteins were not necessary for male fertility or that some compensatory mechanism for their loss may be present (Escoffier et al., 2007; Ino et al., 2001; Saegusa et al., 2000). Second, whole-cell patch-clamp recordings using non-capacitated mouse corpus epididymal sperm showed that sperm do not have detectable Cav currents. Although Cav currents could be detected in spermatocytes, they gradually disappear along spermatogenesis to finally become undetectable in mature sperm (Ren and Xia, 2010). On the other hand, Cohen et al. (2014), recently explained this undetected Cav current as a consequence of the maturational state of the sperm analyzed plus technical limitations. When they used a mouse model null for the α_{1E} subunit of Cav2.3, they observed subfertility in natural matings and severe defects in acrosome reaction and in vitro fertilization of zona-intact oocytes. Removal of the ZP from the oocytes resulted in complete rescue of the phenotype, providing evidence that the defect was in sperm-egg interactions, consistent with a physiologic failure in acrosome reaction and/or penetration of the ZP. Furthermore, sperm lacking α_{1E} subunit showed altered Ca^{2+} responses (Cohen et al., 2014).

5.3.2. CatSper channel

The molecular identity of the first sperm-specific ion channel required for male fertility in mice was determined in 2001 with the cloning of CatSper1 (Ren et al., 2001). The first member of the CatSper family of genes, CatSper1 is the pore forming α subunit of the Cation channel of Sperm (CatSper). Since then, nine CatSper subunits composing the heteromeric CatSper channel have been identified, and at least five of them—CatSper1–4 and CatSperδ—have been shown to be indispensable for proper channel formation and function (Carlson et al., 2005; Chung et al., 2017, 2011; Liu et al., 2007; Lobley et al., 2003; Qi et al., 2007; Quill et al., 2003; Wang et al., 2009). CatSper's pore is formed by four α subunits (CatSper 1–4) and contains five auxiliary subunits (CatSper β, CatSper γ, CatSper δ, CatSper ε and CatSper ζ). All subunits are sperm-specific proteins located in the principal piece of the sperm flagellum and they express interdependently. Humans with mutations within the CatSper1 or CatSper2 genes were also shown to be infertile (Avidan et al., 2003; Hildebrand et al., 2010). The CatSper channel forms a quadrilateral arrangement in three dimensions that organizes structurally distinct Ca^{2+} signaling domains along the flagella, which focus protein phosphorylation in time and space and are indispensable for hyperactivated motility (Chung et al., 2014).

CatSper activity was directly recorded by patch-clamp in mice (Kirichok et al., 2006) and human (Lishko et al., 2010) sperm, establishing CatSper as the principal Ca^{2+} channel in both cells. The characteristic current (ICatSper) is lost in mice lacking any of the α subunits (CatSper 1–4) or the accessory subunit δ and, as previously mentioned, are all infertile due to an inability of spermatozoa to hyperactivate resulting in failure of sperm to pass into the oviduct to reach the egg (Miller et al., 2015). CatSper current is weakly voltage-dependent with a slope factor (k) of 30 in mice and 20 in human, where true voltage-gated ion channels must have much steeper $k \sim 4$, and potently activated by intracellular alkalinization. This weak voltage dependency is thought to be the result of heterogeneity of the arginine and lysine composition of the putative voltage sensor domains within the CatSper α subunits. Importantly, the $V_{1/2}$ (the voltage at which half of the channels are activated) of human CatSper is +85 mV versus +11 mV of mouse CatSper at the same pHi (pHi = 7.5) (Kirichok et al., 2006), leading to the question of how human CatSper might be activated at such high membrane potentials. Progesterone, a major steroid hormone released by the ovaries and the cumulus cells surrounding the egg, induces robust Ca^{2+} influx into both human and mouse sperm cells (Blackmore et al., 1990; Romarowski et al., 2016; Thomas and Meizel, 1989). However, progesterone (P4) activates human CatSper, but not murine, at low concentrations by shifting the voltage dependency of the human CatSper channel into the physiological range (Lishko et al., 2011). In human sperm, the alpha/beta hydrolase domain containing protein 2 (ABHD2) serves as the progesterone flagellar receptor. It functions as a lipid hydrolase by removing endogenous CatSper inhibitors, endocannabinoids 1AG/2AG, upon association with P4 (Miller et al., 2016). Interestingly, ABHD2 expression was also detected in mouse epididymal sperm cells in spite of murine CatSper being insensitive to P4. Murine and human ABHD2 proteins share a high degree

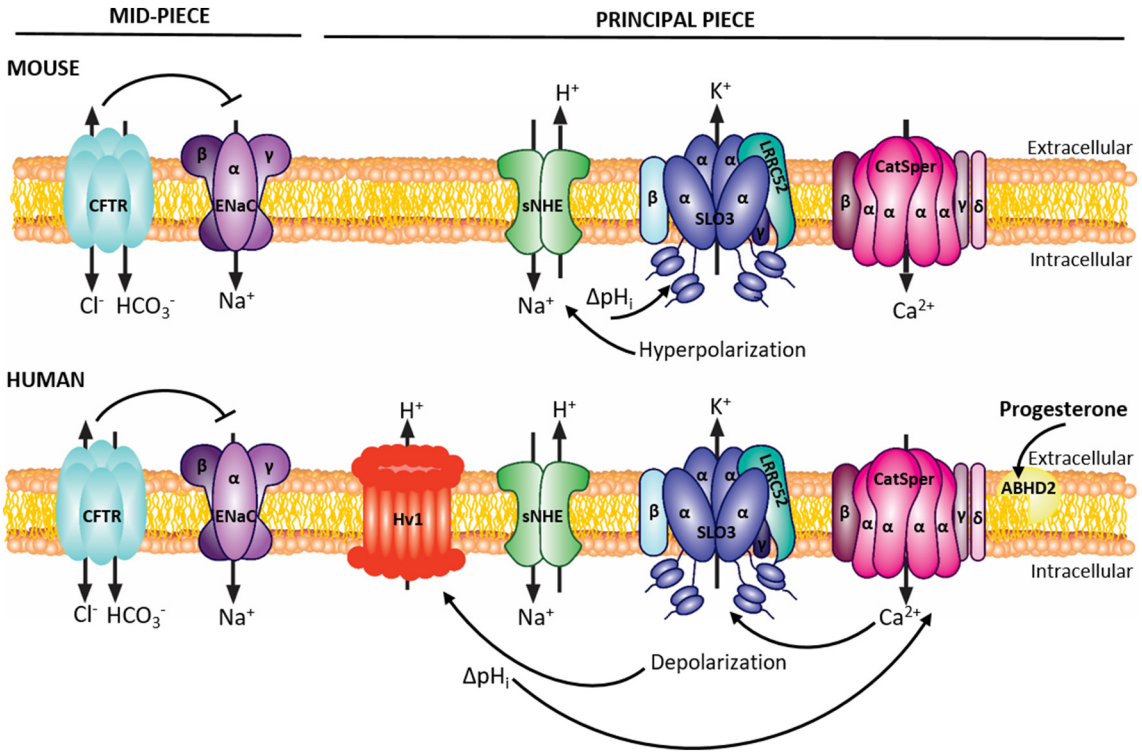

Fig. 3. Schematic model representing sperm channels involved in membrane potential regulation in mouse and human sperm. The model shows the channels involved in the determination of the membrane potential and summarizes their regulation as well as mechanisms involved, as described in the text. Arrows inside channels indicate the direction of ion movement. The connecting arrows indicate inhibition (bar-headed) or activation (arrow-headed). See text for details.

of sequence similarity, but they have different subcellular localizations, which could explain the absence of CatSper P4 sensitivity. While in human sperm, ABHD2 locates in the flagellum where it co-localizes with CatSper, in murine sperm it is restricted to the acrosomal region, and is absent from the flagellum (Miller et al., 2016).

In order to reveal the role of SLO3 channels in activating CatSper channels, Chávez et al. (2014) showed that the Ca^{2+} increase that fails to occur in capacitated *Slo3*$^{-/-}$ mutant sperm in response to high KCl, can be restored by two different experimental treatments: 1) artificial hyperpolarization using the K^+-ionophore valinomycin, and 2) increasing extracellular pH, which results in an increase in intracellular pH. Therefore, in mice, SLO3 channels activate CatSper channels indirectly by promoting a rise in intracellular pH through a voltage-dependent mechanism. Since the treatment of sperm with valinomycin to achieve hyperpolarization in itself elevates internal pH, a plausible hypothesis is that hyperpolarization per se, which increases the inward driving force of Na^+, is contributing to intracellular alkalization, possibly by augmenting the efficiency of H^+ export by sNHE and/or bicarbonate transport (Chávez et al., 2014).

5.4. Acrosome reaction and changes in Em

The acrosome reaction (AR) is the fusion between the outer acrosomal membrane and the overlying plasma membrane, at multiple points, resulting in vesiculation and loss of the fused outer acrosomal membrane/plasma membrane. This allows the acrosomal content to be released and the inner acrosomal membrane becomes the new cell surface. Membrane fusion proteins from the SNARE family are present in the acrosomal region and may be integrated into microdomains that facilitate Ca^{2+}-regulated membrane fusion (Belmonte et al., 2016; Correia et al., 2015; Mayorga et al., 2007). The release of acrosomal content at the surface of the zona may, in combination with hyperactivated motility, facilitate zona penetration. However, observation of mouse IVF using sperm with GFP-labelled acrosomes showed that, in addition to cells that undergo AR at the surface of the zona, sperm which arrive having already lost their acrosome may go on to penetrate the zona and fertilize (Jin et al., 2011). In this regard, hyperpolarization was proven to be necessary and sufficient for agonist-induced AR (De La Vega-Beltran et al., 2012). Considering that the Em from a capacitated sperm population is typically taken as the average between two subpopulations, the status of the acrosome in these subpopulations was evaluated using genetically modified mice that carry Acr-GFP in their sperm acrosomes and lose it after the AR. Acrosome-reacted sperm are composed almost exclusively of the depolarized population, and that almost all hyperpolarized sperm have an intact acrosome (Escoffier et al., 2015).

6. Concluding remarks

Fig. 3 shows a schematic model representing all the sperm channels discussed in this review that are involved in membrane potential regulation. Despite all the above-cited work, the mechanisms involved in *Em* hyperpolarization regulation of acquisition of acrosomal responsiveness are still poorly understood. Although there is no doubt that the two main physiological hallmarks of capacitation are hyperactivation and acrosomal responsiveness, whether these processes are completely independent or somehow coordinately regulated is still known. In the present review we analyzed evidence that points towards a signaling flow where changes in the flagellum originate acrosomal responsiveness in the head. These changes in the flagellum correspond to changes of *Em* that would propagate to the head. However, how this *Em* shifts in the flagellum signals the preparation for the acrosome reaction is an unanswered question. On one hand, the answer could be found in the role of *Em* changes on inactive/active switching state of T Ca^{2+} channels (Arnoult et al., 1999). Another possibility relates to favoring a closed primed state of the outer acrosomal membrane to the plasma membrane as seen using electron microscopy (Zanetti and Mayorga, 2009). A third unexplored possibility could reside in actin dynamics as

a result of *Em* fluctuations. There is a clear need of work to be done in order to answer these unsolved pathways. Added to this complexity, conservation of pathways among species seems nowadays more the exception than the rule, when talking about sperm capacitation. New technical advances such as super resolution microscopy (Alvau et al., 2016; Chung et al., 2014), image-based flow cytometry (Matamoros-Volante et al., 2017) and genome editing techniques (Abbasi et al., 2017) promise exciting developments on our knowledge of how these pathways interplay to achieve capacitation.

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