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## Intracellular pH in Sperm Physiology

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### Abstract

Intracellular pH (pHi) regulation is essential for cell function. Notably, several unique sperm ion transporters and enzymes whose elimination causes infertility are either pHi dependent or somehow related to pHi regulation. Amongst them are: CatSper, a Ca<sup>2+</sup> channel; Slo3, a K<sup>+</sup> channel; the sperm-specific Na<sup>+</sup>/H<sup>+</sup> exchanger and the soluble adenylyl cyclase. It is thus clear that pHi regulation is of the utmost importance for sperm physiology. This review briefly summarizes the key components involved in pHi regulation, their characteristics and participation in fundamental sperm functions such as motility, maturation and the acrosome reaction.

### Keywords

sperm; intracellular pH; sperm motility; sperm chemotaxis

## INTRODUCTION

Maintaining intracellular pH (cytoplasmic pH or pHi), within physiological boundaries is fundamental for cell function. Most cellular processes are influenced and operate within a restricted pHi range. Changes in pHi affect the ionization state of weak acids and bases which are present in most proteins and many biomolecules [1]. Therefore, as metabolic activity acidifies the cytosol and cells can be exposed to a varying external pH (pHe), lack of pHi regulation can have severe functional consequences. In general, cells finely tune their pHi employing several dynamic mechanisms whose balance in terms of production, elimination, transport and buffering of H<sup>+</sup> determine its value at a certain time. Cells slow down or speed up the activity of a wide range of plasma membrane ion transporters that move acids and/or bases [2]. As anticipated, HCO<sub>3</sub><sup>-</sup> and H<sup>+</sup> transporters are amongst the key players in pHi regulation.

Cells can exhibit drastic changes in transit from a dormant to an active state, a condition which often entails a sudden pH change. Spermatozoa (sperm) are a clear example as they

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are exposed dramatic changes in the ionic composition of their surroundings during their journey towards fertilizing the egg. Mammalian sperm encounter an acidic media with a low  $\text{HCO}_3^-$  concentration in the epididymis, which keeps them quiescent, while in the female uterus and oviduct the luminal fluid contains a high  $\text{HCO}_3^-$  concentration and becomes increasingly alkaline, a condition required for fertilization (reviewed in [3]). It is worth noting that several unique sperm ion transporters and enzymes, whose elimination cause infertility, are either pHi dependent or somehow related to pHi regulation. Amongst them are: CatSper, a  $\text{Ca}^{2+}$  channel [4]; Slo3, a  $\text{K}^+$  channel [5]; the sperm specific  $\text{Na}^+/\text{H}^+$  exchanger [6] and the soluble adenylyl cyclase [7], [8] (Fig. 1). Thus, pHi regulation is of the utmost importance for sperm. Numerous strategies have evolved in the last 30 years to measure pHi. Their advantages and limitations are being continuously revised. These techniques involve determinations in cell populations or in single cells and include: 1)  $^{31}\text{P}$  nuclear magnetic resonance (NMR), 2) weak acid-base distribution, and 3) optical absorbance and fluorescence techniques [9], [10]. In the last years pHi is being studied in sperm mostly using fluorescent probes. Here we will attempt to give a summarized critical panorama of the field with focus on the main molecular players of pHi regulation in sperm and on the functions where they participate.

### Key molecules involved in sperm pHi regulation

The main ion transporters in charge of pHi regulation can be arranged into two groups: 1) membrane  $\text{H}^+$  transporters and 2)  $\text{HCO}_3^-$  transporters. The first group includes  $\text{Na}^+/\text{H}^+$  exchangers of the solute carrier 9 family (SLC9) [11] and  $\text{H}^+$  channels [12], [13]. The second group is constituted by the  $\text{HCO}_3^-$  transporters of the SLC4 [14], [15], SLC26 families [16]. and the cystic fibrosis transmembrane conductance regulator (CFTR) is able to conduct  $\text{HCO}_3^-$  [17]–[21]. As carbonic anhydrases (CAs) catalyze  $\text{CO}_2/\text{HCO}_3^-$  equilibrium, they are also considered.

**Soluble Adenylyl Cyclase**—The discovery of soluble adenylyl cyclase (sAC) has allowed a better understanding of how cells locally produce the fundamental second messenger cAMP. Additionally, as  $\text{HCO}_3^-$  directly regulates sAC, this enzyme is able to translate  $\text{CO}_2/\text{HCO}_3^-/\text{pH}$  changes into cAMP levels. Furthermore, the sperm specific  $\text{Na}^+/\text{H}^+$  exchanger described below has a cAMP binding site and forms a stable complex with sAC thus it is intimately related to cAMP metabolism (reviewed in [22]).

Unlike transmembrane ACs (tmACs), sAC is insensitive to forskolin (independent of G-protein) and activated by  $\text{HCO}_3^-$  [23] and  $\text{Ca}^{2+}$  [24]. In 1999, the primary structure of sAC from rat was determined, which lacks transmembrane segments [25]. Curiously, the catalytic domains of sAC are more similar to those of cyanobacteria ACs than to those found in eukaryotic tmACs. As anticipated, the AC activity of cyanobacteria is also up-regulated by  $\text{HCO}_3^-$  [26].

A careful study of rat and mouse testis mRNA revealed that there are two alternative splicing products, which independently encode  $\text{sAC}_t$  and  $\text{sAC}_f$  [27]. It has been reported that sAC is essential for sperm function since sAC-null male mice are infertile due to a severe defect in sperm motility [7], [8]. Although sAC gene is preferentially expressed in

testis, sAC<sub>t</sub> is also found in somatic cells. It is localized in cytoplasm and/or intracellular organelles such as mitochondria and considered to play a role to monitor CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>/pH [28].

**Na<sup>+</sup>/H<sup>+</sup> exchangers**—Na<sup>+</sup>/H<sup>+</sup> exchangers (NHEs), also known as Na<sup>+</sup>/H<sup>+</sup> antiporters (NHA), are integral membrane proteins that catalyze the exchange of Na<sup>+</sup> for H<sup>+</sup> across lipid bilayers and are ubiquitously distributed in almost all living organisms. They contribute to the basic homeostatic mechanisms that control pHi, cellular volume and intra-organelle pH [29]. In mammals, the SLC9 gene family encodes 13 evolutionarily conserved NHEs divided into three subgroups (SLC9A, SLC9B and SLC9C) [30]. The subfamily SLC9A encompasses nine isoforms (NHE1-9), the SLC9B subgroup consists of two isoforms (NHA1 and NHA2) and the SLC9C subgroup consists of a sperm-specific NHE (sNHE) and a putative NHE (NHE11). It has been reported that mammalian spermatozoa express four isoforms of NHEs, NHE1 (SLC9A1), NHE5 (SLC9A5) [31], [32], sNHE (SLC9C1) [6] and the testis-specific NHE (NHA1/SLC9B1) [33].

In general, NHEs are composed of 12 transmembrane segments which catalyze the ion exchange and a cytoplasmic carboxyl terminal regulatory domain which interacts with several proteins including cytoskeleton-associated components, kinases and Ca<sup>2+</sup> binding proteins [34]. In contrast, the sNHE is constituted by 14 putative transmembrane segments and a long cytoplasmic carboxyl terminus [6]. The sNHE lacks the first two transmembrane segments found in the other NHEs; instead it has 4 subsequent extra transmembrane segments, which are structurally similar to the voltage sensor domain found in voltage-gated ion channels [6]. This is of particular interest given that sea urchin spermatozoa have a voltage-dependent NHE in the flagellar plasma membrane [35], [36]. In addition, the sNHE has a putative cyclic nucleotide binding consensus domain at the carboxyl terminus [6].

Although NHE1 was found in human and rat spermatozoa and NHE5 in rat spermatozoa [31], [32], the physiological roles of these NHEs are unknown. On the other hand, sNHE was demonstrated to be essential for sperm function since its elimination causes infertility in male mice due to a severe defect on sperm motility [6]. Intracellular alkalization by NH<sub>4</sub>Cl partially recovered sperm motility of sNHE-null mice. In contrast, addition of membrane-permeable cAMP analogues almost fully recovered the sperm motility of the mutant sperm [6]. A careful study of sperm from sNHE-null mice revealed that their sAC activity was highly diminished [37]. Notably, immunoblot analysis indicated that sAC<sub>fl</sub> is missing in this mutant although sAC<sub>t</sub> is apparently not affected. Further experiments suggested that sNHE is physically associated with sAC and both proteins reciprocally stabilize their protein expression in HEK293 cells [37]. In addition, proteomic analysis of sea urchin sAC-associated proteins revealed that sNHE is one of the major sAC-associated proteins [38]. These experimental results suggest that sAC and sNHE form a functional complex. Nevertheless, the localization of sAC reported so far, the midpiece of sperm flagellum [8], does not correspond to the localization of sNHE, the principal piece of sperm flagellum [6]. This discrepancy could be explained by the specificity of the antibody used for sAC immunostaining, which detects preferentially sAC<sub>t</sub> [28]. If this is the case, sAC<sub>t</sub> and sAC<sub>fl</sub> would be differentially localized in mammalian spermatozoa.

The fact that sNHE possesses a predicted cyclic nucleotide binding domain (CNBD) together with the physical interaction between sNHE and sAC suggests that cAMP regulates the activity of sNHE. However, some proteins found in prokaryotes and eukaryotes have a structurally almost identical domain to the CNBD that does not interact with cyclic nucleotides (cyclic nucleotide binding homology domains, CNBHD) [39], [40]. Therefore, biochemical experiments are required to examine if sNHE indeed has a functional CNBD. Interestingly, human sNHE lacks an arginine residue present in most CNBDs, which plays a critical role for the interaction with the phosphate group of a cyclic nucleotide [40]. Mouse and sea urchin sperm conserve this arginine residing in each predicted CNBD of sNHE, suggesting some functional differences between human sNHE and those of mouse and sea urchin. Further investigations are required to clarify these issues.

The testis-specific NHE identified by Liu et al. in 2010 [33] is now classified into a new family of NHE, NHA1 (SLC9B1). NHA1 has 12 transmembrane segments as other NHEs, but lacks the long cytoplasmic C-terminal. Immunofluorescence demonstrated that NHA1 is localized in the principal piece of sperm flagellum in mouse spermatozoa. Furthermore, anti-NHEA1 antibody reduced sperm motility and the rate of *in vitro* fertilization. Therefore, NHA1 is proposed to regulate sperm motility. In order to define the physiological function of NHA1 in spermatozoa, it is desirable to analyze sperm function of NHA-1 knockout animals.

**Voltage gated H<sup>+</sup> channel**—In 2006, two groups independently identified a voltage-gated H<sup>+</sup> channel (H<sub>v</sub> channel) [41], [42], which is composed of 4 transmembrane segments structurally similar to the voltage sensor domain commonly found in other voltage-gated ion channels. This channel exists as a homodimer with two H<sup>+</sup> pores, since each subunit forms a H<sup>+</sup> permeable pore. The H<sub>v</sub> channel is activated by membrane potential depolarization and an outward H<sup>+</sup> gradient across the plasma membrane. In addition, unsaturated fatty acids such as arachidonic acid enhance the H<sub>v</sub> channel activity while Zn<sup>2+</sup> potently inhibits it [13].

Whole-cell patch-clamp recordings from human sperm revealed a relatively large voltage-dependent H<sup>+</sup> conductance [12]. Western blotting and immunostaining confirmed that sperm possess H<sub>v</sub> channels in the principal piece of the flagellum. The sperm H<sub>v</sub> channel conserves all the biophysical properties displayed in somatic cells or heterologous systems, including being potently inhibited by Zn<sup>2+</sup>. This last property is particularly important for sperm pH<sub>i</sub> regulation since it is known that human seminal fluid contains a millimolar range of Zn<sup>2+</sup>, which should completely block the H<sup>+</sup> conductance of the H<sub>v</sub> channel, and it may account for the action of Zn<sup>2+</sup> as a decapacitation factor [43]. In addition, sperm H<sub>v</sub> channels are activated by the endocannabinoid anandamide, which may explain part of its positive effects on mammalian reproduction [12].

Although human sperm display large H<sup>+</sup> currents (100 pA/pF at +100 mV), those of mouse sperm are quite small (<10 pA/pF) [12]. These results indicate that human and mouse sperm regulate their pH<sub>i</sub> differently.

**Carbonic anhydrases**—Besides the H<sup>+</sup> carriers and the HCO<sub>3</sub><sup>-</sup> transporters, the cells also depend on carbonic anhydrases (CAs) to properly regulate their pH<sub>i</sub>. CAs are

ubiquitous metalloenzymes (depending on the isoform, they require  $Zn^{2+}$ ,  $Fe^{2+}$ ,  $Co^{2+}$  or  $Cd^{2+}$  as cofactor) present in the three life domains (bacteria, archaea and eukarya). The principal function of CAs is to catalyze the reversible reaction of carbon dioxide hydration to bicarbonate and proton ( $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ ). CAs are encoded by five gene families without apparent evolutionary relationship:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\delta$ . The sixteen isoforms of  $\alpha$ CAs (ACI-ACXV) are the only CAs present in mammals showing distinct subcellular and tissue distribution, kinetic properties and sensitivity to inhibitors [44]. Despite the importance of CAs in the regulation of pHi in all living organisms, so far little information is available about their presence in mammalian sperm and even less is known about their function in the physiology of these cells (see Table 1).

The presence of different isoforms of  $\alpha$ CAs in mammalian spermatozoa suggests a possible role of these metalloenzymes throughout the development of male gametes or during the physiological events that occur prior to fertilization. In fact this seems to be the case at least for motility as a  $HCO_3^-$  intracellular increase, as well as a  $H^+$  decrease (both ions produced by the CAs' reaction) are fundamental to this process in mammalian sperm [45]. However, the available studies that suggest the direct participation of CAs in the balance of these ions during motility are still scarce. It has been shown that mouse and human spermatozoa increase their flagellar beat frequency when exposed to  $CO_2$  and this effect is inhibited by ethoxzolamide (EZA; a specific CAs inhibitor) [46], [47]. It was also found that  $CAIV^{-/-}$  mouse sperm display a decrease in the total activity of CAs as well as a decrease in their response to  $CO_2$  [46].

It is worth mentioning that in marine organisms the participation of CAs in sperm motility has also been studied. In flatfish spermatozoa a 29 kDa CA isoform (probably CAII, considering its aminoacid sequence) was detected in high amounts. Application of EZA strongly affected sperm motility [48]. On the other hand, in spermatozoa from a certain type of squid, the production of  $H^+$  by a plasma membrane-anchored CA (along with an intracellular increase of  $Ca^{2+}$ ) is crucial in the chemotactic signaling pathway of these cells [49].

In many cell types certain CAs isoforms can form complexes with  $HCO_3^-$  transporters. In recent years this association has been called the bicarbonate transport metabolon, a complex of enzymes spatially close to each other that catalyze a series of reactions in a metabolic pathway; in this way the product of one enzyme is the substrate of the next, which allows the reaction to proceed efficiently [50].

**Bicarbonate ( $HCO_3^-$ ) transporters**— $HCO_3^-$  transporters constitute one of the main families of transmembrane proteins involved in the pHi regulation of mammalian cells. As mentioned previously, this family includes  $HCO_3^-$  transporters SLC4, SLC26, and CFTR [3]. The SLC4 transporter family includes the products of ten genes (SLC4A1-5 and SLC4A7-11). Nine SLC4 family members are integral membrane proteins that carry  $HCO_3^-$  (or carbonate) and at least one monoatomic ion (typically  $Na^+$  or  $Cl^-$ ), across the plasma membrane [51]. The physiological function of eight family members is already defined and they fall into three major phylogenetic subfamilies: 1) the  $Cl^-/HCO_3^-$  anion exchangers AE1-3 (SLC4A1-3), 2) the electrogenic  $Na^+/HCO_3^-$  cotransporters NBCe1 (SLC4A4) and

NBCe2 (SLC4A5) and 3) the electroneutral  $\text{Na}^+/\text{HCO}_3^-$  cotransporters NBCn1 (SLC4A7), NDCBE (SLC4A8) and NBCn2 (SLC4A10). The function of the two remaining SLC4 members, AE4 (SLC4A9) and BTR1 (SLC4A11) is still not fully understood [51].

On the other hand, the mammalian SLC26 gene family comprises 11 genes (SLC26A1-A11) that encodes multifunctional anion exchangers and anion channels transporting a broad range of substrates including  $\text{HCO}_3^-$ ,  $\text{Cl}^-$ ,  $\text{OH}^-$ ,  $\text{I}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCOO}^-$  and  $\text{C}_2\text{O}_4^{2-}$ . Among SLC26A family,  $\text{HCO}_3^-$  permeable members represent the second major subfamily of  $\text{HCO}_3^-$  transporters: SLCA3-A4, SLCA6-A9 and SLCA11 [52].

The CFTR is the only member of the ABC family of transporters that acts as an ion channel; it has been shown to be permeable to  $\text{HCO}_3^-$  [53]. CFTR is found commonly in the apical membranes of epithelial cells and its mutations give rise to cystic fibrosis (CF), one of the most common genetic diseases in some human groups.

Although so far the available information about the molecular entities, the distribution and function of  $\text{HCO}_3^-$  transporters in mammalian sperm physiology is very limited, in the last years a few studies have improved our knowledge on this subject. AE2 (SLC4A2) is localized in the equatorial segment of mammalian sperm head [54], [55]. On the other hand, mutations in human SLC26A3 produce congenital chloride diarrhea (CLD) and patients with CLD are subfertile, suggesting this transporter is important for sperm physiology [56]. In fact, SLC26A8, SLC26A3, as well as SLC26A6 and CFTR, have been identified in the midpiece of mouse/guinea pig spermatozoa [18], [21], [57]–[59]. These transporters physically interact and participate in the pHi increase that takes place during capacitation [21]. Results from another study strongly suggest that an electrogenic  $\text{Na}^+/\text{HCO}_3^-$  cotransporter may also participate in the regulation of capacitation in mouse sperm [60]. In that report it was shown that  $\text{HCO}_3^-$  induces a  $\text{Na}^+$ -dependent hyperpolarization of the mouse sperm plasma membrane and this leads to a pHi increase which is sensitive to DIDS (an anion exchanger inhibitor). In the same species it was demonstrated that a  $\text{Na}^+$ -driven  $\text{Cl}^-/\text{HCO}_3^-$  exchange is involved in the pHi regulation, possibly during capacitation [61].

In human spermatozoa the identification of  $\text{HCO}_3^-$  transporters remains unexplored.

**pHi dependent ion channels , CatSper and Slo3**—For over three decades it was known that progesterone and a component of the zona pellucida (ZP3) induce the acrosome reaction (AR, see below) with a strict requirement for an intracellular  $\text{Ca}^{2+}$  rise. It was later established that the  $\text{Ca}^{2+}$  increase triggered by these biomolecules during the AR is influenced by pHi. Interestingly, artificial intracellular alkalinization of mouse sperm produced a  $\text{Ca}^{2+}$  increase [62] and addition of  $\text{HCO}_3^-$  increased the beat frequency of sperm [63]. Although it was not completely clear how pHi and  $\text{Ca}^{2+}$  changes influence each other to regulate sperm functions, it was proposed that there were channels sensitive to membrane potential and pHi. This notion was later confirmed and the importance of pHi regulation in sperm was strongly highlighted with the discovery of two pHi and voltage sensitive ion channels, CatSper and Slo3, that are exclusively expressed in spermatozoa and whose absence produces male infertility. CatSper was identified in mouse sperm as a putative  $\text{Ca}^{2+}$  channel in 2001 [4], [64]. It was then shown that the activity of CatSper is required for



hyperactivated motility and fertility although sperm from CatSper null mice can undergo the AR and fertilize zona free eggs [4], [64], [65]. After different approaches and strong efforts, whole-cell patch-clamp recordings in mature sperm became a reality in 2006 [66], such technique thus far has not detected other  $\text{Ca}^{2+}$  currents but those mediated by CatSper [67]. The characterization of CatSper has proven difficult due to the high promiscuity among its agonists and the lack of specific antagonists [68] and because heterologous expression has not been successful. However, it is clear that alkalization increases CatSper activity and that progesterone activates it directly; this is true for human sperm. The ligands for CatSper channels from other species remain to be established.

In 1998 a pHi and voltage sensitive  $\text{K}^+$  channel (Slo3) highly expressed in mouse testis was described [69]. It was later reported that spermatozoa from Slo3 null mice exhibit several defects, including altered morphology, reduced motility and decreased AR efficiency [5], [70]. Recently, a  $\text{K}^+$  channel was also recorded in human sperm by two different groups; one claimed that the molecular identity of this channel is Slo3 [71] but the other presented evidence that this current resembled more closely the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel, Slo1 [72]. New results from our lab in human sperm revealed that there is a plasma membrane hyperpolarization associated to the capacitation process (see below) and that more than one type of  $\text{K}^+$  channel may be involved, possibly Slo1 and Slo3 [73]. Regardless of the molecular identity, these sperm specific channels may contribute to the changes on membrane potential, intracellular  $\text{Ca}^{2+}$  and pHi, parameters that control not only the AR but other sperm functions. Further investigation is required to understand how and if these channels interact with each other forming a macro protein complex, a possibility that may explain recent findings of antagonist cross sensitivity displayed by CatSper and Slo3 and/or the molecular identity of the  $\text{K}^+$  current in human sperm [74].

### Sperm functions where pHi regulation is critical

**Initiation of motility**—In most species, spermatozoa are stored immotile in the testis (or epididymis in the case of mammals). In general, it is believed that this quiescent sperm state is maintained by acidification of sperm pHi since the sperm dynein ATPases, motor proteins that generate the flagellar beat, are highly pH dependent [75], [76]. The pH-motility regulation is relatively well studied in sea urchin sperm.

Sea urchin sperm remain immotile in the gonads due to a low pHi maintained by a high  $\text{CO}_2$  tension and initiate flagellar beating upon spawning [77]. When sperm are released into seawater (pH 8.0), a rapid  $\text{H}^+$  efflux occurs [78], elevating pHi from 7.2 to 7.6 [78]. This pHi increase activates axonemal dynein ATPases, which are highly active at alkaline pHi (7.5 – 8.0) [75]. As the mitochondrion is the only ATP source in sea urchin sperm and dynein ATPases are the main consumers, motility and respiration are tightly linked and regulated by pHi [79], [80]. Both sperm pHi and motility depend on external ionic composition; for example low pH (5.0), high  $[\text{K}^+]$  (200 mM) or  $\text{Na}^+$ -free seawater lower sperm pHi to 5.7 – 6.6 [79] and prevent its motility. Notably, pHi alkalization by  $\text{NH}_4\text{Cl}$  addition restores sperm motility under these conditions [78], establishing the direct involvement of pHi as regulator of motility.

The pHi dependence on external pH and Na<sup>+</sup>, and the inhibition of motility by high external K<sup>+</sup> can be explained by the participation of the voltage-sensitive NHE present in the sea urchin sperm flagellum, which interestingly, can be activated by a membrane hyperpolarization [35], [36]. Since sNHE (SLC9C1) possesses a voltage sensor domain [6] and peptide fragments of sNHE were detected in a proteomic analysis of sAC-associated proteins of sea urchin sperm [38], it is more likely that sNHE is the NHE that elevates pHi upon motility initiation in this species. Removal of tracer elements such as Zn<sup>2+</sup> also lowers pHi retarding sperm motility initiation [81], which suggests that Zn<sup>2+</sup> is involved, directly or indirectly, in the regulation of sNHE activity.

**Sea urchin sperm chemotaxis**—Sperm chemotaxis has been extensively studied in sea urchin spermatozoa. In this marine invertebrates, sperm are attracted toward the egg by sperm-activating peptides (SAPs) which are diffusible peptides released from the external coat of the egg (Review at [82]) SAPs stimulate sea urchin sperm motility and respiration in slightly acidified seawater (pH 6.2 – 6.8) [83], by increasing pHi [84], [85]. This property has been successfully used to screen for SAPs, and to date has led to the identification of hundreds of SAPs from numerous sea urchin species [82].

Currently, it is believed that mainly SAPs guide sperm toward the egg modulating their swimming behavior and, as a result, increasing the fertilization probability (review in [86]). Resact and speract are two of the best characterized SAPs that are chemotactic for two sea urchin species, *A. punctulata* [87] and *L. pictus*, respectively [88]. Both trigger a chemotactic response in a Ca<sup>2+</sup> dependent manner [87]–[89]. In particular, Ca<sup>2+</sup> influx into the flagellum is critical to shape the flagellar beat and the sperm swimming trajectory [90]–[94]. This Ca<sup>2+</sup> influx is comprised of [Ca<sup>2+</sup>]<sub>i</sub> oscillations mounted on a sustained increase that initiate in the sperm flagella and travel toward the head [95]. Each oscillation transiently increases the flagellar asymmetry and causes the sperm to turn [90]. An orchestrated sequence of turns interspersed with periods of straighter swimming allows sperm to swim towards the chemoattractant source [87]–[89] and allows the sperm to locate the egg (Fig. 2, inset).

However, [Ca<sup>2+</sup>]<sub>i</sub> changes are the result of a complex signaling cascade shared, with some differences, by the sea urchin species thus far studied [89], [96]. In the signaling cascade of SAPs (Fig. 2), the cGMP-induced K<sup>+</sup> efflux through a TetraKCNG/CNGK channel [97], [98] is an initial and fundamental step. This K<sup>+</sup> efflux leads to hyperpolarization of the sperm membrane potential (Em) and modulates the function of several ion transporters and enzymes as shown in Fig. 2. Indeed, when this K<sup>+</sup> efflux is inhibited by high K<sup>+</sup> seawater, all the sperm responses to SAPs, except for an enhanced accumulation of cGMP, are completely inhibited [99].

One important consequence of this Em hyperpolarization is the pHi increase. This alkalization, as motility initiation, is pHe dependent and inhibited by high [K<sup>+</sup>]<sub>e</sub> and by the absence of [Na<sup>+</sup>]<sub>e</sub> [85], [100], thus the Em-sensitive NHE (probably sNHE) is likely involved in the speract response. Our time-resolved kinetic measurements of speract induced pHi and [Ca<sup>2+</sup>]<sub>i</sub> changes using fast mixing device [101] and caged compounds (cGMP and speract) [102] revealed that the pHi increase induced by speract and cGMP occurs with a



short delay (50 – 200 ms) always preceding the  $[Ca^{2+}]_i$  increase, supporting the idea that Em hyperpolarization activates the sNHE. However, the physiological relevance of the pHi increase induced by SAPs on sperm chemotaxis is under debate.

At present, it is considered that the rate of  $[Ca^{2+}]_i$  change ( $d [Ca^{2+}]_i / d t$ ) determines the sea urchin sperm flagellar beating mode and swimming pattern [93]. It has been proposed that voltage-gated  $Ca^{2+}$  channels ( $Ca_v$  channels) are involved in the speract response [90], [103]. Both high voltage-activated ( $Ca_v1$  and  $Ca_v2$  families) and low voltage-activated ( $Ca_v3$  family) transcripts were detected in sea urchin testis; specifically  $Ca_v1.2$  was localized in the sperm flagellum by immunofluorescence [104]. At a first glance, if only typical  $Ca_v$  channels were involved in sea urchin sperm chemotaxis, the change in pHi induced by SAPs might not have a great impact on  $[Ca^{2+}]_i$  regulation. Actually, Solzin et al. (2004) [105] showed that the chemotactic behavior of *A. punctulata* sperm to resact was not altered in the presence of imidazol (10 mM), which was proposed to function as a pHi buffer. In addition, the authors did not detect an increase in sperm pHi by uncaging cGMP. However, we observed the opposite result, namely, an increase in pHi induced by uncaging cGMP in *A. punctulata* as well as *S. purpuratus* sperm [96]. It must be noted also that pHi influences speract-receptor interaction [106], the activity of both GC and sAC [107], [108] and the activity of PDE5 [109]. Therefore, pHi could modulate sea urchin sperm swimming in several ways.

On the other hand, in recent years CatSper, the sperm specific  $Ca^{2+}$  channel activated by alkaline pHi, has been shown to be essential for mouse and human fertility [110], [111] reviewed in [4]. This channel is present in the *S. purpuratus* genome [113] and sperm from this species display a pHi dependent  $[Ca^{2+}]_i$  increase [114]. Therefore, it is worth exploring if CatSper participates in the speract cascade and if it is regulated by the pHi increase as in other species. The participation of pHi in chemotaxis could still turn out to be important. Consequently, further investigation is required, in particular single cell fast measurements, to reveal when and where the speract-induced pHi and  $[Ca^{2+}]_i$  increases occur within the flagellum and how they are related. Furthermore, the lack of specific sNHE inhibitors has hampered examining the role of pHi in motility regulation and generally in sperm physiology, thus identifying such compounds is imperative. Since sNHE is essential for mouse fertilization, specific sNHE inhibitors might also function as contraceptives.

**Capacitation and Hyperactivation**—Although mammalian spermatozoa acquire the capacity of flagellar motility in the epididymis [115], they remain quiescent in its acidic lumen which results from the V-ATPase activity found in its apical plasma membrane [116], [117]. Upon ejaculation, sperm are exposed to the seminal fluid (pH around 7.5) which ensures sperm motility [116]. However, ejaculated mammalian spermatozoa are unable to fertilize the egg until a process of maturation, named capacitation, takes place. Under physiological condition, spermatozoa complete capacitation in the female reproductive tract, but it is possible to reproduce this process *in vitro* in an appropriate medium, containing  $HCO_3^-$ ,  $Ca^{2+}$  and albumin. Soon after ejaculation, sperm swim progressively with symmetric, low amplitude and high frequency flagellar beat. In contrast, spermatozoa recovered from the ampulla of the oviduct, the site of fertilization, show vigorous motility with asymmetric, high amplitude and low frequency flagellar beat, termed hyperactivation

[118]. Hyperactivation is essential for spermatozoa to achieve fertilization *in vivo* since it is necessary for their migration towards the ampulla in the oviduct [119]. This type of motility is also necessary to penetrate ZP during *in vitro* fertilization [4]. In this sense, hyperactivation can be considered as part of capacitation, though capacitation is more usually defined as a preparation for sperm to undergo the AR. It has been established that  $\text{Ca}^{2+}$  is fundamental for hyperactivation and CatSper is an essential channel in this process. However, it is still not clear what the physiological signals that induce hyperactivation are [118], but it is known that  $\text{pH}_e$  and  $\text{HCO}_3^-$  changes are crucial signals. In the rhesus monkey, the  $\text{pH}$  and  $\text{HCO}_3^-$  concentration in the lumen of the oviduct during the follicular phase are 7.2 and 35 mM, respectively, and they increase to 7.6 and 90 mM during ovulation [120]. Therefore,  $\text{HCO}_3^-$  and  $\text{H}^+$  transporters as well as CAs are considered crucial players for both capacitation and hyperactivation, as illustrated in Fig. 1. Since *in vitro* capacitation requires relatively long periods (30 min in mouse and several hours in human), the metabolic state, such as mitochondrial  $\text{CO}_2$  could have an important role in this process, although currently there is no experimental evidence.

### The acrosome reaction

The acrosome is a secretory vesicle localized in the apical region of the sperm head in most species, except for some such as teleosts. When sperm receive an appropriate stimulus, this vesicle undergoes exocytosis and releases its acrosomal contents including hydrolytic enzymes; this process is called the acrosome reaction (AR). The AR is a unique single exocytotic event since the outer acrosomal membrane and the plasma membrane fuse at multiple sites and are released from the cell as hybrid vesicles. As a consequence, only the inner acrosomal membrane becomes a new plasma membrane. It is believed that only the sperm that has undergone the AR can penetrate the egg coat and complete sperm-egg fusion [121]. It is known that  $\text{Ca}^{2+}$  is one of the key elements needed for AR since its discovery in marine invertebrates [122], though  $\text{pH}_i$  is also crucial to achieve this reaction [123], [124].

In mammals, a  $\text{pH}_i$  rise is fundamental for the ZP-induced AR [124]. This  $\text{pH}_i$  change is inhibited by pertussis toxin, suggesting the involvement of a G-protein (Gi). However, how this  $\text{pH}_i$  increase is achieved remains unknown. On the other hand, recent studies using genetically manipulated mice, which express enhanced green fluorescence protein (EGFP) in the acrosome, revealed that most fertilizing spermatozoa had undergone AR before their contact with ZP [125], questioning if ZP is the physiological inducer of the AR. As mentioned previously, CatSper, Slo3 and the sAC-sNHE complex have been described as sperm-specific important players related to  $\text{pH}_i$  in mammalian spermatozoa. Particularly, CatSper is proposed to participate in the AR besides its role in the hyperactivated sperm motility [126]–[128]. Differences between *in vitro* and *in vivo* fertilization have been accumulating. Indeed, many proteins proposed to be involved in the AR are not essential for *in vivo* fertilization since their elimination using gene-targeted male mice does not result in infertility [129]. Therefore, although technically difficult, understanding the physiological process of mammalian internal fertilization requires examining it *in vivo*.

In contrast to mammalian AR, it is clear that in the sea urchin, the physiological inducer of the AR is a fucose sulfate polymer (FSP), a major component of egg jelly coat [130]. When

FSP binds to its receptor, spermatozoa undergo exocytosis of the acrosomal vesicle and expose the internal face of the acrosomal membrane (now the membrane of the acrosomal tubule). Subsequently, polymerization of actin takes place in the subacrosomal space leading to the formation of the 1  $\mu\text{m}$  long acrosomal tubule. This process depends on the finely coordinated ionic flux changes evoked by FSP (reviewed in [45], [131], [132]).

FSP binding to its receptor, suREJ1, evokes  $E_m$  changes and increases in  $[\text{Ca}^{2+}]_i$ ,  $\text{pHi}$ ,  $[\text{Na}^+]_i$ , cAMP,  $\text{IP}_3$  and NAADP (reviewed in [45]). External  $\text{Ca}^{2+}$  is necessary for the  $[\text{Ca}^{2+}]_i$  increase and the internal alkalization (0.1 – 0.2 pH units), which are prerequisites for the AR to occur [122], [123], [133]–[135]. It is thought that at least two different  $\text{Ca}^{2+}$  channels, acting sequentially, mediate the  $\text{Ca}^{2+}$  entry necessary to induce the AR. The first one is probably a  $\text{Ca}_v$  channel considering its sensitivity to verapamil and dihydropyridines [123], [136] and the second, which opens 5 s later, is a  $\text{pHi}$ -dependent channel activated by an alkalization. Interestingly, this channel is inhibited by low  $[\text{Na}^+]_e$ , high  $[\text{K}^+]_e$  or TEA (a  $\text{K}^+$ -channel blocker) [136], conditions that also inhibit the voltage-sensitive NHE [100], probably sNHE. These latter findings suggest the involvement of sNHE in the AR. However, inhibition of the initial  $\text{Ca}^{2+}$  entry nearly completely blocks the  $\text{pHi}$  increase triggered by FSP, implying the participation of a  $\text{Ca}^{2+}$ -dependent  $\text{H}^+$  extrusion mechanism [123]. As sNHE itself seems to be  $\text{Ca}^{2+}$  independent [84], [85], the  $\text{pHi}$  increase associated to the AR may involve: a) a  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel, which could mediate the sNHE activation through  $E_m$  changes; b) cAMP produced by sAC, which possibly activates sNHE; and c) another type of NHEs.

Besides modulating the activity of the second  $\text{Ca}^{2+}$  channel, the  $\text{pHi}$  increase promotes actin polymerization leading to the formation of the acrosomal tubule [137]. On the other hand, as the  $\text{pHi}$  increase depends on  $\text{pHe}$ , the AR is highly  $\text{pHe}$  dependent, being prevented at  $\text{pHe}$  7.3 – 7.5 and completely induced near physiological  $\text{pHe}$  7.6 – 8.0 [138], which suggests the possible involvement of a  $\text{H}_v$  channel since the  $\text{H}^+$  gradient across the plasma membrane, as well as  $E_m$  depolarization activates this channel. Although the importance of  $\text{pHi}$  in the sea urchin AR has long been known, the molecular mechanism is not fully understood and further investigation is necessary to elicit the details.

## PERSPECTIVES

The regulation of  $\text{pHi}$  is fundamental for sperm function. Now we know that certain sperm-specific proteins essential for fertilization are involved in  $\text{pHi}$  regulation or regulated by  $\text{pHi}$ . However, many questions regarding the physiological function of these proteins are still unanswered. Currently it is clear that there are differences between *in vitro* fertilization and what occurs in the lumen of the oviduct that must be considered to fully understand this fundamental process. In fact, the  $\text{pHi}$  of the oviductal lumen dramatically elevates upon ovulation due to  $\text{HCO}_3^-$  production in the epithelial cells lining it. The  $\text{pHi}$  and ionic changes in the oviduct must be considered to understand the physiological sperm responses. In this sense, we still need to learn a lot about sperm  $\text{pHi}$  regulation by  $\text{CO}_2$ ,  $\text{HCO}_3^-$ ,  $\text{H}^+$ , CAs and  $\text{HCO}_3^-$  and  $\text{H}^+$  transporters, as well as their modulators, to understand how these fundamental cells operate and achieve fertilization.

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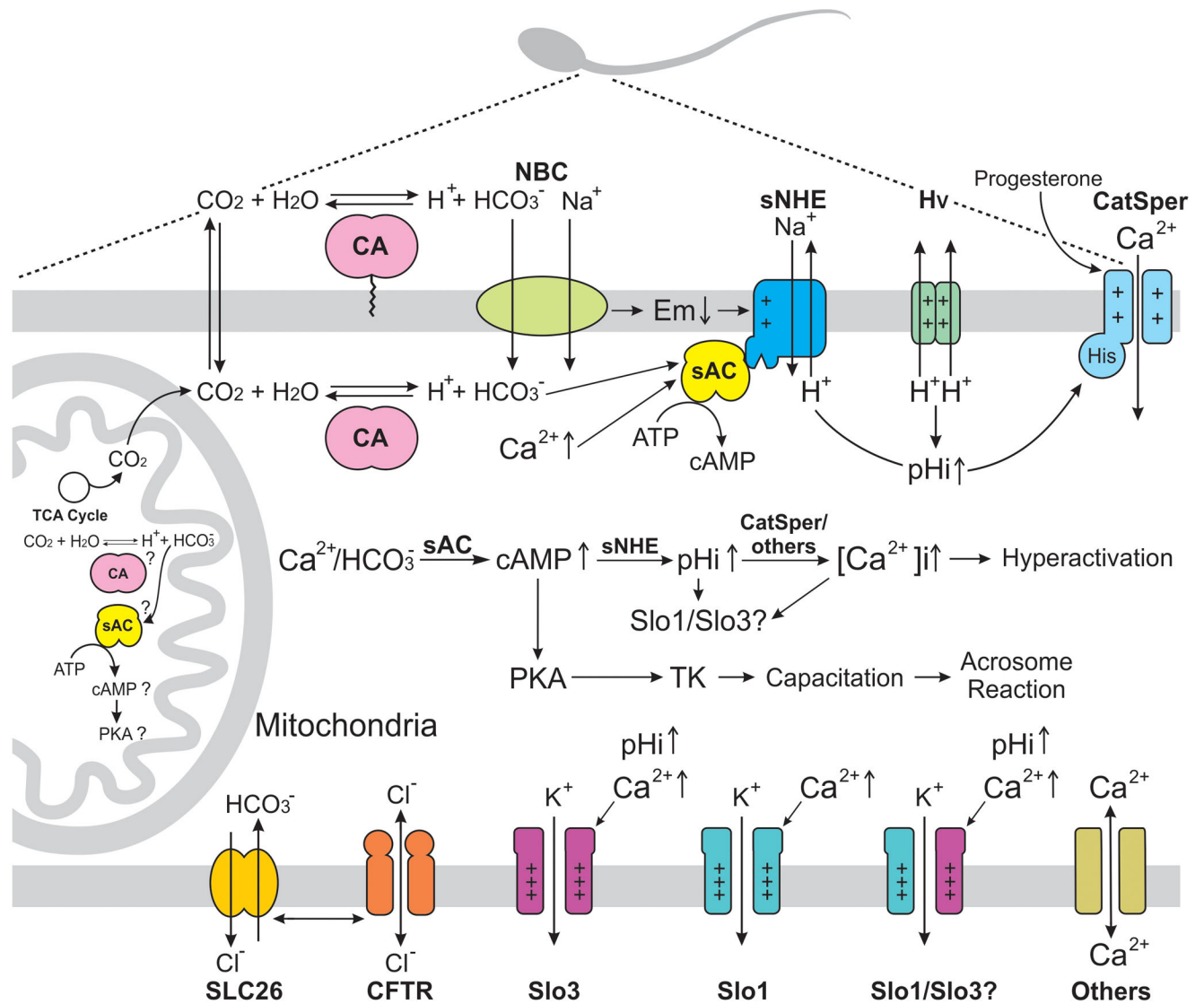
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**Highlights**

- Sperm intracellular pH regulation is crucial for fertilization
- Intracellular pH dependent sperm ion channels
- Importance of sperm specific Na<sup>+</sup>/H<sup>+</sup> exchanger in motility and capacitation
- sperm specific Na<sup>+</sup>/H<sup>+</sup> exchanger interacts with soluble adenylyl cyclase
- carbonic anhydrase/ HCO<sub>3</sub> transporter metabolon in sperm function

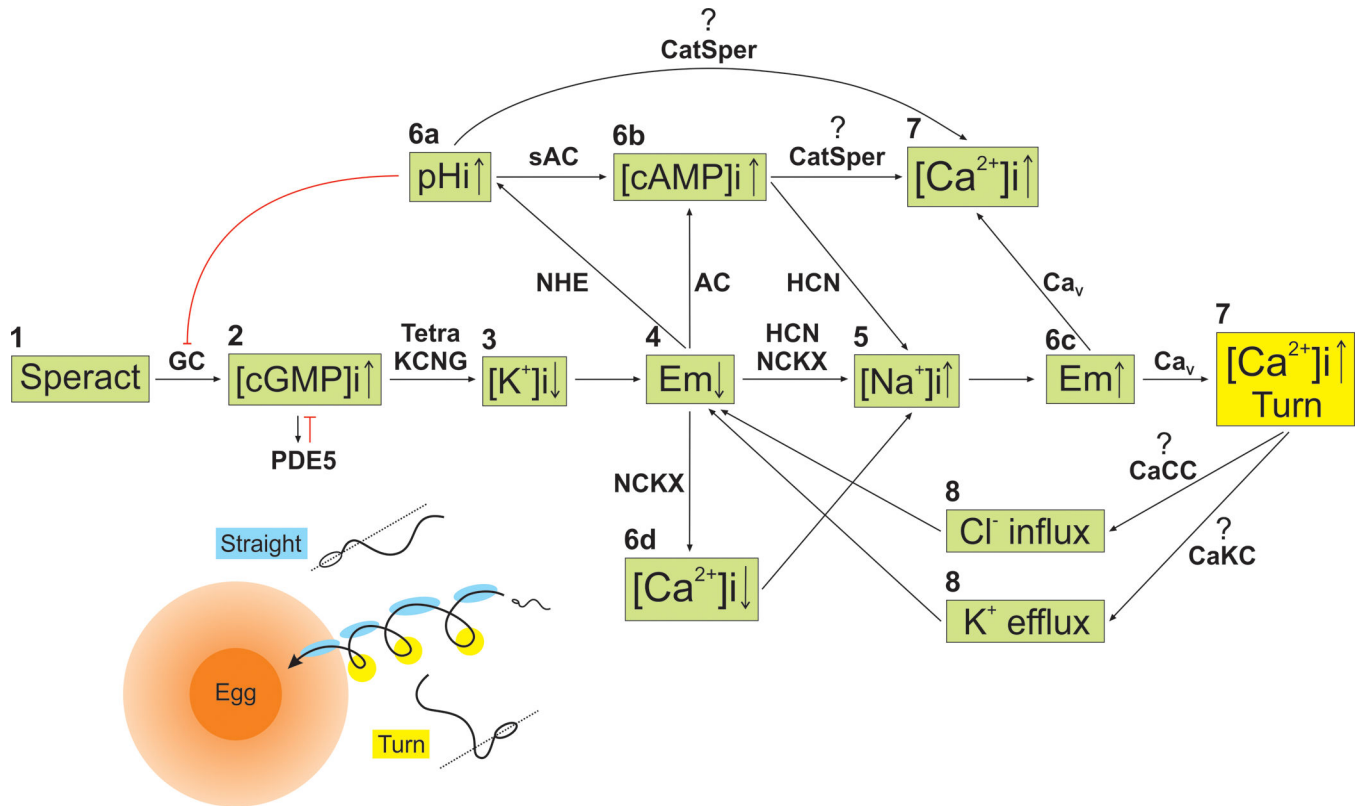




**Figure 1. Model of mammalian sperm pH<sub>i</sub> regulation during capacitation/hyperactivation**

The CO<sub>2</sub> / HCO<sub>3</sub><sup>-</sup> are equilibrated inside the female tract by membrane associated carbonic anhydrases (CAs). The influx of HCO<sub>3</sub><sup>-</sup> into the sperm is mediated by Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporters (NBC) and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers (SLC26). Probably the cytoplasmic CAs also contribute to the HCO<sub>3</sub><sup>-</sup> increase by conversion of cytosolic CO<sub>2</sub>. Specifically, SLC26A3 and A6 isoforms appear to have a physical interaction with CFTR, which may permeate HCO<sub>3</sub><sup>-</sup> besides Cl<sup>-</sup>. Aside, HCO<sub>3</sub><sup>-</sup> influx hyperpolarizes mouse spermatozoa in a [Na<sup>+</sup>]<sub>e</sub>-dependent manner via NBC. HCO<sub>3</sub><sup>-</sup> activates (along with Ca<sup>2+</sup>) a soluble adenylyl cyclase (sAC) leading to a cAMP increase, which activates a PKA (which may also depend on [Ca<sup>2+</sup>]<sub>i</sub>) and also may bind to the cyclic nucleotide binding domain of the sperm specific Na<sup>+</sup>/H<sup>+</sup> exchanger (sNHE) inducing a pH<sub>i</sub> increase in mouse. sNHE may also be stimulated by the hyperpolarization that occurs upon sperm capacitation. Unlike mouse, a voltage-dependent H<sup>+</sup> channel (H<sub>v</sub>) may be involved in human sperm pH<sub>i</sub> increase. In both species, the pH<sub>i</sub> increase (among other factors) activates a sperm specific Ca<sup>2+</sup> permeable channel

known as CatSper, which allows an intracellular  $\text{Ca}^{2+}$  increase essential for hyperactivation. We include an additional  $\text{Ca}^{2+}$  transporter to indicate that other systems participate in  $[\text{Ca}^{2+}]_i$  regulation. On the other hand,  $\text{K}^+$  channels Slo3 and Slo1 (or their heterotetramer) are activated by intracellular alkalinization and/or by  $\text{Ca}^{2+}$  (human sperm), contributing to the hyperpolarization that occurs during capacitation.  $\text{CO}_2$  is also generated inside mitochondria through the tricarboxylic acid cycle (TCA) and it may serve as substrate for intracellular or intramitochondrial CAs. The  $\text{HCO}_3^-$  may also activate intramitochondrial sAC which in turn would trigger protein phosphorylation. Although mammalian sperm possess many mitochondria inside the midpiece, for didactic purposes only one mitochondrion is shown here.



**Figure 2. The speract signaling cascade in sea urchin sperm**

After binding to its receptor (1), speract stimulates a membrane guanylyl cyclase (GC), which elevates cGMP (2) that activates tetrameric cGMP-regulated  $K^+$  channels (tetraKCNG), causing a membrane potential ( $E_m$ ) hyperpolarization (4) due to  $K^+$  efflux (3). This  $E_m$  change may activate a  $Na^+/H^+$  exchanger (sNHE) (5a), remove inactivation from voltage-activated  $Ca^{2+}$  channels ( $Ca_v$ ), enhance hyperpolarization-activated and cyclic nucleotide-gated channels (HCN), and facilitate  $Ca^{2+}$  extrusion (5d) by  $K^+$ -dependent  $Na^+/Ca^{2+}$  exchangers (NCKX). sNHE activation increases pHi (5a). HCN opening causes  $Na^+$  influx (5c) and contributes to depolarize  $E_m$  activating  $Ca_v$ s, which increase  $[Ca^{2+}]_i$  (6) and enhance asymmetric flagellar bending allowing sperm to turn. Possibly, the rise of  $[Ca^{2+}]_i$  also opens  $Ca^{2+}$ -regulated  $K^+$  channels (CaKC) and  $Ca^{2+}$ -regulated Cl channels (CaCC), which then contribute to again hyperpolarize  $E_m$  (7), removing inactivation from  $Ca_v$  channels and opening HCN channels. During this period sperm swim in a straighter trajectory whose regulation is ill defined but critical for chemotaxis. This succession of events occurs cyclically, generating orchestrated transient  $[Ca^{2+}]_i$  increases (6) that produce a sequence of turns until one or more of the second messengers in the pathway are downregulated. The pHi (5a) and cAMP increases (5b) stimulate an undetermined  $Ca^{2+}$  influx pathway that contributes to the sustained  $[Ca^{2+}]_i$  increase (6), and possibly to the depolarization that accompanies the speract response. It is now worth exploring if CatSper participates in this process. Inset: A sperm drifting circular swimming trajectory in a chemoattractant gradient (orange shadow surrounding the egg) is stimulated periodically due to changes in the rate of chemoattractant capture. When swimming towards the egg in an ascending gradient, the onset of  $[Ca^{2+}]_i$  fluctuations is suppressed until the cell detects an

ascending to descending gradient inversion. After a  $\sim 200$  ms delay, the spermatozoon undergoes a transient  $[Ca^{2+}]_i$  increase just before reaching the gradient minima (yellow circles). The rate of change in  $[Ca^{2+}]_i$  ( $d[Ca^{2+}]_i/dt$ ) controls sperm trajectory. As a result, sperm turn (yellow) when they are swimming away from the egg and swim in a straighter trajectory (blue) while coming closer to the chemoattractant source (i. e., the egg jelly).

**Table 1**

Molecules involved in the regulation of pHi in mammalian sperm

Molecule	Species/Localization	Technique	Function	Reference
CAI	H	ELISA	-	[139]
CAII	H	ELISA	-	[139]
	H, M	Northern blot	-	[141]
	M	Western Blot	-	
	H/PA; R/A	IC	-	[140]
	H, R	Western Blot	-	
CAIV	M/A, ES, PA, MP*	SEM	-	
	M	RT-PCR Western Blot	Motility	[46]
	M			
	M/A, ES, PA, MP, PP*	IC		
CAXII	M	PCR	-	[142]
	IC	-		
CAXIII	H, M	PCR	-	[143]
	H	IH	-	
sNHE	M	Northern Blot	Motility	[6]
	M	Western Blot		
	M/PP	IC		
NHA1 H <sub>v</sub>	M/PP	IC	Motility	[33]
	H/PP	Patch Clamp ISH	Motility	[12]
	H	Western Blot		
	IC			
	H/PP			
NBC	M	-	Capacitation	[60]
SLC26A 3	M	RT-PCR	Capacitation	[21]
	M	Western Blot		
	M/MP	IC		
SLC26A 6	M	RT-PCR	Capacitation	[21]
	M	Western Blot		
	M/MP	IC		

\* Only in corpus and cauda spermatozoa.

**Species:** H, Human; M, Mouse; R, Rat. **Cellular localization:** A, Acrosome; ES, Equatorial segment; P, Postacrosomal region; MP, Midpiece; PP, Principal piece. **Technique:** ISH, In situ hybridization; IC, Immunocytochemistry; IH, Immunohistochemistry; SEM, Scanning electron microscopy.