Review

CatSper: The complex main gate of calcium entry in mammalian spermatozoa

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ABSTRACT

Calcium ions (Ca\textsuperscript{2+}) are involved in nearly every aspect of cellular life. They are one of the most abundant elements in mammals and play a vital role in physiological and biochemical processes acting mainly as intracellular messengers. In spermatozoa, several key functions are regulated by cytoplasmic Ca\textsuperscript{2+} concentration such as sperm capacitation, chemotaxis, hyperactive motility, and acrosome reaction. The sperm-specific ion channel CatSper is the principal calcium channel in sperm mediating the calcium influx into the sperm flagellum and acting as an essential modulator of downstream mechanisms involved in fertilization. This review aims to provide insights into the structure, localization, and function of the mammalian CatSper channel, primarily human and mice. The activation of CatSper by progesterone and prostaglandins, as well as the ligand-independent regulation of the channel by a change in the membrane voltage and intracellular pH are going to be addressed. Finally, major questions, challenges, and perspectives are discussed.

1. Introduction

The survival of the animal species relies almost entirely on the ability of a spermatozoon to fertilize an egg. The male germ cell is a unique highly specialized cell that is designed to exert its main function outside of its production site. After being released in the lumen of the seminiferous tubules, sperm cells have to accomplish several challenging tasks before reaching and fertilizing the egg. The journey begins after spermatogenesis has been completed culminating in the formation of highly differentiated spermatozoa that are structurally complete but are still functionally immature. It is through their transit in the epididymis that sperms cells complete important maturation steps crucial for fulfilling their functions. These include sperm chromatin condensation, surface protein modifications such as the incorporation of cholesterol, morphological changes, and most importantly sperm motility initiation and acquisition of coordinated sperm motion (reviewed in Sullivan and Mieusset (2016)). Membrane remodeling and active glycosylation of surface components are believed to stabilize the cell in order to remain in a quiescent state (Cooper, 2007; Sullivan et al., 2019; Sullivan and Mieusset, 2016; Yeung et al., 1993). These changes are already at this stage, highly dependent on the exchange of ions and electrolytes through diverse ion channels (Gupta, 2017). Despite being motile upon ejaculation, sperms are unable to fertilize the oocyte unless they undergo a “priming” step in the female reproductive tract referred to as capacitation (Austin, 1952; Chang, 1951). During this process which is partly dependent on an increase in intracellular calcium concentration ([Ca\textsuperscript{2+}]), extensive metabolic and physiological changes occur in all sperm compartments. These changes involve further membrane remodeling which includes the redistribution, modification, addition, or removal of proteins acquired during epididymal transit. The female reproductive tract is indeed rich in albumin that binds to cholesterol and induces an important cholesterol efflux leading to an increase in the membrane fluidity. In addition, exposure of sperms to high levels of bicarbonate present in the female tract induces activation of soluble adenylate cyclase leading to the production of cyclic adenosine monophosphate (cAMP) and activation of the protein kinase A (PKA) (Gervasi and Visconti, 2017). The phosphorylation of tyrosine residues by PKA, in turn, induces membrane hyperpolarization which later activates diverse calcium channels, key among them are the Caticonic channel of Sperm CatSper and voltage-gated calcium channels Ca\textsubscript{v} leading to an increase in [Ca\textsuperscript{2+}] (Darsonz et al., 2011; De Jonge, 2017, 2005; Puga Molina et al., 2018; Visconti et al., 2011). This increase allows the sperm to
acquire hyperactive motility, a landmark of sperm capacitation, and an absolute prerequisite for fertilization. Hyperactivated sperms are characterized by having a high amplitude whip-like asymmetrical tail bending, are non-progressive in aqueous low-viscosity media, and are thought to be progressive in the physiological viscosity of the upper female reproductive tract (de Lamirande et al., 1997; Kirkman-Brown and Smith, 2011; Mortimer, 1997). Another major consequence of capacitation is the sperm ability to undergo the acrosome reaction (AR) as a result of the acquired increase of membrane fluidity. The AR is characterized by the release of proteolytic enzymes allowing the sperm to penetrate the Zona Pellucida (ZP) surrounding the oocyte and finally fertilize it.

Throughout this long journey, the sperm cell must continuously adapt to new environments to deliver the genetic information needed to create a new individual. From capacitation and navigation to hyperactivation and acrosome reaction, calcium ions play a central role by acting at diverse spatial sites to orchestrate these various events (Publicover et al., 2007, 2008). As the principal calcium channel of sperm, CatSper is the main gate through which extracellular calcium enters the sperm flagellum and is therefore vital for sperm fertility (Lishko and Smith, 2011; Mortimer, 1997). Another major consequence of creating a new life, it is far from being completely understood. Improving our understanding of the complexity of the CatSper channel is thought to be required for the diverse types of stimuli that CatSper can respond to (Visconti et al., 2011).

2. CatSper: the complex, promiscuous and essential calcium channel of the sperm flagellum

2.1. Structure

Being exclusively expressed in spermatozoa and evolutionarily conserved among species from mammals to invertebrates, CatSper acts as a polyvalent, chemosensory calcium channel that is unique in controlling a variety of events leading to fertilization. A combination of mouse genetics, as well as the application of patch-clamp techniques, revealed that CatSper is the principal Ca\(^{2+}\) channel in mouse sperm (Kirichok et al., 2006; Lobley et al., 2003; Qi et al., 2007; Quill et al., 2003; Ren et al., 2001). CatSper is specifically located in the membrane of the flagellar principal piece and is known to be pH-sensitive and weakly voltage-dependent (Lishko et al., 2012). In the absence of extracellular calcium, CatSper also allows the entry of monovalent cations such as Na\(^+\) and Cs\(^+\), as well as divalent cations Ba\(^{2+}\) with a much lower affinity compared to Ca\(^{2+}\) (Sun et al., 2017). The heterogeneous CatSper channel is composed of at least ten subunits with four pore-forming \(\alpha\) subunits (CatSper 1–4) and six auxiliary subunits (\(\beta\), \(\gamma\), \(\delta\), \(\zeta\), \(\epsilon\) and EFCAB9) making it one of the most complex channels ever described (Fig. 1, A) (Carlson et al., 2003; Chung et al., 2017, 2011; Darszon et al., 2011; Hwang et al., 2019; Quill et al., 2001; Ren et al., 2001). The high complexity of CatSper is thought to be required for the activation of various stimuli that CatSper can respond to (Visconti et al., 2011).

2.1.1. Pore-forming \(\alpha\) subunits: CatSper 1-4

In 2001, the first member of CatSper (renamed CatSper 1 after the discovery of other subunits) was identified during sequence homology searches to the voltage-gated Ca\(^{2+}\) channels Ca\(_v\) (Ren et al., 2001). Very shortly afterward, CatSper 2 was identified as another pore-forming \(\alpha\) subunit using a signal peptide trapping method (Quill et al., 2001). CatSper 3 and CatSper 4 were later identified using in silico database mining (Lobley et al., 2003). Structurally, each of the four distinct subunits has six transmembrane segments (TM), unlike the Ca\(_v\) that has 24 TM segments in a single polypeptide (Lobley et al., 2003). Importantly, targeted disruption of each of the four pore-forming \(\alpha\) subunits CatSper 1–4 leads to male infertility caused by loss of channel function and absence of hyperactivated motility (Carlson et al., 2005, 2003; Qi et al., 2007; Quill et al., 2003, 2001). It has also been shown that CatSper2, CatSper3 and CatSper4 co-precipitate with CatSper1 either from mouse testis lysate or in a heterologous system indicating that CatSper pore is formed by all four subunits (Qi et al., 2007). Similarly, humans bearing pathogenic mutations in the CatSper1 and CatSper2 genes suffer from infertility. Like in all other voltage-gated cation channels, CatSper 1 and CatSper 2 have several positively charged residues in the 4th TM segment. However, CatSper 3 and CatSper 4 have only two positively charged residues which might explain the mild voltage dependence of CatSper (Ren et al., 2001). CatSper 1 is different

![Fig. 1. Structure of the mammalian CatSper channel and its localization in the sperm flagellum.](image-url)
than the three other α subunits in the fact that it possesses a histidine-rich large cytoplasmic terminal domain that is speculated to function as a pH sensor (83 histidines in the 446 amino acid composing the N terminus) (Ren et al., 2001). The sequence similarity between the four α subunits is indeed low and ranges between 16 and 22% (Navarro et al., 2008). Similarly, the interspecies homology of the different CatSper subunits between mouse and human, is relatively low, ranging from 50% (CATSPER1) to 69% (CATSPER4) (Brown et al., 2019). This could explain the significant differences in the function and regulation of CatSper in these species.

2.1.2. Auxiliary subunits: CatSper β, γ, δ, ζ, ε and EFCAB9

CatSper β was the first reported auxiliary subunit of CatSper that was identified using database search and qRT-PCR (Liu et al., 2007). It contains two TM segments with two short cytoplasmic domains and a large extracellular domain (Fig. 1, A). In contrast, CatSper γ contains only one TM segment with a large extracellular domain and a short cytoplasmic tail. The large extracellular domains of CatSper β and CatSper γ suggest possible channel modulation by external signals such as ligands and cell-cell interactions during sperm maturation in the epididymis and/or in the female reproductive tract (Darson et al., 2011). CatSper δ has a single TM segment with a large extracellular domain and a short cytoplasmic tail, very similar to CatSper γ (Chung et al., 2011). It has been shown that the sperm plasma membrane of CatSper1 null mice also lacks the other three components of the subunit α (CatSper 2–4), as well as the auxiliary subunits CatSper β, γ and δ (Babcock, 2007; Chung et al., 2011; Qi et al., 2007). CatSper ζ and ε co-localize with the rest of the CatSper complex. However, unlike other CatSper knockout mouse models, lack of CatSperζ disrupts the quadrilateral longitudinal nanodomain arrangement of CatSper complex but the channel remains functional and only leads to subfertility in the mutant mice (Chung et al., 2017, 2011). These studies indicate that CatSper ζ is involved in the compartmentalization of calcium signaling and can modulate the calcium mechanism of CatSper. While the quadrilateral Ca$^{2+}$ signaling threads are disrupted in CATSPER 1 knockout mouse sperm (Chung et al., 2014), they seem not to be in CATSPER 2 deficient human sperm (Schiﬀer et al., 2020). A new member of the CatSper complex was recently identiﬁed as EF-hand calcium-binding domain-containing protein 9 or EFCAB9, a calmodulin-like protein that binds Ca$^{2+}$ and acts as a dual calcium and pH sensor (Hwang et al., 2019). EFCAB9 is an evolutionarily conserved component of the CatSper channel and is a direct binding partner of CatSperζ. The EFCAB9-CatSperζ complex is associated with the channel pore and confers pH-dependent activation and Ca$^{2+}$ sensitivity thus regulating the opening and closing of CatSper channel. Null-mice deﬁcient for CatSperζ, Efcab9 or lacking both subunits, were shown to be severely subfertile with sperms that failed to acquire hyperactivated motility (Chung et al., 2017; Hwang et al., 2019). The absence of EFCAB9 and/or CatSperζ renders the channel much less responsive to alkalization in the presence of physiologically relevant Ca$^{2+}$, ultimately making the channel a less efﬁcient Ca$^{2+}$ conductor. While CatSper channel organizes in four doublet bands in the ﬂagellar membrane, loss of Efcab9 alters these doublet tracks as well as sperm surface nanoarchitecture (Chung et al., 2017, 2014; Hwang et al., 2019). Interestingly, the loss of CatSperζ in mice delayed the targeting of the CatSper complex to the ﬂagellum, but also led to a periodic thinning and disruption of the linear pattern. Nine outer dense ﬁbers (ODF) and the FS closely lying around the axoneme or linearly in the same column to structurally and functionally link the CatSper complexes (Bystroff, 2018; Vylicka and Lishko, 2020). These hypothetical arrangements could be responsible for the synchronization of the CatSper opening as well as a more efﬁcient propagation of Ca$^{2+}$ waves over the length of ﬂagella (Fig. 1, B).

2.3. Function

The undeniable importance of CatSper during fertilization lies in the fact that null mice lacking any of CatSper isoforms are infertile (Jin et al., 2007; Quill et al., 2003, 2001; Ren et al., 2001) and men with mutations or deletions in CATSPER1, CATSPER2 and CATSPERE suffer from infertility (Avenarius et al., 2009; Avidan et al., 2003; Brown et al., 2018; Hildebrand et al., 2010; Luo et al., 2019; Smith et al., 2013; Williams et al., 2015; Zhang et al., 2007). To understand what makes CatSper-deﬁcient males infertile, it is important to summarize our current understanding of the role of CatSper in sperm fertilizing capacities.

2.3.1. Rheotaxis, thermotaxis, and chemotaxis

During their journey from ejaculation to fertilization, spermatozoa must travel a long distance and survive in a variety of environments characterized by diverse physical and chemical conditions (Fig. 2). Because of the long distance and the complexity of the convoluted ductal oviduct, several sperm guidance mechanisms exist employing short- and long-range cues enabling capacitated sperm to reach the oocyte. Most of these mechanisms were well described in sea urchin sperm from Arbacia Punctulata but our current knowledge on mammalian guidance mechanisms is still very sparse (Kaupp et al., 2006). Spermatozoa employ at least three different guidance mechanisms: (i) rheotaxis that refers to the directed movement against the fluid flow (Miki and Clapham, 2013), (ii) thermotaxis that refer to directed movement in a temperature gradient (Bahat et al., 2003) and (iii) chemotaxis that refers to the directed movement of a cell in a gradient of chemoattractant (Pabro et al., 2002; Oliveira et al., 1999; Ralt et al., 1994). While chemotaxis is a short-range mechanism acting at the order of the millimeter, rheotaxis and thermotaxis are long-range mechanisms guiding spermatozoa along the microtubule-based axoneme which comprises two central pairs of microtubules doublets and nine outer microtubule doublets forming the “9 + 2” pattern. Nine outer dense fibers (ODF) and the FS closely lying under the plasma membrane surrounding the axoneme as well as two peripheral longitudinal columns (LC) connected by circumferential ribs (Fig. 1, B).

Even though it is situated in the principal piece of the sperm flagellum, CatSper mediates calcium influx that propagates within seconds through the midpiece and the head (Xia et al., 2007). This calcium propagation is sustained in the sperm head and is thought to be implicated in the acrosome reaction (discussed in paragraph 2.3.3) (Xia and Ren, 2009a). A mathematical model developed to investigate this propagation proposes that CatSper mediated calcium influx induces additional calcium release from internal stores located in the sperm midpiece (Olson et al., 2010). A similar pattern has been observed when human sperm are stimulated with odorant such as bourgeois (Spehr et al., 2005). The physiologically relevance of this calcium propagation, in addition to being associated with acrosome reaction, is thought to induce hyperactive motility and regulate chemotaxis (discussed in paragraph 2.3). Using super-resolution ﬂuorescent microscopy, Chung and colleagues determined the distribution of CatSper proteins at a nanometer-scale resolution (Chung et al., 2017, 2014). CatSper channel complex is organized in quadrilateral longitudinal nanodomains that form a unique pattern of four linear ‘stripes’ running down the principal piece of the flagellum (Fig. 1, B). Each of these domains runs along each side of the two longitudinal columns of the sperm flagella. In addition, each of these four lines was found to be formed by two rows of CatSper complexes. It has been hypothesized that within a nanodomain, the CatSperζ and EFCAB9 subunits connect and interact with the neighboring CatSper complex either in a zigzag manner to the adjacent column or linearly in the same column to structurally and functionally link the CatSper complexes (Bystroff, 2018; Vylicka and Lishko, 2020). These hypothetical arrangements could be responsible for the synchronization of the CatSper opening as well as a more efﬁcient propagation of Ca$^{2+}$ waves over the length of ﬂagella (Fig. 1, B).
A: The female reproductive tract is composed of different regions and the sperm cells must constantly adapt to different environments throughout their journey from the vagina to the ampulla. The vagina is the site of deposition of semen and where sperms begin to migrate out of the seminal plasma and into the cervical mucus. Out of the millions of spermatozoa deposited in the vagina, only an estimated number of one hundred capacitated sperms will meet the egg. Capacitation is mainly induced by exposure of sperms to high concentrations of bicarbonate (HCO$_3^-$) and serum albumin. After this first selection process, most of the sperm cohort continues their way up to the uterus where sperm transport is facilitated via peristaltic contractions and rheotaxis. As they approach the egg at the ampulla, sperms are exposed to warmer temperature at the site of fertilization (37 °C at the ampulla), a guiding mechanism referred to as thermotaxis. B: High concentrations of progesterone secreted by the cumulus cells forms a gradient that guides the sperm when in close proximity to the egg through chemotaxis. Progesterone, as well as prostaglandins, activate CatSper leading to a rapid transient increase in intracellular calcium concentration ([Ca$^{2+}$]). CatSper activation is implicated in hyperactivated motility characterized by a star shaped, non-progressive trajectory in aqueous media. This will facilitate sperm penetration through the extracellular matrix of cumulus cells by mechanical shear forces. Sperms will then release their acrosomal content to digest the Zona Pellucida during the acrosome reaction and finally fertilize the egg.

2.3.2. Hyperactivation

Calcium influx through CatSper and mobilization of intracellular calcium stores are thought to be one of the key events triggering sperm hyperactivation (Lishko et al., 2011; Marquez et al., 2007a; Mortimer, 1997; Publicover et al., 2008; Strünker et al., 2011) (Fig. 2, B). After ovulation, the egg is stored for a short period in the ampulla of the oviduct, the location where fertilization occurs. The acquisition of hyperactive motility is essential for enabling the sperms to detach from the ciliary oviductal epithelium of the isthmus where they tend to form a reservoir, arrive at the ampulla at the right time and overcome the protective vestment of the oocyte (De Jonge, 2005; Demott and Suarez, 1992; Suarez, 2008). The current model explaining this high-amplitude
The complex CatSper channel is composed of ten subunits and is activated in a ligand dependent fashion – by steroids and prostaglandins – as well as in a ligand independent fashion via the diversified crosstalk with other channels and ion exchangers. In order to function properly, CatSper requires three concomitant activation mechanisms: 1) membrane depolarization, 2) intracellular alkalization and 3) presence of progesterone. As sperms approach to the site of fertilization, sperms are exposed to elevated temperature (37°C) which induce the activation of sodium channel TRPV4 (also called D sper) leading to membrane depolarization following sodium efflux. This activates Hv1 and CatSper leading to extrusion of protons by Hv1 and further activation of Catsper due to intracellular alkalization. Concomitantly, progesterone binds to ABHD2 and releases CatSper inhibition by 2-AG promoting the calcium influx through CatSper and the potassium efflux through the potassium channel Slo3 (also called KSper). Influx of calcium induces hyperactive motility and efflux of potassium, leads to membrane hyperpolarization and regulation of CatSper in a negative feedback loop mechanism.

2.3.3. Acrosome reaction

The AR allows the sperm, by the release of proteolytic enzymes, to penetrate the cumulus oophorus and the ZP surrounding the oocyte (Fig. 2, B) (Jin et al., 2011). This reaction is a prerequisite for spermatozooa to successfully fertilize the egg and it is known to be strictly dependent on an increase in $[Ca^{2+}]_i$ (Yanagimachi, 1994). Different theories exist on the nature of the inducers of the human acrosome reaction physiologically. It is generally believed that AR is triggered by sperm contact with the zona pellucida, although it has been shown that most fertilizing spermatozoa in mouse undergo the AR already within the cumulus oophorus that surrounds the newly ovulated mouse egg (Bedford, 2011; Inoue et al., 2011; Jin et al., 2011; Sun et al., 2011). However, it seems that in humans only acrosome intact sperm cells can undergo hyperactivation in human sperm suggesting that CatSper is involved in their ability to acquire the whip-lash movement (Rennhack et al., 2018).

Murine sperms incubated with a calcium-free medium are able to undergo hyperactivation by mobilization of stored calcium through the IP3R that were shown to localize in the sperm neck region (Marquez et al., 2007b). A plasma membrane calcium-ATPase 4 (PMCA 4), localized in the principal piece of the flagellum, is responsible for eliminating calcium that is also crucial for sperm motility and acquiring hyperactivation. Indeed, mitochondrial abnormalities found in PMCA 4-deficient spermatozoa lead to a calcium overload resulting in male infertility due to defective calcium extrusion (Okunade et al., 2004). Calcium is therefore not only required for the initiation of hyperactivation but also to its maintenance by directly regulating components of the acrosomal machinery (Darson et al., 2007).
bind to the ZP (Liu et al., 2006). While the classical view proposes the zona pellucida proteins, mainly ZP3, to be the main inducer of mammalian AR, picomolar concentrations of progesterone secreted by the cumulus cells is also known to trigger a wave of increase in \([Ca^{2+}]_i\) and promote priming and AR (Meizel et al., 1997; Unates et al., 2014). We can distinguish two types of calcium signaling during this unique, tightly regulated and irreversible exocytotic process; initial calcium rise upon binding of sperm to ZP or in response to progesterone activation followed by another sustained calcium rise due to calcium release from intracellular stores (Darszon et al., 2011). It has been proposed that CatSper is responsible for the initial transient \(Ca^{2+}\) rise (Singh and Rajender, 2015). CatSper mediated calcium influx into the flagellum then leads to a calcium elevation in the sperm head probably by causing calcium-dependent release from the intracellular store located in the sperm neck (Singh and Rajender, 2015; Xia et al., 2007). The role of the CatSper channel in inducing mammalian AR is still however highly debatable. In a study showing a tail-to-head \(Ca^{2+}\) propagation in mouse sperm, the CatSper-mediated \([Ca^{2+}]_{i}\) increase seemed not to be required for AR (Xia et al., 2007). In another more recent study aiming at understanding the relation between acrosome reaction and CatSper-mediated calcium influx in human sperm, the effect of progesterone on AR was shown to be blunted in the presence of T-type channel blockers NNC at the same concentrations that inhibit 80% of the progesterone-stimulated calcium influx (Tamburrino et al., 2014). This suggests that CatSper-mediated calcium influx could be regulating the acrosome reaction in humans but more experiments are needed to establish the role of CatSper in this process (Beltrán et al., 2016).

### 2.4. Regulation and cross-talks

The application of patch-clamp techniques to measure CatSper currents that were adapted to human sperm less than a decade ago paved the way to the elucidation of the regulation of this highly complex channel (Kirchok et al., 2006; Kirchok and Lishko, 2011; Lishko et al., 2011; Strünker et al., 2011). We can divide the mechanisms leading to CatSper activation in two fashions, the first is ligand-dependent and the second is ligand-independent. Both mechanisms discussed below are activated simultaneously and are highly interconnected.

#### 2.4.1. Ligand-dependent pathway

Progesterone: the nanomolar concentrations of progesterone produced and released by the cumulus cells surrounding the oocyte has long been known to play a central role in human sperm fertilizing capacities by acting primarily on acrosome reaction and sperm motility (Blackmore et al., 1990; Forti et al., 1999). However, the unknown mechanisms by which progesterone act on sperm have fascinated and, at the same time, frustrated reproductive scientists. Progesterone classically acts in a genomic fashion by binding to a nuclear receptor and initiating gene transcription, but spermatozoa have a highly condensed genome and are transcriptionally inactive (Baldi et al., 2009). Various mechanisms have been proposed to explain this “non-genomic” action of progesterone on sperm. The mechanisms mainly involved G-protein coupled progestin receptors (mPR), a putative membrane-associated progesterone steroid receptor PGRMC1 (Progesterone Receptor Membrane Component 1), as well as second messengers such as cAMP, cGMP, PKA, PKG - induced calcium release from intracellular stores, SOC and cGMP channels. However, the molecular mechanisms by which progesterone acts on sperm have remained elusive despite considerable efforts by the scientific community (Baldi et al., 2009). The discovery of CatSper as the principal calcium channel in sperm (Ren et al., 2001) along with the application of patch-clamp techniques in human sperm have finally led to the discovery of the progesterone’s gateway into sperm (Publicover and Barratt, 2011). Progesterone, along with an alkaline pH, stimulates a rapid calcium entry with almost no latency that is incompatible with a signaling pathway involving metabotropic receptors and second messengers. Using the patch-clamp technique as well as optical fluorimetry in sperms loaded with a calcium indicator, progesterone was shown to induce a biphasic signal of a rapid and transient calcium increase followed by a slower sustained calcium elevation (Strünker et al., 2011). The T-type channel blockers, mibebradil, and NNC, significantly altered the progesterone-induced calcium response. The constants of half-maximal activation \((K_i)\) of progesterone-induced increase in calcium were shifted to lower concentration in capacitated sperm suggesting that capacitation renders sperm more sensitive to progesterone (Strünker et al., 2011). Using the patch-clamp, Lishko and colleagues demonstrated that the addition of progesterone dramatically increased the amplitude of human monovalent CatSper current \((I_{catSper})\) (Lishko et al., 2011). After the action of progesterone and PGE1 on human sperm was proved to be “non-genomic” because of the rapid activation rate, it became clear that progesterone either binds directly to CatSper or to a receptor that is directly associated with the channel (Strünker et al., 2011). Progesterone was later proved to act on CatSper via a membrane endocannabinoid signaling pathway through the \(\alpha/\beta\) hydrolase domain-containing protein 2 (ABHD2). At rest, CatSper is thought to be inhibited by the endocannabinoid 2-arachidonoylglycerol (2-AG) in the flagellar membrane. Upon binding of progesterone, ABHD2 degrades 2-AG and thereby relieves CatSper from inhibition (Miller et al., 2016). It is noteworthy to mention that progesterone activates CatSper only in human and macaque sperm and does not induce any increase in \([Ca^{2+}]_i\) in mouse sperm (Lishko et al., 2011; Strünker et al., 2011; Sumigama et al., 2015).

Prostaglandins (PGs): these compounds derived from arachidonic fatty acids are abundant in the seminal plasma and are secreted by the oviduct and cumulus cells surrounding the oocyte. In addition to progesterone, prostaglandin E1 (PGE1) has also been shown to act through CatSper and increase intracellular calcium concentration in a very similar biphasic manner with similar amplitude and potentiates the \(I_{catSper}\) (Lishko et al., 2011; Strünker et al., 2011). The large current recorded in the presence of PGE1 was fully inhibited by NNC similarly to the calcium influx measured with fluorimetry. The addition of saturating concentration of PGE1 (2 \(\mu\)M) to sperm cells already potentiated by progesterone resulted in a further increase in the amplitude of \(I_{catSper}\) and vice versa. This suggests that although progesterone and PGE1 activate the same channel, they have two distinct binding sites (Kaupp and Strünker, 2017; Lishko et al., 2011; Lishko and Mannowetz, 2018; Miller et al., 2015; Strünker et al., 2011). Consistently, it has been recently reported that PGs and progesterone act in a highly synergistic manner (Brenker et al., 2018a). Other PGs also induced an increase in intracellular calcium concentration but with different signal amplitudes such as PGE1 and PGD2. The relative effects of ligands activating human CatSper can be classified as follows: Progesterone > PGE1 > PGD2 > PGF2 (Lishko et al., 2011; Miller et al., 2015). Similarly to progesterone, PGE1 does not induce a calcium influx in mouse sperm underlining once more the important differences between human and mouse CatSper regulation (Lishko and Mannowetz, 2018). For a long time, PGs were thought to bind to a prostanoid receptor on human sperm (Schaefer et al., 1998) but this is incompatible with the biphasic signal of PG-induced intracellular calcium increase and the pharmacology of the PGE1 response as measured by fluorimetry (Strünker et al., 2011). The mechanism of action by which PGs act on CatSper thus remains elusive and does not involve ABHD2 (Kaupp and Strünker, 2017).

Cyclic nucleotide cAMP and cNG: in intact mouse spermatozoa, membrane-permeable analogs of cAMP and cGMP (such as 8-Br-cAMP and 8-Br-cGMP) have been shown to activate CatSper-dependent calcium entry and neither cAMP nor cGMP was shown to elevate \([Ca^{2+}]_i\) in CatSper-null mice (Ren et al., 2001). It was therefore originally proposed that the CatSper channel can be directly or indirectly activated by cyclic nucleotides (Ren et al., 2001). The following discoveries could not demonstrate a direct \([Ca^{2+}]_i\) increase in response to cyclic nucleotides, to photolysis of caged cAMP or in response to bicomponent-induced elevation of cAMP (Brenker et al., 2012; Strünker et al., 2011; Wennemuth et al., 2003). CatSper-dependent increase in \([Ca^{2+}]_i\) induced by
alkalization and high [K⁺] was however strongly facilitated by the presence of bicarbonate that is known to increase cAMP levels through sAC (Carlson et al., 2003; Xie et al., 2006). In mouse, patch-clamp recordings of CatSper currents were not affected by cyclic nucleotides (Kirichok et al., 2006) suggesting that the facilitation of CatSper by cyclic nucleotides is indirect and involves an intermediary signaling cascade that is disrupted during patch-clamp recordings (Kirichok and Lishko, 2011). It has been therefore proposed that cAMP facilitates CatSper-dependent calcium increase via PKA-dependent phosphorylation (Orta et al., 2018).

Albumin and Zona Pellucida glycoproteins: cholesterol efflux by serum albumin is a key component in sperm cell capacitation and the influx of calcium during this process has been linked directly to the presence of CatSper within sperm cells (Xia and Ren, 2009b). Indeed, Bovine Serum Albumin (BSA) - induced [Ca²⁺]i increase was shown by patch-clamp recordings to be absent in CatSper-deficient sperm. The mechanisms by which BSA is coupled to the CatSper channel are still largely unknown (Ren and Xia, 2010; Sun et al., 2017). Application of solubilized ZP glycoproteins induces an intracellular calcium increase that is known to be involved in inducing a change in motility and allowing the release of the sperm acrosomal content, which is crucial for penetrating the egg. A suggested role for CatSper in the early phase of ZP-induced increase in [Ca²⁺]i, was proposed. In CatSper 1-deficient mice sperm, the early calcium response induced by solubilized ZP glycoproteins is absent and can be restored with exogenous expression of Green Fluorescent Proteins (GFP)-tagged CatSper 1 protein (Xia and Ren, 2009a). The mechanisms involved in transducing ZP-induction to the opening of CatSper channels are currently unknown (Ren and Xia, 2010).

2.4.2. Ligand-independent pathway

Intracellular alkaline pH: intracellular pH is a critical regulator of sperm activity, from the moment they are released in the lumen of the seminiferous tubules until they reach and fertilize the egg. While stored in the epididymis, the sperm bath in an acidic milieu (pH = 5.5–6.8) making their intracellular pH (pHi) also acidic (~6) that helps them remain quiescent before ejaculation (Acott and Carr, 1984; Jones and Bavister, 2000). Upon entering the female reproductive tract, spermatozoa are exposed to a higher extracellular pH (pHe) of around 7 due to the presence of a high concentration of Na⁺ that elevates the sperm pHi to about 6.5 as they first become motile (Carr and Acott, 1989; Kirichok and Lishko, 2011). During their subsequent transit, pHi further increases as a result of sperm capacituation but remains below pHe. This increase in pH has been shown to activate the CatSper channel in mice and humans (Kirichok et al., 2006; Lishko et al., 2011; Strümker et al., 2011). Application of the patch-clamp technique revealed that intracellular alkaline pH strongly potentiates the murine CatSper current (ICatSper) by inducing a seven-fold increase leading to an increase in flagellar [Ca²⁺]f and sperm hyperactivation (Kirichok et al., 2006). In humans, intracellular alkalization by the simple addition of NH₄Cl is sufficient to induce calcium influx and potentiate ICatSper independently of progesterone that does not alter pHi (Strümker et al., 2011). Even if murine and human CatSper1 proteins are highly enriched in histidine which is an indication of a channel’s pH sensitivity, intracellular alkalization alone seems to be sufficient to open murine CatSper but not human CatSper which also depends on activation by ligands as previously discussed. One of the major players regulating the intracellular pH in human but not in mouse sperm is the voltage-gated proton channel Hv1 that shows a pH-dependent inward rectification (Kirichok et al., 2006). Hv1 is the dominant proton conductance channel (Lishko et al., 2010). It allows only outward transport of protons and is therefore dedicated to inducing intracellular alkalization. Human sperm was also shown to hold a shorter isoform of Hv1 termed Hv1Sper (Berger et al., 2017). It is generated from Hv1 by the removal of 68 amino acids from the N-terminus by post-translational proteolytic cleavage. In both channels, the conductance-voltage relationship is determined by the pH difference across the membrane (ΔpH). However, a constant ΔpH activates only Hv1Sper but not Hv1. This suggests that cleavage of the N-terminus regulates pH sensing in Hv1 (Berger et al., 2017). Hv1 is activated by diverse mechanisms: 1) membrane depolarization, 2) an alkaline extracellular environment similar to the one in the female reproductive tract (that can reach up to pH = 8), 3) endocannabinoid anandamide, and 4) removal of extracellular zinc that is a potent Hv1 blocker (Lishko et al., 2010; Lishko and Kirichok, 2010). In mouse, the sperm-specific sodium/proton exchanger (sNEH) is activated by membrane hyperpolarization and seems to be responsible for intracellular alkalization and consequently CatSper activation (Wang et al., 2007, 2003).

Change in the membrane voltage: the same initial patch-clamp recordings of the CatSper currents also revealed that murine CatSper is weakly voltage-dependent with a slope factor (k) of 30 compared to a much steeper k of about 4 in solely voltage-gated ion channels (Kirichok et al., 2006). The low voltage sensitivity of CatSper can be explained by the low number of positively charged residues in the S4 transmembrane domain of the other CatSper subunits in contrast to CatSper 1 (Navarro et al., 2009; Qi et al., 2007). Human ICatSper recordings revealed fundamental differences with their rodent’s counterpart. The voltage dependence of human CatSper is slightly steeper (k = 20) and half activation voltage in human sperm is strikingly higher with V1/2human = +85 mV compared to V1/2mouse = +11 mV at the same intracellular pH (7.5) (Kirichok et al., 2006; Lishko et al., 2011). This again suggests that the regulation of mouse and human CatSper is fundamentally different. Although the CatSper channel has only weak voltage dependence, it is still very important to the optimal functioning of the channel since it is directly connected to the channel’s pH sensitivity and the activation of pH-regulating channels such as Hv1. At low intracellular pH, the CatSper voltage dependence regulates the channel status by keeping it closed at sperm physiological membrane potentials (from ~70 mV to 0 mV). An increase in intracellular pH significantly shifts murine CatSper voltage dependence towards the negative membrane potentials allowing the channel to open by lower potentials in the physiological range (Kirichok et al., 2006). The principal regulator of membrane potential is attributed to the potassium permeability that is regulated by two members of the Slo family of potassium channels: Slo1 and Slo3 (Mannowetz et al., 2013; Navarro et al., 2008; Schreiber et al., 1998). The principal potassium channel in mouse - Slo3 - is pH-sensitive, calcium-independent, and voltage-sensitive (Brenker et al., 2014; Santi et al., 2010; Schreiber et al., 1998). Slo3 deficient mice display severely reduced male fertility (Santi et al., 2010; Zhang et al., 2006). In contrast to mouse, human sperm potassium current (KSper) is pH-independent, sensitive to [Ca²⁺]f and can be inhibited by progesterone (Mannowetz et al., 2013). In humans, calcium rather than pHi was shown to control KSper suggesting that the prototypial Ca²⁺ - regulated K⁺ channel regulating human KSper is Slo1. However, human Slo3 is also activated by calcium rather than alkalization and is abundantly present in the human sperm flagellum. This suggests that it is rather Slo3 that represents the principal K⁺ channel in human sperm that carries the Ca²⁺-activated IKsper Current (Brenker et al., 2014). The efflux of potassium through Slo3 was therefore proposed to regulate CatSper by leading to the membrane hyperpolarization that in turn inhibits the progesterone-evoked Ca²⁺ influx through CatSper (Brenker et al., 2014).

2.4.3. CatSper is promiscuous

Under physiological conditions, progesterone, and PGs, together with membrane voltage and intracellular pH, act collectively to regulate calcium entry into sperm via the human CatSper. However, CatSper is also modulated by various endogenous or exogenous compounds and is known for its promiscuity. In particular, CatSper appears to be regulated by several endogenous steroids. Pregnenolone sulfate exerts effects similar to those of progesterone, while physiological concentrations of testosterone, hydrocortisone and estradiol were first shown to act as antagonists to CatSper and to reduce or prevent CatSper activation by progesterone (Mannowetz et al., 2017). However, these results could not be reproduced by Bremer et al. whose data lead to entirely different
conclusions. Testosterone, hydrocortisone, and estradiol were shown to enhance CatSper currents, with varying potency and efficacy. In addition, the three steroids were shown not to antagonize the progesterone-induced CatSper currents (Brenker et al., 2018b). Similarly, it has been shown that in human follicular fluid unidentified compounds other than progesterone contribute to the increase of \([\text{Ca}^{2+}]_i\) and modulate CatSper activation (Brown et al., 2019). Concerning exogenous ligands, molecules as diverse as a variety of odorants can directly act on CatSper without involving G protein-coupled receptors (GPCRs) or cAMP (Brenker et al., 2012). It has also been shown that endocrine-disrupting chemicals (EDCs) such as chemical UV filters act directly on CatSper and induce an increase in \([\text{Ca}^{2+}]_i\) (Brenker et al., 2018b; Rehfeld et al., 2017, 2016; Schiffer et al., 2014; Shannon et al., 2016; Tavares et al., 2013; Yuan et al., 2020). Premature activation of the CatSper channel by EDCs can potentially desensitize the sperm to physiological ligands such as progesterone and prostaglandins and may, therefore, alter the precisely coordinated sequence of fertilization. With this in mind, taking advantage of the promiscuous binding to a wide range of compounds renders CatSper an excellent target for new non-hormonal contraceptives. Two potential examples were thought to be good candidates such as the plant triterpenoids Pristimerine and Lupeol, which are similar in structure to steroid hormones. Both compounds were shown to inhibit CatSper activation by progesterone, sperm hyperactivation, and slightly reduce basal motility of capacitated human sperms (Mannowetz et al., 2017; Brenker et al., 2018b). However, these observations could not be reproduced by Brenker et al. who showed that both compounds did not affect the progesterone-induced \(\text{Ca}^{2+}\) influx (Brenker et al., 2018b). This indicates that further studies are needed to address the promiscuous steroid-binding side controlling CatSper. Regulation of CatSper is critical for proper sperm function and compounds that directly affect CatSper or influence calcium signaling pose a genuine threat to sperm fertilization potential (Miller et al., 2015).

2.4.4. Integrated model

As sperms enter the female reproductive tract, they are first exposed to a high concentration of bicarbonate and albumin, two main players in initiating capacitation by stimulating activation of the sAC/PKA phosphorylation pathway and cholesterol efflux, respectively. At the time of ejaculation, sperms are combined with the seminal fluid, which contains high enough concentration of zinc to block the activity of the Hv1 channel, by binding to two histidine residues that stabilize the channel in the closed state (Cherny and DeCoursey, 1999; Lishko and Kirichok, 2010). As sperms ascend from the vagina through the cervix and into the upper reproductive tract, divalent zinc ions are chelated by proteins present in the oviducal fluid resulting in gradual activation of Hv1 (Lishko et al., 2010; Lu et al., 2008). PKA also phosphorylates Hv1 further activating the channel and potentiating the efflux of protons (Puga Molina et al., 2018). In order to function properly, CatSper of human sperm requires three concomitant activation mechanisms: 1) membrane depolarization, 2) intracellular alkalinization and 3) the presence of prostaglandin. As sperms approach the site of fertilization, they are first exposed to high concentrations of progesterone close to a high concentration of bicarbonate and albumin. As sperms approach the site of fertilization, they are first exposed to high concentrations of progesterone close to a high concentration of bicarbonate and albumin. This leads to membrane hyperpolarization and regulation of CatSper in a negative feedback loop mechanism (Fig. 3).

3. Major challenges, remaining questions, and perspectives

3.1. Challenge: functional expression of CatSper

The regulation of mammalian CatSper by the various stimuli as well as the mechanisms by which channel activity is regulated is far from being completely understood for a variety of reasons. First because the functional expression of CatSper in any heterologous systems has not yet been achieved since the channel is resistant to functional expression (Darzson et al., 2011). This inability to express the channel in other cell types may be related to the complexity of the multi-subunit constituents of the channel. Alternatively, it is also possible that this channel requires a yet unknown essential subunit for the assembly of a functional CatSper complex. Occurrence and localization of CatSper were indeed shown to be coordinated with the assembly of the FS proteins along the axoneme (Chung et al., 2017; Smith et al., 2013). It has been also shown that it is only after the different CatSper subunits are properly positioned on the flagellum and are docked to underlying axonemal structures that the channel becomes functional (Lishko et al., 2012). The last two CatSper subunits - EFCAB9 and CatSper \(\zeta\) - were relatively recently identified (Chung et al., 2017; Hwang et al., 2019). Second, the human and murine CatSper shows significant differences in both their regulation and ligand bindings, which certainly reflects an adaptation of sperm to the local environment of the female reproductive tract. While both murine and human CatSper are activated at alkaline pH and are weakly voltage-sensitive (Lishko et al., 2011; Strünker et al., 2011), only the human and macaque CatSper are sensitive to progesterone (EC50 = 10 nM) and prostaglandins (EC50 = 8 nM) ((Lishko et al., 2012; Sumigama et al., 2015). Third, the application of the patch-clamp technique was first made possible in 2006 on mouse sperm and was only introduced to human sperm less than a decade ago which is relatively a short period of time (Kirichok et al., 2006; Lishko et al., 2011; Strünker et al., 2011). Finally, the lack of suitable pharmacological tools has always hindered the elucidation of CatSper function. It is only until recently that a specific inhibitor of CatSper (RU1968) was introduced to help better understand the pharmacological regulation of this complex channel (Rennhack et al., 2018).

3.2. CatSper as a pharmacological target for male-directed contraception

Due to its unique composition, specific expression in sperm, and central role in male fertility, the CatSper channel is a prime target for infertility treatment or male contraception. Early studies have already explored the contraceptive potential of Anti-CatSper1 IgG (Li et al., 2009) or the Ca\(^{2+}\) channel blocker HC-056456 that has been shown to block CatSper channel activity and reversibly prevented hyperactivated motility of capacitated sperm (Carlson et al., 2009). We can expect that in the coming years several studies testing the ability of pharmacological or natural compounds to act as CatSper agonists or inhibitors will be identified and made available to the scientific community. Using a high-throughput screening technique, thousands of ion channels ligands were assessed for their ability to induce calcium signaling and improve sperm motility (Martins Da Silva et al., 2017). This allowed the identification of a phosphodiesterase inhibitor (PDE3), the trequinsin hydrochloride, that was shown to act as an efficacious agonist of CatSper, and increase sperm hyperactivation and penetration into viscous medium (McBrinn et al., 2019). These pharmacological tools will not only provide additional insights into the properties, regulation, and role of the CatSper channel, but also offer a potential approach for male contraception.
CatSper channel but will also allow us to evaluate their potential as CatSper-targeting drugs for the treatment of infertility or as non-hormonal male contraceptives.

3.3. Remaining questions

Although the role of [Ca\textsuperscript{2+}]\textsubscript{i} signaling in sperm physiology is central, several questions remain unanswered regarding CatSper and its role in mediating sperm function. Future studies should address the precise organization of the CatSper channels along the four longitudinal nanodomains in the principal piece of the flagellum and how this arrangement allows synchronization of CatSper opening, making efficient propagation of Ca\textsuperscript{2+} waves possible. The translation of these waves into phosphorylation cascades and structural changes underlying hyperactivated asymmetrical motility are still also to be clarified. It is also important to better understand the species differences between human and rodent CatSper and to identify the other endogenous CatSper ligands acting as a chemoattractant for sperm chemotaxis. In terms of channel regulation, both progesterone and PGs were shown to act on CatSper synergistically and are equally important in inducing calcium downstream events leading to fertilization (Brenker et al., 2018a).

The mechanism of action by which progesterone acts on CatSper to elevate [Ca\textsuperscript{2+}]\textsubscript{i} has been previously described and is thought to be mediated through ABHD2 (Miller et al., 2016). However, the mechanism of CatSper activation by prostaglandins remains elusive and the relation with the pH-control of the channel is not yet understood. Indeed, the specific role of both progesterone and PGE1 during fertilization has not yet been fully established (Baldi et al., 2009). This is mainly due to the experimental challenges faced in reproducing the chemical composition of the female reproductive tract on one hand and the lack of appropriate pharmacological tools to decipher the complex crosstalk of ion channels involved in regulating sperm physiology on the other hand (Suarez and Pacey, 2006; Suarez, 2008; Barratt and Publicover, 2012). In addition, the precise role of membrane potential in regulating CatSper in human sperm is still not yet elucidated and needs further exploration (Brenker et al., 2014; Mannowitz et al., 2013). Mechanisms of CatSper silencing in the male reproductive tract and activation in the female genital tract also need further investigation.

Acknowledgments

The authors are grateful to the graphic designer Valentin Durand for assistance with the artwork. This work was supported by the Swiss Centre for Applied Human Toxicology (SCAHT) and by the Département de l’Instruction Publique of the State of Geneva.

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