

Rediscovering sperm ion channels with the patch-clamp technique

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ABSTRACT: Upon ejaculation, mammalian spermatozoa have to undergo a sequence of physiological transformations within the female reproductive tract that will allow them to reach and fertilize the egg. These include initiation of motility, hyperactivation of motility and perhaps chemotaxis toward the egg, and culminate in the acrosome reaction that permits sperm to penetrate the protective vestments of the egg. These physiological responses are triggered through the activation of sperm ion channels that cause elevations of sperm intracellular pH and Ca^{2+} in response to certain cues within the female reproductive tract. Despite their key role in sperm physiology and their absolute requirement for the process of fertilization, sperm ion channels remain poorly understood due to the extreme difficulty in application of the patch-clamp technique to spermatozoa. This review covers the topic of sperm ion channels in the following order: first, we discuss how the intracellular Ca^{2+} and pH signaling mediated by sperm ion channels controls sperm behavior during the process of fertilization. Then, we briefly cover the history of the methodology to study sperm ion channels, which culminated in the recent development of a reproducible whole-cell patch-clamp technique for mouse and human cells. We further discuss the main approaches used to patch-clamp mature mouse and human spermatozoa. Finally, we focus on the newly discovered sperm ion channels CatSper, KSper (Slo3) and HSper (H_v1), identified by the sperm patch-clamp technique. We conclude that the patch-clamp technique has markedly improved and shifted our understanding of the sperm ion channels, in addition to revealing significant species-specific differences in these channels. This method is critical for identification of the molecular mechanisms that control sperm behavior within the female reproductive tract and make fertilization possible.

Key words: sperm motility / patch clamp / Ca^{2+} / H^+ / K^+ ion channels / hyperactivation / acrosome reaction

Introduction

Intracellular Ca^{2+} and intracellular H^+ are two key messengers with opposite effects on sperm activity and fertilizing ability: increasing intracellular $[\text{Ca}^{2+}]$ stimulates sperm motility and fertility (Darszon *et al.*, 2005; Publicover *et al.*, 2007; Fraser, 2010), whereas increasing intracellular $[\text{H}^+]$ inhibits them (Babcock *et al.*, 1983; Carr and Acott, 1989; Florman *et al.*, 1989, 1992; Hamamah and Gatti, 1998; Suarez, 2008). Together with intracellular cAMP (Morton *et al.*, 1974; Tash and Means, 1983; Okamura *et al.*, 1985; Brokaw, 1987; Visconti *et al.*, 1995; Fraser, 2010), these two antagonistic intracellular messengers control the most important aspects of sperm behavior in the female reproductive tract: initiation of sperm motility upon ejaculation, sperm capacitation, hyperactivation, chemotaxis and the acrosome reaction. Sperm intracellular $[\text{Ca}^{2+}]$ and $[\text{H}^+]$ are primarily determined by Ca^{2+} and H^+ channels and transporters of the sperm plasma membrane (Darszon *et al.*, 2006a); however, intracellular Ca^{2+} stores such as the acrosome vesicle and the redundant nuclear envelope may also contribute to sperm intracellular Ca^{2+} signaling (Ho and Suarez, 2003; Costello *et al.*, 2009). Unfortunately, the

lack of direct methods to study ion channels and transporters of the sperm plasma membrane has hampered our understanding of the molecular mechanisms that regulate sperm activity and male fertility.

The invention of the patch-clamp technique by Neher and Sakmann by the beginning of the 1980s (Hamill *et al.*, 1981) initiated a revolution in our understanding of plasma membrane ion channels and transporters of somatic cells. However, a similar routine application of the patch-clamp technique to spermatozoa was considered impossible due to their small size, vigorous motility and tight association of the plasma membrane with rigid intracellular structures. Only recently, in 2006, was this technical problem resolved for mouse sperm cells with the development of a method that allowed reproducible patch-clamp recording from whole sperm plasma membrane (Kirichok *et al.*, 2006). A few years later, a similar approach was successfully used to patch-clamp human spermatozoa, opening new opportunities to study the molecular mechanisms of male fertility, specifically in humans (Lishko *et al.*, 2010).

After an overview of sperm Ca^{2+} and H^+ signaling, this review will summarize the main approaches used to study the ion channels responsible for this signaling. We will specifically focus on the recently

developed sperm patch-clamp technique and how it has changed our understanding of sperm ion channels. Because the application of the patch-clamp technique has demonstrated that properties of sperm ion channels differ significantly between species, in this review, we focused on the physiology of human spermatozoa. Physiological properties of sperm cells from other species are considered only when they are likely to apply to human spermatozoa and no data on human cells are available.

General principles of sperm intracellular H^+ and Ca^{2+} signaling

Resting intracellular concentrations of Ca^{2+} and H^+ within the sperm cell are primarily set by ion pumps that use the energy stored in ATP (directly or indirectly) to establish concentration gradients for these two ions across the plasma membrane. Thus, while the concentration of Ca^{2+} in the extracellular medium is $\sim 1\text{--}2$ mM, its resting concentration in the sperm cytosol is only $\sim 100\text{--}200$ nM (Babcock and Pfeiffer, 1987; Linares-Hernandez *et al.*, 1998; Wennemuth *et al.*, 2000). In contrast, the concentration of protons seems to be always higher inside the sperm cell than outside with resting $\Delta pH \approx 0.4\text{--}1.0$ (Babcock and Pfeiffer, 1987; Carr and Acott, 1989; Florman *et al.*, 1989; Vredenburg-Wilberg and Parrish, 1995; Hamamah *et al.*, 1996; Zeng *et al.*, 1996; Hamamah and Gatti, 1998). While mammalian spermatozoa are stored in the caudal epididymis before ejaculation, pH of the extracellular fluid varies between 5.5 and 6.8 depending on species (Acott and Carr, 1984). Since sperm pH_i is always more acidic, it should be round 6.0 or lower ($[H^+]_i > 1 \mu M$) for the majority of mammalian species in this portion of the male reproductive tract. These pH values are significantly lower than those normally found within somatic cells (Roos and Boron, 1981), and help in rendering spermatozoa quiescent before ejaculation. Upon ejaculation spermatozoa are exposed to higher extracellular pH (average $pH_o \approx 7.0$ or higher), and their pH_i is also elevated to about 6.5 ($[H^+]_i \approx 0.3 \mu M$), as they become motile for the first time (Babcock *et al.*, 1983; Carr and Acott, 1989; Hamamah *et al.*, 1996; Zeng *et al.*, 1996). During subsequent transit through the female reproductive tract, the sperm pH_i further increases as the result of sperm capacitation (Parrish *et al.*, 1989; Vredenburg-Wilberg and Parrish, 1995; Zeng *et al.*, 1996; Suarez, 2008), but it remains lower than pH_o . In the female reproductive tract, pH_o is alkaline (except for vagina) and can reach up to 8.4 ($[H^+]_o = 4$ nM) in cervical mucus and 7.8 ($[H^+]_o = 16$ nM) in the Fallopian tubes depending on species and the phase of the female cycle (Acott and Carr, 1984).

The high resting $[H^+]_i$ and low resting $[Ca^{2+}]_i$, established by H^+ and Ca^{2+} pumps of the sperm plasma membrane suppress the activity of the sperm cell. Activation of spermatozoa requires reduction of $[H^+]_i$ and elevation of $[Ca^{2+}]_i$, which is easy to achieve due to the steep resting concentration gradients for H^+ and Ca^{2+} across the sperm plasma membrane. Indeed, merely opening the H^+ and Ca^{2+} ion channels of the sperm plasma membrane would be sufficient to allow H^+ to exit and Ca^{2+} to enter the sperm cytosol down their respective concentration gradients. The opening of the sperm H^+ and Ca^{2+} channels must be controlled by specific cues of the female reproductive tract to regulate the activity of the sperm cells

in accordance with their position within the tract and depending on the phase of the menstrual cycle. Among the cues of the female reproductive tract that can activate sperm ion channels are progesterone released by ovaries and cumulus cells surrounding the egg, glycoproteins of the zona pellucida and the major protein of the oviductal fluid, albumin (Darszon *et al.*, 2005, 2006a; Publicover *et al.*, 2007; Xia and Ren, 2009a, b; Fraser, 2010; Ren and Xia, 2010). However, there are probably more factors of the female reproductive tract yet to be discovered that directly or indirectly control the activity of sperm ion channels and help us to synchronize the arrival of the egg and the sperm at the site of fertilization (the ampulla of the oviduct).

Both sperm ion pumps and ion channels that participate in controlling $[H^+]_i$ and $[Ca^{2+}]_i$ are required for the regulation of sperm function. For example, sperm contain two flagellar Ca^{2+} transport proteins: a Ca^{2+} -ATPase (PMCA4) that pumps Ca^{2+} out of the sperm cell to support low resting intraflagellar $[Ca^{2+}]_i$ (Okunade *et al.*, 2004; Schuh *et al.*, 2004) and a Ca^{2+} channel CatSper (Ren *et al.*, 2001; Kirichok *et al.*, 2006; Qi *et al.*, 2007) that allows extracellular Ca^{2+} to enter the sperm flagellum. The balance between the activities of these two proteins sets the concentration of Ca^{2+} in the sperm flagellum, and male mice deficient in either PMCA4 or CatSper are infertile and have impaired sperm motility (Ren *et al.*, 2001; Okunade *et al.*, 2004; Schuh *et al.*, 2004; Qi *et al.*, 2007). However, pumps transport ions much more slowly than channels and their activity can be overwhelmed by ion channels that operate orders of magnitude faster (Hille, 1992). Pumps also cause relatively slow changes in the intracellular ion concentrations, whereas ion channels are primarily responsible for rapid signaling events.

It should also be noted that due to the significant length (usually $> 50 \mu m$) and miniscule cytoplasmic cross-section (much $< 1 \mu m^2$ throughout) of the sperm cell, diffusion between different parts of the sperm cell, such as the head and the principal piece, is limited. An extremely small amount of sperm cytosol squeezed in between the plasma membrane and the intracellular structures further contributes to the spatial separation of the different cellular compartments. Moreover, it has been demonstrated that the expression of sperm ion channels is highly compartmentalized and differs significantly between the head and the principal piece (Ren *et al.*, 2001; Navarro *et al.*, 2007; Qi *et al.*, 2007; Lishko *et al.*, 2010). Thus, intracellular H^+ and Ca^{2+} signaling within the sperm head and the flagellum are controlled by different mechanisms and are spatially isolated from each other to allow for the independent control of Ca^{2+} and pH-dependent physiological responses occurring in the head and the flagellum, such as the acrosome reaction and hyperactivation.

Intracellular Ca^{2+} and H^+ as regulators of sperm activity in the female reproductive tract

The female reproductive tract presents multiple barriers for the sperm cells on their route to the egg (Suarez and Pacey, 2006). Female immune responses, viscous cervical mucus, the thin uterotubal junction, sticky oviductal epithelium covered with mucus, the maze of the oviductal epithelial folds near the site of fertilization and egg's protective vestments all markedly reduce a sperm's chances to reach and fertilize the egg (Suarez and Pacey, 2006). Although millions of

spermatozoa are introduced into the female reproductive tract, only a few will eventually traverse the viscous environment of the female reproductive tract to arrive at the site of fertilization, and only one will penetrate through the egg's cumulus oophorus (CO) and zona pellucida to fertilize the egg. To reach and fertilize the egg, spermatozoa have to (Fig. 1): (1) become motile for the first time; (2) develop hyperactivated motility to overcome the viscous mucus and sticky epithelium of the Fallopian tube and the protective vestments of the egg; (3) use chemotaxis to find the egg among the labyrinthine folds of the oviductal epithelium and (4) undergo acrosomal exocytosis to release the hydrolytic enzymes that cleave glycoproteins of the zona pellucida to allow the penetration of the zona. Below we discuss these four processes essential for successful fertilization and demonstrate that they require elevation of sperm pH_i and $[\text{Ca}^{2+}]_i$.

Before ejaculation, morphologically mature, densely packed mammalian spermatozoa are stored in a quiescent state in the caudal

portion of the epididymis and vas deferens (Acott and Carr, 1984; Carr and Acott, 1984; Hamamah and Gatti, 1998). This quiescence is likely to help transcriptionally silent spermatozoa preserve their molecular and functional integrity for a longer time. Upon ejaculation, spermatozoa are mixed with the seminal plasma and initiate their motility for the first time. Sperm motility at this time is characterized by relatively low-amplitude, symmetrical tail bending when compared with the high-amplitude, asymmetrical tail bending characteristic of hyperactivation and observed close to the site of fertilization (Suarez and Pacey, 2006; Suarez, 2008). Elevations of sperm intracellular pH and cAMP in response to a new extracellular environment have been directly implicated in the initiation of sperm motility.

Although our understanding of sperm quiescence is limited, in many species the acidic environment of cauda epididymis keeps spermatozoa immotile by lowering their intracellular pH (Acott and Carr, 1984; Carr and Acott, 1984; Hamamah and Gatti, 1998). High viscosity of the cauda epididymal fluid may also help us to immobilize spermatozoa (Usselman and Cone, 1983; Carr *et al.*, 1985). The exact mechanism by which low extracellular pH within the cauda epididymis causes low sperm intracellular pH remains unclear, but lactate and other weak acids that can shuttle protons across the cell membrane have been implicated (Acott and Carr, 1984; Carr and Acott, 1984; Hamamah and Gatti, 1998). In some species such as humans, lactate may not be essential, since the plasma membrane seems to have a significant passive H^+ conductance (Hamamah *et al.*, 1996; Hamamah and Gatti, 1998). Finally, the ion pumps of the sperm plasma membrane keep intracellular pH lower than the extracellular pH (see earlier). During ejaculation, spermatozoa are diluted in the seminal plasma that has a higher pH . Intracellular pH is thought to follow the increase in the extracellular pH , and sperm motility is initiated due to the direct stimulation of axonemal proteins by higher pH .

Another factor important for the activation of sperm motility, intracellular cAMP, stimulates the axoneme indirectly: it activates protein kinase A (PKA) which is thought to cause phosphorylation of the axoneme (Harrison, 2004; Nolan *et al.*, 2004). Upon ejaculation, sperm intracellular cAMP is elevated due to the activation of sperm soluble adenylyl cyclase (sACY) by the high bicarbonate concentration in the seminal plasma and the female reproductive tract when compared with the epididymal fluid (Okamura *et al.*, 1985; Chen *et al.*, 2000). Male mice deficient for sperm sACY (Esposito *et al.*, 2004; Hess *et al.*, 2005; Xie *et al.*, 2006) and PKA (Nolan *et al.*, 2004) are infertile and have impaired sperm motility. However, it should be noted that PKA and sACY do not seem to be required for the initiation of sperm motility, but rather increase the frequency of sperm tail beating to improve progressive motility (Wennemuth *et al.*, 2003; Nolan *et al.*, 2004; Xie *et al.*, 2006). Interestingly, recombinant SACY or SACY in particulate fractions of sperm is also activated by Ca^{2+} (Jaiswal and Conti, 2003), and the presence of Ca^{2+} in the extracellular medium is required for sACY-dependent increase in the frequency of flagellar beat triggered by bicarbonate (Carlson *et al.*, 2007).

Normal sperm motility is characterized by low-amplitude symmetrical tail beating. This type of motility allows spermatozoa to traverse the watery cervical mucus and uterus; however, it should be noted that contractile activity of uterine muscle may facilitate transport of spermatozoa through the cervix and uterus (Suarez and Pacey,

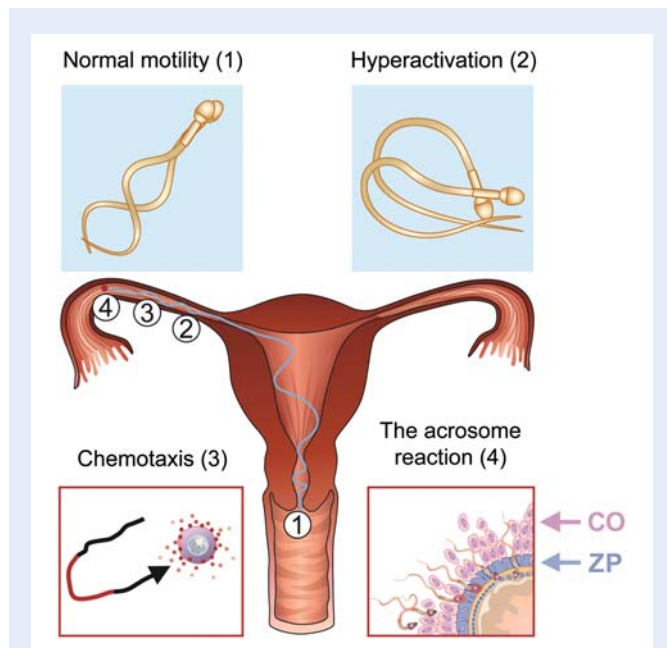


Figure 1 The milestones of sperm journey toward the egg. The approximate locations where normal sperm motility (1), hyperactivated sperm motility (2), chemotaxis (3) and the acrosome reaction (4) occur within the female reproductive tract are shown by the corresponding numbers. Normal motility (1) is triggered upon ejaculation in the anterior vagina and is characterized by the low amplitude, symmetrical tail bending. Hyperactive motility (2) occurs after spermatozoa enter Fallopian tubes. It is characterized by the high-amplitude asymmetrical tail bending and develops much higher thrust to penetrate through the viscous environment of the female reproductive tract. Sperm chemotaxis (3) is believed to occur close to the site of fertilization in the ampulla of Fallopian tubes. Chemoattractant(s) (shown by the red dots) released by the cumulus cells surrounding the egg (or by the egg itself) help spermatozoa to find the egg among the epithelial folds of the Fallopian tube. The acrosome reaction (4) occurs either within the CO or upon the contact with zona pellucida. The hydrolytic enzymes released from the sperm head as the result of the acrosome reaction help spermatozoa to penetrate these egg's protective vestments.

2006). After sperm entry into the Fallopian tubes, normal motility is unlikely to provide sufficient thrust to overcome sticky oviductal epithelium covered with dense mucus and, eventually, egg's protective vestments. To overcome these barriers, spermatozoa have to develop hyperactivation, a special type of motility characterized by high-amplitude, asymmetrical tail bending that develops a significantly higher swimming force than the normal motility (Suarez and Pacey, 2006; Suarez, 2008).

After the activation of motility, all further milestones of the sperm's journey toward the egg (including hyperactivation, chemotaxis and the acrosome reaction) require capacitation. Capacitation is defined as the functional maturation of sperm cells within the female reproductive tract (Austin, 1951; Chang, 1951; Yanagimachi, 1994). Since capacitated spermatozoa acquire hyperactive motility and become able to undergo chemotaxis and the acrosome reaction, they can now reach and fertilize the egg. Capacitation is completed in the Fallopian tubes, after spermatozoa have spent several hours in the female reproductive tract. As assessed *in vitro*, extracellular Ca^{2+} , bicarbonate and albumin in the oviductal fluid are considered essential for capacitation (Visconti *et al.*, 2002). Capacitation is a complex and poorly understood process accompanied by a wave of protein phosphorylation caused by cAMP-dependent PKA directly and through recruitment of tyrosine kinases (Visconti *et al.*, 1995, 2002; Harrison, 2004; Harrison and Gadella, 2005) as well as by the elevation of basal levels of sperm pH_i and $[\text{Ca}^{2+}]_i$ (Parrish *et al.*, 1989; Yanagimachi, 1994; Baldi *et al.*, 1996; Zeng *et al.*, 1996). These higher basal levels of pH_i and $[\text{Ca}^{2+}]_i$ are likely to be sufficient to cause the hyperactivation of sperm motility (Suarez, 2008). Chemotactic navigation toward the egg and the acrosome reaction occur later with already capacitated spermatozoa (Eisenbach and Giojalas, 2006; Florman *et al.*, 2008) and require further elevations of pH_i and $[\text{Ca}^{2+}]_i$ above the new basal levels achieved during capacitation. We will now further consider hyperactivation, chemotaxis and the acrosome reaction in detail.

As mentioned earlier, hyperactivation is characterized by large-amplitude, whip-like asymmetrical tail beating that results in significantly stronger swimming force than in normal sperm motility (Suarez, 2008). Hyperactivation not only allows spermatozoa to move along the sticky oviductal epithelium covered with mucus, but is also essential for the penetration of protective vestments of the egg, the CO and zona pellucida (Suarez and Pacey, 2006; Suarez, 2008). Hyperactivation requires both elevation of intracellular pH and elevation of intracellular Ca^{2+} (Suarez, 2008), which are achieved as a result of sperm capacitation. Elevated intracellular pH and Ca^{2+} directly affect the axoneme and, in combination, cause the high-amplitude, asymmetrical flagellar beating, characteristic of hyperactivation (Ho *et al.*, 2002). It has been demonstrated that the flagellar Ca^{2+} channel CatSper is required for hyperactivation, certainly in mice and probably humans (Carlson *et al.*, 2003, 2005; Qi *et al.*, 2007). The mechanism for H^+ extrusion is less certain and may differ between species, even within mammals. In human spermatozoa, the dominant H^+ conductor of the plasma membrane is the flagellar H^+ channel H_v1 (Lishko and Kirichok, 2010; Lishko *et al.*, 2010). Activity of both human CatSper and H_v1 is enhanced by capacitation (Lishko *et al.*, 2010, 2011). Since CatSper can be further potentiated by progesterone (Lishko *et al.*, 2011; Strünker *et al.*, 2011) and H_v1 —by anandamide (Lishko and Kirichok, 2010), both released by cumulus cells

surrounding the egg, human spermatozoa may achieve an even higher degree of hyperactivation during penetration through the CO and zona pellucida.

As spermatozoa approach the site of fertilization, they have to find the egg among the labyrinthine folds of the Fallopian tubes (Suarez and Pacey, 2006). Several studies have reported that progesterone released by the cumulus cells surrounding the egg is likely to serve as the principal sperm chemoattractant in humans; however, other possible chemoattractants have been proposed, and the very existence of sperm chemotaxis in vertebrates remains controversial (Eisenbach and Giojalas, 2006; Kaupp *et al.*, 2008). In some marine invertebrates, sperm chemotaxis has been convincingly demonstrated. For the sea urchin *Arbacia punctulata*, spermatozoa perform chemotactic turns that result in swimming trajectories directed toward a source of the resact peptide that is released from the egg (Suzuki and Garbers, 1984). Each turn is caused by a brief asymmetrical tail motion that is triggered by a transient elevation of Ca^{2+} in the sperm flagellum (Kaupp *et al.*, 2008; Guerrero *et al.*, 2010). The Ca^{2+} required for chemotactic turns in *A. punctulata* comes from the extracellular medium via an unidentified flagellar Ca^{2+} channel directly or indirectly activated by resact.

Although the chemotaxis of human sperm toward progesterone is still being debated, human spermatozoa do possess a flagellar Ca^{2+} channel activated by progesterone: the CatSper channel (Lishko *et al.*, 2011; Strünker *et al.*, 2011). As will be discussed later, the CatSper channel is potently activated by low nanomolar concentrations of progesterone, and the CatSper-mediated Ca^{2+} influx can induce asymmetrical tail beating (Lishko *et al.*, 2011; Strünker *et al.*, 2011). Thus, Ca^{2+} influx through the CatSper channel appears to be essential for both sperm hyperactivation and chemotaxis.

Finally, when the sperm cell finds the egg, it has to overcome its protective vestments, the CO and the zona pellucida. The CO, the outermost vestment of the egg, consists of the cumulus cells (follicular cells retained around the oocyte) suspended in a viscoelastic matrix formed primarily by hyaluronic acid secreted by the same cumulus cells (Dandekar *et al.*, 1992; Suarez, 2008). The dense layer of zona pellucida lies under the CO and is formed by glycoproteins ZP1, ZP2, ZP3 and ZP4 (ZP1–ZP3 in mice) secreted by the egg itself (Wassarman and Litscher, 2008; Gupta *et al.*, 2009). To penetrate through the protective vestments of the egg, the sperm cells have to be hyperactivated (Suarez, 2008). It should be noted that in the CO, the concentration of progesterone released by the cumulus cells is in the micromolar range (Osman *et al.*, 1989). Since sperm cells are completely buried in the CO during the penetration through the protective vestments of the egg and subjected to high concentrations of progesterone, their motility may reach the highest degree of hyperactivation.

However, the matrix of the zona pellucida is so dense that hyperactivation alone does not seem to be sufficient to get the sperm through this layer. To penetrate the zona, spermatozoa complement the mechanical force of hyperactivation with enzymatic disruption of the zona's matrix by hydrolytic enzymes released from the acrosome vesicle located at the tip of the sperm head. The release of hydrolytic enzymes, called the acrosome reaction, occurs upon contact of the sperm head with the zona pellucida and is caused by exocytotic fusion of the acrosomal vesicle with the sperm plasma membrane (Yanagimachi, 1994). The acrosome reaction is thought to be

triggered by elevation of sperm $[Ca^{2+}]_i$ and pH_i upon binding of the yet unidentified sperm head receptor to glycoproteins of the zona pellucida (ZP3 in mice and ZP1, ZP3, ZP4 in humans) (Dean, 2007; Florman *et al.*, 2008; Wassarman and Litscher, 2008; Gupta *et al.*, 2009). However, the sperm receptor for the glycoproteins of the zona pellucida as well as the identity of the Ca^{2+} and H^+ transport mechanisms associated with this receptor remains elusive.

Although the importance of intracellular Ca^{2+} and H^+ signaling in sperm physiology has been recognized for a long time, until very recently the Ca^{2+} and H^+ ion channels of the plasma membrane that cause elevations of pH_i and $[Ca^{2+}]_i$ have remained unknown despite significant efforts in this direction. In the following section, we briefly summarize the methods that have been used to study sperm intracellular pH and Ca^{2+} signaling and to identify the sperm ion channels involved. To explain why the identities of sperm Ca^{2+} and H^+ channels remained elusive until very recently, we consider the relative effectiveness of every method discussed in identification and characterization of the ion channels of mature spermatozoa.

Overview of experimental approaches to study sperm intracellular Ca^{2+} and pH signaling

The first evidence that Ca^{2+} and H^+ are important for sperm physiology came early from the observation that changing the level of these two ions in extracellular medium strongly affects sperm motility, chemotaxis and acrosome reaction. Extracellular Ca^{2+} was demonstrated to be essential for sperm capacitation, hyperactivation, chemotaxis and the acrosome reaction (Iwamatsu and Chang, 1971; Yanagimachi, 1994; Eisenbach and Giojalas, 2006; Florman *et al.*, 2008; Suarez, 2008). It has also been convincingly demonstrated in demembrated spermatozoa that Ca^{2+} and pH directly affect the activity of axoneme (Lindemann *et al.*, 1987; Goltz *et al.*, 1988; Ho *et al.*, 2002). Acidic pH suppressed the motility of demembrated bull sperm, and flagellum beating was activated only when pH of the incubation medium was 7.0 or higher (Ho *et al.*, 2002). Hyperactivation required an even more alkaline pH of 7.9–8.5 (Ho *et al.*, 2002). Hyperactivation of demembrated sperm also required the elevation of $[Ca^{2+}]_i$ in the incubation medium from 50 nM to 400 nM (Ho *et al.*, 2002). Unfortunately, the next logical step, identification of the membrane transport mechanisms that control sperm pH_i and $[Ca^{2+}]_i$, turned out to be extremely challenging.

Development of ion-sensitive fluorescent probes for Ca^{2+} and pH provided a tool to approach this problem for the first time. These probes allowed the observation of changes in $[Ca^{2+}]_i$ and $[H^+]_i$ in live, motile spermatozoa (Babcock, 1983; Babcock *et al.*, 1983; Irvine and Aitken, 1986; Babcock and Pfeiffer, 1987) as they underwent crucial physiological responses such as motility initiation, hyperactivation, chemotaxis and acrosome reaction. These studies demonstrated that when spermatozoa were incubated for several hours *in vitro* under conditions similar to those found within the female reproductive tract and acquired fertilizing competence (*in vitro* capacitation), their pH_i and $[Ca^{2+}]_i$ rose (Parrish *et al.*,

1989; White and Aitken, 1989; Baldi *et al.*, 1991, 1996; Zeng *et al.*, 1996). Comparison of hamster spermatozoa exhibiting normal or hyperactivated motility demonstrated that $[Ca^{2+}]_i$ is elevated in hyperactivated sperm (Suarez *et al.*, 1993). Replication of the acrosome reaction *in vitro* by treating bovine spermatozoa with solubilized zona pellucida induced the elevation of both $[Ca^{2+}]_i$ and $[H^+]_i$ (Florman *et al.*, 1989, 2008). Furthermore, in sea urchin, resact (the peptide-chemoattractant released by the egg) induced elevation of intracellular pH and Ca^{2+} (Lee and Garbers, 1986; Schackmann and Chock, 1986; Cook *et al.*, 1994). In a resact gradient, freely swimming *A. punctulata* spermatozoa generated flagellar Ca^{2+} spikes that triggered chemotactic turns to guide spermatozoa toward higher concentrations of the chemoattractant (Bohmer *et al.*, 2005; Wood *et al.*, 2007). The likely chemoattractant of human spermatozoa, progesterone, also induced massive Ca^{2+} influx into human sperm cells (Thomas and Meizel, 1989; Blackmore *et al.*, 1990). When human spermatozoa were exposed to a gradient of progesterone, they generated a series of Ca^{2+} spikes similar to those observed during chemotaxis of *A. punctulata* sperm (Harper *et al.*, 2004). No progesterone-induced elevation of intracellular pH has been reported in human spermatozoa.

Although optical methods for measuring intracellular $[Ca^{2+}]_i$ and pH_i demonstrated the existence of sperm intracellular Ca^{2+} and pH signaling for the first time, they failed to provide functional and molecular characterization of the Ca^{2+} and H^+ transport mechanisms of the sperm plasma membrane responsible for these signaling events. The principal reason for this was that the technique did not provide reliable control of the membrane voltage and the composition of the intracellular medium and ionic gradients across the sperm plasma membrane. Since these are essential determinants of the ion channel activity, the interpretation of experimental results was difficult, and there was no guarantee that only the single ion channel/transporter that is being studied contributes to the observed changes in $[Ca^{2+}]_i$ and $[H^+]_i$. Another substantial experimental difficulty was associated with the fact that sperm cells have an extremely low cytosolic volume, and the level of fluorescent signals originating from a single spermatozoon was weak. For this reason, the majority of optical experiments with sperm cells were performed by measuring fluorescent signals from sperm suspensions (Darszon *et al.*, 2005). This method detected signals averaged over large populations of cells, which reduced the already low time resolution of the optical methods and might mask fast signaling events. Although single-cell measurements have sometimes been performed, primarily in recent years, the fluorescent signal from the principal piece was often too weak to be detected, and signals from the head and midpiece (that probably have larger amounts of cytosol than the principal piece) were primarily measured (Darszon *et al.*, 2005). Unfortunately, even measurements of $[Ca^{2+}]_i$ and $[H^+]_i$ in sperm suspensions did not solve the problem of weak fluorescence from the principal piece, and about 85% of the population signal originated from the head and midpiece (Darszon *et al.*, 2005). Thus, the optical methods provided little information on $[Ca^{2+}]_i$ and $[H^+]_i$ within the principal piece, where the regulation of sperm motility occurs. A few recent optical studies that succeeded in measuring $[Ca^{2+}]_i$ from the sperm flagellum have demonstrated a complex pattern of Ca^{2+} transients in this cellular domain (Bohmer *et al.*, 2005; Wood *et al.*, 2007; Xia *et al.*, 2007).

The low volume of sperm cytoplasm can cause another problem for optical measurements of sperm $[Ca^{2+}]_i$ and $[H^+]_i$. Even a minute plasma membrane disruption that results in only a few Ca^{2+} or H^+ ions entering the cell will lead to a significant change in $[Ca^{2+}]_i$ and $[H^+]_i$. The probability of such membrane disruption during the experiment is especially high when motile sperm cells are attached to the bottom of the recording chamber or when lipophilic compounds such as progesterone are applied in relatively high concentrations. If overlooked, this problem could lead to substantial experimental artifacts and incorrect conclusions regarding the mechanisms of sperm $[Ca^{2+}]_i$ and $[H^+]_i$ signaling. In contrast, unspecific membrane leak is instantly recognized during the patch-clamp experiment, since its current–voltage relationship and ion selectivity is different from those of Ca^{2+} or H^+ currents.

In the beginning of the 1980s, concurrent with the introduction of ion-sensitive fluorescent probes to study sperm intracellular Ca^{2+} and pH signaling, the patch-clamp technique was revealed as the cutting-edge method to study plasma membrane ion channels (Hamill *et al.*, 1981). Using glass micropipettes that can form a very tight seal with lipid membranes, the patch-clamp technique made possible the direct measurement of ion currents across the whole plasma membrane (whole-cell configuration) and even detection of activity of a single ion channels (cell-attached and inside-out configurations) (Fig. 2). The whole-cell configuration proved to be the most

useful, although single-channel patch-clamp measurements were also often employed to obtain additional mechanistic insight into the observed phenomena.

For ion channel research, the patch-clamp technique provided three important advantages over the optical methods. First, it directly measured ion channel activity—the transmembrane ion current—rather than the result—the change in the intracellular concentration of an ion. Second, it provided superior time resolution of < 1 ms. Third, it allowed for very precise control of the conditions that affect ion channel activity, namely the membrane voltage, which was directly controlled by the patch-clamp amplifier, and the composition of the solution on both sides of the plasma membrane, including the concentration of the permeable ion and that of potential ion channel regulators such as ATP, neurotransmitters and cAMP. The latter provided the ability to adjust the compositions of the bath and intracellular recording solutions through manipulation with ions, channel activators and inhibitors so that the current through a single type of ion channel was recorded and studied in isolation from other ion channels present in the same membrane.

Due to these advantages, the patch-clamp technique produced a marked advance in our understanding of the ion channels of somatic cells, especially cells that depend heavily on electrical signaling such as neurons or cardiomyocytes. Since spermatozoa apparently rely on ion channels as heavily as neurons or cardiomyocytes, numerous

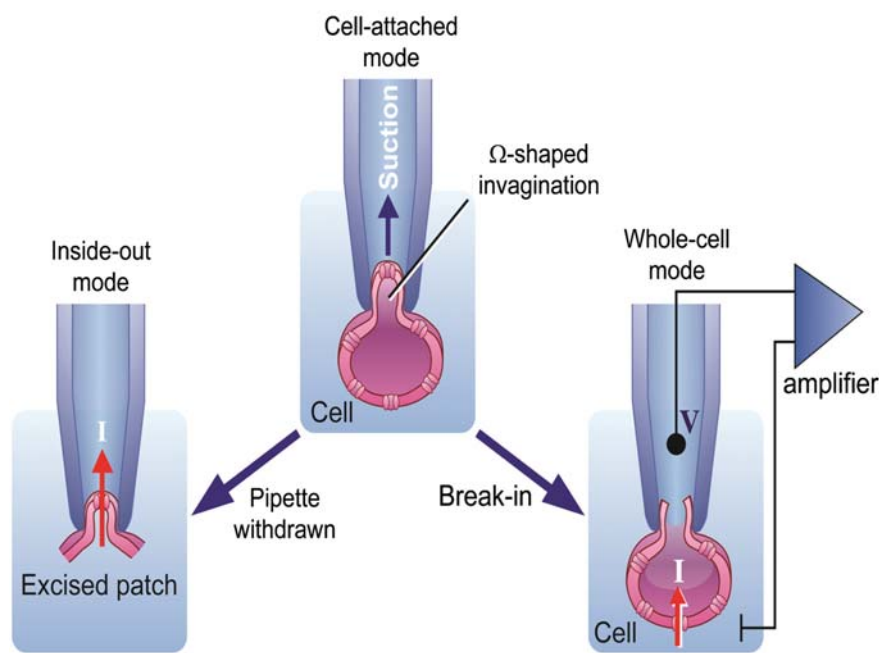


Figure 2 The modes of the patch-clamp technique. To form the cell-attached mode of the patch-clamp technique, a portion of the plasma membrane must be gently driven into the tip of the patch pipette by light suction so that it forms an Ω -shaped invagination within the tip of the pipette and establishes a tight seal with the internal walls of the tip. Since the cell-attached configuration allows only for a limited control of the membrane potential and the recording solutions, it is rarely used for electrophysiological measurements. After formation of the cell-attached mode, the membrane patch under the patch pipette can be destroyed by high-amplitude voltage pulses (up to 1 V) and/or suction to form the whole-mitoplast mode of the patch-clamp technique that allows the recording from the whole-cell plasma membrane. Alternatively, the patch pipette can be withdrawn from the cell to form the inside-out mode of the patch-clamp technique to record single channels. Two electrodes, one in the pipette and one in the bath solution, are connected to the patch-clamp amplifier that controls potential across the cell membrane (V) and measures transmembrane currents (I). The amplifier is shown only for the whole-cell mode.

attempts were made to apply the patch-clamp technique to sperm cells from different species. Unfortunately, all early attempts to achieve sperm patch-clamp recording were frustrated by the obvious inability to form a tight seal between the glass patch pipette and the sperm plasma membrane, the essential prerequisite of patch-clamp recording. The special composition of the sperm plasma membrane and the small size of sperm cells were usually given as the primary reasons for the inability to achieve the seal.

Nevertheless, the formation of tight high-resistance seals between the patch pipette and the sperm head, albeit with a very low probability, was reported (Guerrero *et al.*, 1987). It was found that two techniques improved the probability of seal formation: a perpendicular approach between the pipette and the surface of the sperm head (Espinosa *et al.*, 1998; Gorelik *et al.*, 2002; Gu *et al.*, 2004; Jimenez-Gonzalez *et al.*, 2007), and swelling of the sperm cells in hypotonic medium (Babcock *et al.*, 1992; Sanchez *et al.*, 2001). Unfortunately, successful break-in into sperm cells to transition into the whole-cell mode has never been achieved with these techniques and the authors only reported recording of single-channel activity in the cell-attached configuration, which has very limited application. These experiments certainly whet the appetite for studying sperm ion channels, but the low success rate and inability to record in the whole-cell mode made these approaches impractical.

To circumvent the problems associated with patch-clamping sperm cells, attention was directed toward recording from spermatogenic cells. The fact that all ion channels of transcriptionally and translationally silent mature spermatozoa are synthesized during spermatogenesis seemed to indicate that at least some of these channels might be functional in spermatogenic cells. Spermatogenic cells for the patch-clamp experiments were obtained from mouse testes by mechanical and enzymatic dissociation of the seminiferous tubes (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996; Lievano *et al.*, 1996; Santi *et al.*, 1996). Three cell types were most commonly present in such preparations and were primarily used in electrophysiological recordings: pachytene spermatocytes, round spermatids and condensing spermatids (Darszon *et al.*, 2005). The morphology of these cells is quite distinct from that of mature spermatozoa, as they have a mostly round body and a significant amount of cytoplasm. Although this markedly different morphology indicated a potential difference in ion channel expression, it also simplified the application of the patch-clamp technique since the formation of the tight seal between the patch pipette and the spermatogenic cell and breaking-in to record whole-cell currents were straightforward.

Fast inactivating T-type voltage-gated Ca^{2+} channels were identified as the main Ca^{2+} conductance of spermatogenic cells (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996; Lievano *et al.*, 1996; Santi *et al.*, 1996), and it was postulated that they also play a key role in Ca^{2+} signaling in mature spermatozoa (Florman *et al.*, 1998; Darszon *et al.*, 1999, 2006b). The notion that voltage-gated Ca^{2+} channels (Ca_v) are the primary Ca^{2+} conductance of sperm was also supported by optical methods that seemingly identified a membrane depolarization-induced Ca^{2+} influx into the sperm cells in response to application of extracellular medium containing a high concentration of K^+ (elevation of extracellular K^+ causes membrane depolarization) (Wennemuth *et al.*, 2000). Although this putative voltage-gated Ca^{2+} influx also required a simultaneous marked elevation of the extracellular pH (to about 8.6) and thus did not behave exactly like regular Ca_v

channels (Wennemuth *et al.*, 2000), the substantial agreement between electrophysiological experiments on spermatogenic cells and Ca^{2+} imaging in mature sperm cells convinced the field that Ca_v channels are the principal Ca^{2+} entry pathway into the sperm cell. Various Ca_v subunits were also detected in mature spermatozoa by immunocytochemistry (Carlson *et al.*, 2003; Wennemuth *et al.*, 2000; Darszon *et al.*, 2006b).

In spite of this conviction that Ca_v channels play a key role in sperm physiology, genetic evidence that Ca_v channels (or any other type of ion channel) are required for male fertility was lacking. In 2001, progress in this field advanced markedly with the identification of CatSper (Cationic Channel of Sperm)—a putative cationic ion channel specifically expressed in the principal piece of the sperm flagellum (Ren *et al.*, 2001). Male CatSper-deficient mice were completely infertile while being otherwise normal, and their spermatozoa seemed to have reduced motility (Ren *et al.*, 2001).

CatSper (later renamed CatSper1 after identification of other subunits of the same channel) belongs to the voltage-gated cation channels superfamily and has six predicted transmembrane domains (6TM), similar to transient receptor potential (TRP) channels or voltage-gated K^+ channels (Ren *et al.*, 2001) (Fig. 3A). However, the predicted pore region and overall homology of CatSper1 is closest to that of a single 6TM repeat of Ca_v channels consisting of four 6TM repeats (24 transmembrane domains total). Surprisingly, stimulation of CatSper1-deficient mouse spermatozoa with the classic high- K^+ high-pH extracellular medium to induce the Ca^{2+} influx mediated by putative sperm Ca_v channels gave no elevation of $[\text{Ca}^{2+}]_i$ as measured by optical methods (Carlson *et al.*, 2003). Moreover, CatSper1 null spermatozoa were not able to develop hyperactivation after incubation under capacitating conditions (Carlson *et al.*, 2003). These observations seemed to indicate that CatSper1 forms a voltage-gated Ca^{2+} channel that provides Ca^{2+} essential for hyperactivation. However, since conclusions about the nature of the CatSper1 channel were based on indirect optical methods, there was still doubt regarding the ion selectivity of the CatSper channel and its mode of activation. It was not even clear whether CatSper1 could form a functional channel, or just served as a regulatory subunit. Thus, although the discovery of CatSper1 provided the first genetic evidence for the importance of ion channels in sperm physiology, it simultaneously emphasized the need for a method for application of the whole-cell patch-clamp technique to mature spermatozoa.

Soon after the discovery of CatSper1, in 2006, such a method was finally developed for mouse spermatozoa (Kirichok *et al.*, 2006). A few years later, in 2010, it was reported that a similar approach can be used to patch-clamp human spermatozoa (Lishko *et al.*, 2010). As expected, the whole-cell patch-clamp technique for sperm brought about a quantum leap in our understanding of sperm ion channels. First, it allowed comprehensive characterization of the CatSper channel, the principal Ca^{2+} conductance of the sperm plasma membrane, which resulted in a better understanding of the molecular mechanism of sperm hyperactivation (Kirichok *et al.*, 2006). Second, it shed light on the non-genomic mechanism by which the female hormone progesterone induces massive Ca^{2+} influx into human spermatozoa and stimulates their activity (Lishko *et al.*, 2011; Strünker *et al.*, 2011). Third, the principal H^+ conductance of the human sperm plasma membrane was identified as the voltage-gated H^+ channel H_v1 (Lishko *et al.*, 2010). Fourth, the

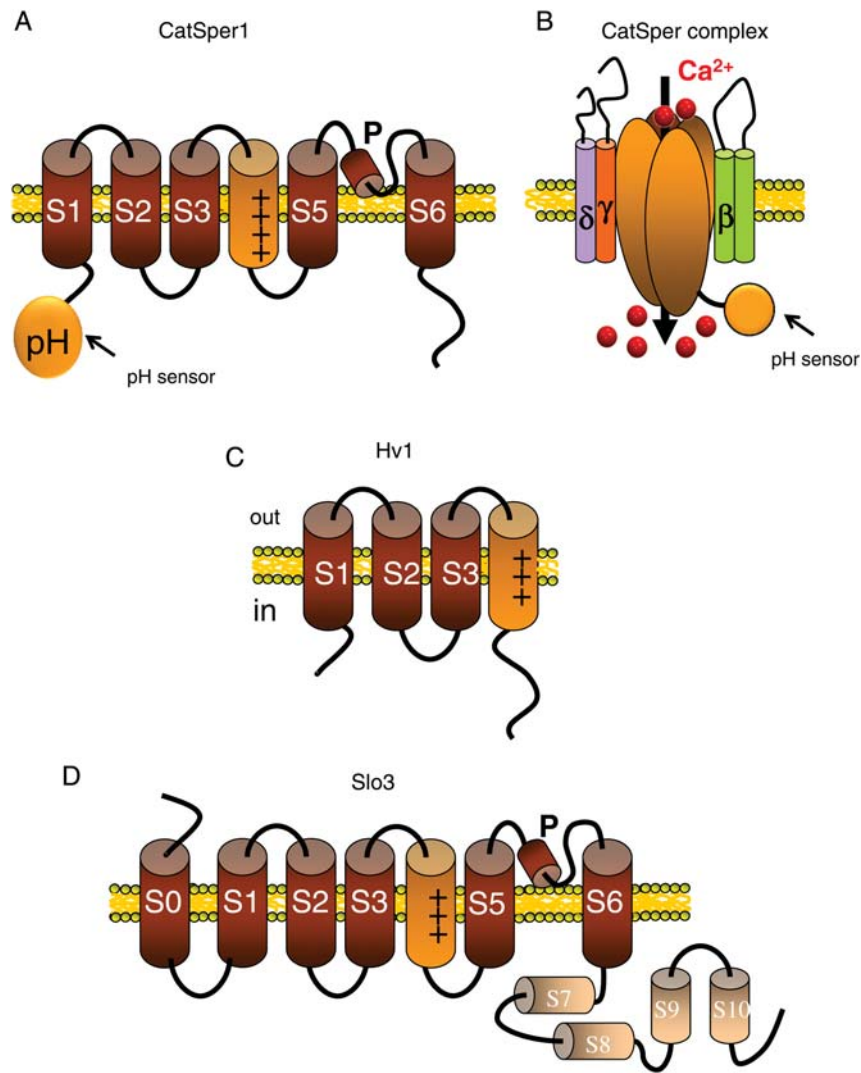


Figure 3 Molecular architecture of sperm ion channels characterized with the patch-clamp technique. (A) Predicted membrane topology of the pore-forming CatSper1 subunit of CatSper channel. Note the classic six transmembrane helix structure (S1–S6) with the positively charged voltage sensor helix S4 and the pore region (P) between transmembrane helices S5 and S6. The putative pH sensor is located in the histidine-rich N-terminal domain of CatSper1. Other pore-forming subunits of the CatSper channel (CatSper2–4) have similar membrane topology, but contain less charge in the S4 transmembrane helix and lack the putative pH-sensor in the N-terminal domain. (B) The molecular composition of CatSper channel complex. The Ca^{2+} -selective pore is formed by the four different CatSper1–4 subunits. The auxiliary CatSper subunits have one (CatSper γ and CatSper δ) or two (CatSper β) transmembrane helices and large extracellular domains that may be involved in regulation of the CatSper channel by cues of the female reproductive tract. (C) Predicted membrane topology of the H_v1 channel. H_v1 consists of four-transmembrane helices (S1–S4) homologous to the voltage-sensor domain S1–S4 of voltage-gated ion channels, but it lacks the pore forming S5–S6 segment of these channels. The remaining voltage-sensor domain only S1–S4 structure mediates transmembrane proton transport (Ramsey *et al.*, 2010). (D) Predicted membrane topology of the Slo3 channel. It has seven predicted transmembrane helices S0–S6, with S1–S6 helices homologous to classic voltage-gated ion channels. The large intracellular C-terminal domain is likely to be involved in the pH-sensitivity of Slo3 (Xia *et al.*, 2004).

principal K^+ conductance of the sperm plasma membrane that controls membrane potential and thus should regulate the activity of CatSper and H_v1 was identified and characterized (Navarro *et al.*, 2007; Santi *et al.*, 2010; Zeng *et al.*, 2011). Finally, significant differences in the Ca^{2+} and H^+ ion channels were identified between mouse and human spermatozoa (Lishko *et al.*, 2010, 2011). This stressed the importance of studying ion channel signaling specifically

in human spermatozoa if we want to understand the molecular mechanisms of male infertility and develop new methods of contraception. As a result of these discoveries, a coherent mechanistic picture of regulation of sperm activity in the female reproductive tract is taking shape. The sperm patch-clamp technique and the discoveries it brought about are discussed in more detail in the following section.

Sperm patch-clamp recording: the current approach

A prerequisite for successful transition into the whole-cell mode of the patch-clamp technique is the formation of a reliable seal between the patch-pipette and the cell plasma membrane (Hamill *et al.*, 1981). If the strength of the seal is not sufficient, the seal will be disrupted during the break-in (which is performed by the application of negative pressure into the pipette or by application of high transmembrane voltage, or both) and the cell will be lost. Unfortunately, formation of a tight seal ($> 10 \text{ G}\Omega$, when the seal strength is expressed as electrical resistance) was considered impossible in the case of sperm cells. Moreover, if the seal was formed, as in the case of the vertical approach to the sperm head (Gu *et al.*, 2004; Jimenez-Gonzalez *et al.*, 2007), its strength was probably not sufficient to allow successful break-in.

When we first attempted patch-clamping of mouse epididymal spermatozoa to provide electrophysiological characterization of the CatSper channel (Kirichok *et al.*, 2006), after numerous unsuccessful attempts to form a $\text{G}\Omega$ seal we were completely convinced that the plasma membrane of intact spermatozoa is extremely rigid and that the formation of the seal is impossible. However, after swelling mouse spermatozoa in a hypotonic solution (half normal tonicity) at 37°C for 45 min, during which some cells turned into a membrane 'pouch' with the axoneme wound up inside and loosely attached to the plasma membrane, we were able to form $\text{G}\Omega$ seals almost spontaneously (unpublished data). Furthermore, it was possible to break-in into swollen spermatozoa and record whole-cell currents. After this observation, we surmized that the apparent rigidity of the sperm plasma membrane that prevented seal formation was not a native property, but was caused by the tight association of the membrane with the underlying intracellular structures. For the formation of the $\text{G}\Omega$ seal, a portion of the plasma membrane must be gently driven into the tip of the patch pipette by light suction so that it forms an Ω -shaped invagination within the tip of the pipette and establishes a tight seal with the internal walls of the tip (Fig. 2). Only pre-swollen spermatozoa were able to 'give' a portion of the plasma membrane for the formation of the $\text{G}\Omega$ seal; the intact spermatozoa had no 'spare' plasma membrane.

Unfortunately, swelling spermatozoa in hypotonic medium at 37°C could lead to a release of hydrolytic enzymes and degradation of membrane proteins. Furthermore, disruption of the natural association between the plasma membrane and the underlying structures could alter the properties of sperm ion channels. Therefore, we decided to identify potential regions of the sperm plasma membrane loosely attached to the intracellular structures in intact spermatozoa. After studying electronic microphotographs of mouse sperm cells, we identified the cytoplasmic droplet as the only region of the plasma membrane loosely associated with intracellular structures (Fig. 4A).

During the process of spermatogenesis in the testis, the 'nurturing' Sertoli cell shapes the spermatozoon into its final slim flagellated form by phagocytosing the residual cytosol of the initially round spermatogenic cell. This phagocytosis continues until the spermatozoon is left with a small (normally $1\text{--}3 \mu\text{m}$ in diameter) droplet of the cytoplasm in the neck region (Cooper, 2011). In the majority of species, including

mice, during the transit of sperm through the epididymis, the cytoplasmic droplet migrates down the midpiece and eventually reaches the connection between the midpiece and the principal piece (the annulus) in the cauda epididymis (Cooper, 2011) (Fig. 4B). The exact function of the cytoplasmic droplet is unknown, but it likely helps in preventing damage to the sperm plasma membrane caused by the sudden decrease in the osmolarity of the extracellular medium and the associated increase in the volume of the sperm cytosol during ejaculation (Cooper, 2011). Upon ejaculation, the droplet is normally shed without disrupting the integrity of the plasma membrane, and its presence on the spermatozoa beyond this point may be associated with infertility at least in male mice, bulls and boars (Cooper, 2011). Surprisingly, the only species in which the cytoplasmic droplet is preserved upon ejaculation is humans. Human spermatozoa preserve the droplet within the female reproductive tract, and it does not interfere with the process of fertilization (Cooper, 2011). Another difference in humans is that the cytoplasmic droplet does not migrate down the midpiece toward the annulus during the epididymal maturation, but always stays in the neck region (Cooper, 2011) (Fig. 4C).

As mentioned earlier, the cytoplasmic droplet is the only region of the sperm plasma membrane that is not tightly associated with the rigid intracellular structures and can be driven into the tip of the patch pipette by gentle suction to form a reliable $\text{G}\Omega$ seal (Kirichok *et al.*, 2006). To study electrophysiological properties of mouse spermatozoa, completely morphologically mature cells isolated from cauda epididymis should ideally be used (Lishko *et al.*, 2010, 2011). However, patch-clamp recording from mouse spermatozoa isolated from the corpus epididymis is easier, since the droplets are less fragile (Kirichok *et al.*, 2006; Navarro *et al.*, 2007; Qi *et al.*, 2007). Unfortunately, since spermatozoa from the corpus epididymis are not quite mature and cannot fertilize the egg, it is important to ensure that the properties of the currents recorded match those from the cauda epididymis sperm.

Human epididymal spermatozoa are much harder to obtain, but the fact that human spermatozoa preserve the droplets after ejaculation opens the door to study the molecular mechanisms of fertilization in humans. In human sperm, the cytoplasmic droplet is located in the neck region (Cooper, 2011). To the inexperienced eye it may seem less conspicuous than the cytoplasmic droplet of mice, since it can be treated as a continuation of the head (Fig. 4C). However, even though the cytoplasmic droplet of a human spermatozoon is small and barely visible, the placement of the patch pipette in the neck region will often lead to the formation of the $\text{G}\Omega$ seal (Lishko *et al.*, 2010).

After the formation of seal with the cytoplasmic droplet of a mouse or human spermatozoon, it is possible to disrupt the small portion of the sperm plasma membrane just under the pipette (break-in) and attain electrical access into the cell (Fig. 2). Breaking-in is best achieved by simultaneous application of high voltage ($\sim 0.6\text{--}1 \text{ V}$) and negative pressure to the pipette. After breaking-in, electrical access to all portions of the sperm plasma is gained, as demonstrated by rapid distribution of the fluorescent dye Lucifer Yellow through the whole interior of the sperm cell (Kirichok *et al.*, 2006; Lishko *et al.*, 2010).

After the formation of the whole-cell mode of the patch-clamp technique, recording from sperm cells is performed exactly as in any

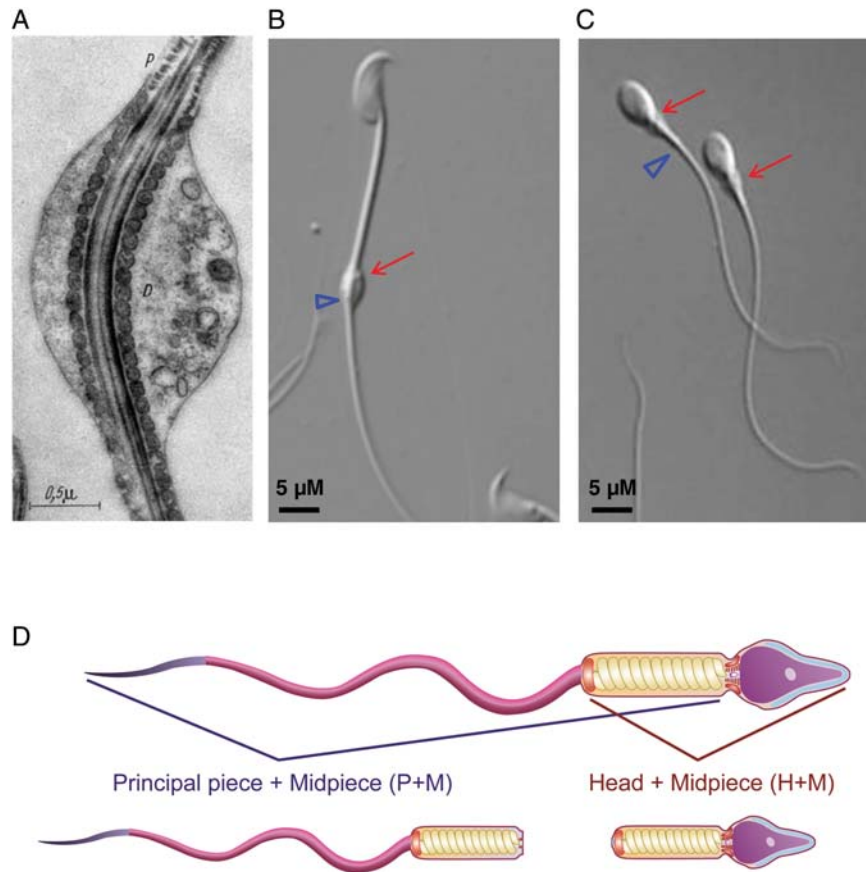
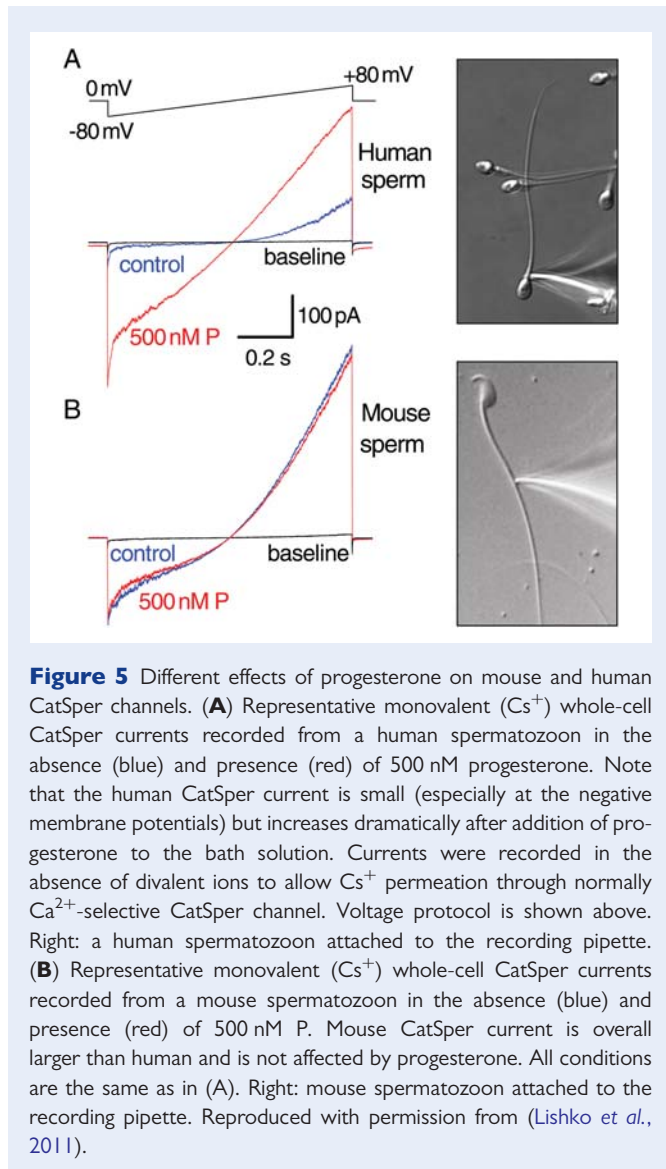


Figure 4 The sperm cytoplasmic droplet: the only gateway for sperm patch-clamp. **(A)** Electron microphotograph of the cytoplasmic droplet of a ram spermatozoon isolated from cauda epididymis. Note the loose association of the plasma membrane with the underlying mitochondria and the axoneme. The most anterior part of the principal piece is indicated with 'P'. Reproduced from (Bloom and Nicander 1961; Figure 3) with kind permission of Springer Science + Business Media. **(B)** Mouse spermatozoon isolated from corpus epididymis. Cytoplasmic droplet is indicated with the red arrow. Annulus (connection between the principal piece and the midpiece) is indicated with the blue arrow. **(C)** Ejaculated human spermatozoa. Cytoplasmic droplet is indicated with the red arrow. Annulus (connection between the principal piece and the midpiece) is indicated with the blue arrow. **(D)** Diagram demonstrating fractionation of the spermatozoon for patch-clamp recording.

other cell type (Fig. 5). Despite the long flagellum, we did not notice significant problems with spatial voltage clamp (about the same voltage was applied to all portions of the sperm flagellum) as judged by the lack of error in reversal potentials of whole-cell currents. However, precautions should be taken to ensure that concentrations of the permeable ions are well controlled within the flagellum. Although after breaking-in, the sperm cell is perfused well with the pipette solution and the intraflagellar ion concentrations reach the same values as within the pipette, ion currents across the flagellar plasma membrane can significantly change the concentration of the permeable ion in the flagellum (either decrease or increase, depending on the direction of the current). This is demonstrated well by the recording of Ba^{2+} currents through the flagellar CatSper channel. Although the pipette solution contains no Ba^{2+} , the Ba^{2+} accumulates inside the flagellum during the negative part of the voltage ramp protocol and causes an outward Ba^{2+} current through the CatSper channel during the positive part of the voltage ramp (Fig. 6). The limited ability to clamp the intraflagellar concentration of permeable ions is the only peculiarity of the sperm whole-cell patch-clamp technique. This problem can be

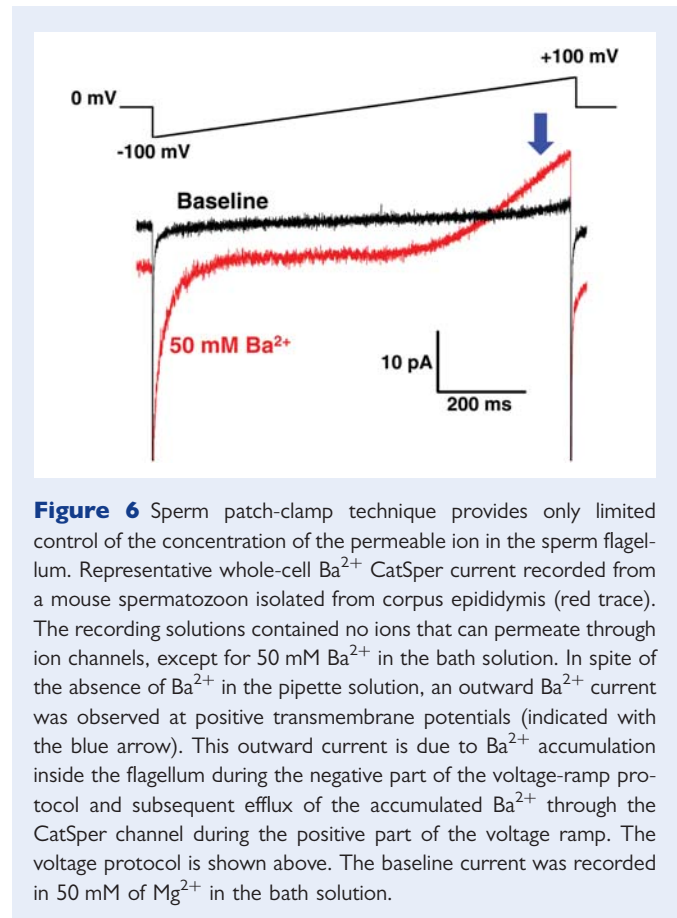
resolved by holding the cell at membrane potentials that cause minimal transmembrane current (close to the reversal potential for the permeable ion), and measuring the current amplitude at the very beginning of the test voltage step while the intraflagellar concentration of the permeable ion is still unaltered.

Patch-clamp recording can be performed not only from the whole spermatozoon, but also from its fragments such as the flagellum to study subcellular localization of ion channels. Upon treatment with trypsin and gentle trituration, mouse sperm cells separate into two parts either at the connection between the head and the midpiece (neck) or at the connection between the midpiece and the principal piece (annulus) (Kirichok *et al.*, 2006; Navarro *et al.*, 2007) (Fig. 4D). Often, such separation does not result in the loss of integrity of the plasma membrane, which closes at the site of separation. Since the two resultant fragments, the 'head plus midpiece' fragment (H+M) and the 'principal piece plus midpiece' fragment (P+M), both contain the cytoplasmic droplet, it is possible to characterize ion channels located on these fragments using the whole-cell patch-clamp technique. Such recordings led to the conclusion that the



CatSper current originated only from the principal piece of the sperm flagellum since it could be recorded from the P + M fragment, and not from the H + M fragment (Kirichok *et al.*, 2006).

A similar separation technique can be applied to human spermatozoa. Although trypsin does not seem to help us to fragment human spermatozoa, simple trituration with a micropipette leads to the separation of a few spermatozoa into head and M + P fragments at the neck region (Lishko *et al.*, 2011). The P + M fragment contains the cytoplasmic droplet and can be used for whole-cell recording. Due to the resistance of human spermatozoa to trypsin treatment, we failed to separate human spermatozoa at the connection between the midpiece and the principal piece (annulus) to obtain H + M fragments that contained the cytoplasmic droplet and were therefore suitable for patch-clamp recording. Although this complicated the electrophysiological analysis of ion channel distribution in human spermatozoa, it was still possible to compare currents recorded from the whole human spermatozoon with those from the P + M fragment. This approach led us to conclude that the current activated by



progesterone in human spermatozoa originates from the flagellum (midpiece plus principal piece) and not from the head (Lishko *et al.*, 2011).

Although the whole-cell patch-clamp technique is the principal patch-clamp mode used for characterization of ion channels, single-channel recording is sometimes desirable in cell-attached and especially the inside-out configuration that allows easy access to the cytoplasmic face of the plasma membrane. However, the fact that reproducible formation of the $\text{G}\Omega$ seal is only possible at the cytoplasmic droplet makes it impossible to record single-channel activity from the domains most interesting in terms of Ca^{2+} and H^+ signaling: the head and the principal piece. This problem can potentially be resolved by a perpendicular approach between the pipette and the surface of the sperm head (Gu *et al.*, 2004; Jimenez-Gonzalez *et al.*, 2007), but the formation of $\text{G}\Omega$ seal with the principal piece of the intact spermatozoa is impossible due to its extremely small cross-section and tight association of the plasma membrane with the intracellular structures. The only possible approach to single-channel recording from the principal piece seems to be pre-swelling of spermatozoa in a hypotonic solution to disrupt this association and convert the flagellar plasma membrane into a 'pouch' with the axoneme wound up inside. Formation of the $\text{G}\Omega$ seal with this pouch is easy, and excision of the patch to form the outside-out mode could be possible. Unfortunately, as mentioned earlier, swelling and disruption of the plasma membrane-axoneme connections might alter the properties of the flagellar ion channels.

In conclusion, the sperm whole-cell patch-clamp technique represents a powerful tool for characterization of sperm ion channels. Its application will further advance our knowledge of molecular mechanisms that control sperm activity and male fertility. Below we discuss sperm ion channels that have been identified with the patch-clamp technique and their physiological relevance.

CatSper channel: the principal Ca^{2+} channel of sperm

By 2001, when the first pore-forming subunit of the CatSper (Cationic Channel of Sperm) channel was identified (Ren *et al.*, 2001), the general agreement in the field was that voltage-gated Ca^{2+} channels (Ca_v) constitute the principal Ca^{2+} conductance of sperm (Florman *et al.*, 1998; Darszon *et al.*, 1999; Publicover and Barratt, 1999). This notion was supported by electrophysiological identification of Ca_v channels in spermatogenic cells using the patch-clamp technique (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996; Lievano *et al.*, 1996; Santi *et al.*, 1996) and by the observation of a putative voltage-gated Ca^{2+} influx into mature sperm cells in response to application of high K^+ /high-pH extracellular medium (Wennemuth *et al.*, 2000) (see earlier). The only unresolved issue seemed to be the molecular identity of the sperm Ca_v channels. There was little doubt that T-type Ca^{2+} channels are involved (Florman *et al.*, 1998; Darszon *et al.*, 1999; Jagannathan *et al.*, 2002a, b), but N- and R-type voltage-gated channels also appeared to be implicated (Wennemuth *et al.*, 2000).

The discovery of CatSper1, a putative Ca^{2+} ion channel required for male fertility (Ren *et al.*, 2001) (Fig. 3A), provided a strong candidate molecule to explain the putative voltage-gated Ca^{2+} influx into sperm cells. Among all known channel proteins, CatSper1 had highest homology to Ca_v channels and its S1–S4 voltage-sensor domain contained a sufficient amount of positively charged lysine/arginine residues in the S4 transmembrane helix to impart strong voltage sensitivity (Ren *et al.*, 2001). Indeed, it was soon demonstrated that the elevation of $[\text{Ca}^{2+}]_i$ in response to high K^+ /high-pH extracellular medium that was initially assigned to the sperm Ca_v channels was completely abolished in CatSper1(–/–) spermatozoa (Carlson *et al.*, 2003). The tempting interpretation of this experiment was that CatSper1 is the long-sought sperm voltage-gated Ca^{2+} channel.

However, since these indirect experiments potentially allowed multiple interpretations (not only regarding the mode of activation of CatSper1, but also its ion selectivity), the whole-cell patch-clamp technique was applied to mouse spermatozoa to provide direct electrophysiological characterization of CatSper1 (Kirichok *et al.*, 2006). Comparison of ion currents recorded from wild type and CatSper1(–/–) spermatozoa confirmed that CatSper1 is indeed required for a highly selective Ca^{2+} current. By recording from fragments of mouse spermatozoa, it was also established that this current originated from the principal piece of the sperm flagellum where CatSper1 protein is located. However, the voltage-dependence of this current was very weak: the slope factor of the voltage activation curve was 30, compared with four in strongly voltage-activated channels. Thus, the CatSper1-dependent channel was not voltage-gated. Instead, it was potently activated by intracellular alkalinization: the current was potentiated ~7-fold when intracellular pH was increased from 6.0 to 7.0, the range of pH_i considered the most physiologically

relevant for spermatozoa (Kirichok *et al.*, 2006). Notably, the amino terminus of CatSper1 is highly histidine-rich (83 histidines in the 446-residue N-terminus), suggesting that the N-terminus plays a role in sensing intracellular pH (Ren *et al.*, 2001) (Fig. 3A).

The pH sensitivity of CatSper can potentially explain why activation of this Ca^{2+} channel in optical experiments required extracellular alkalization. We can hypothesize (although this would not be the only possible explanation) that the membrane depolarization induced by the elevation of extracellular K^+ causes an increase in the permeability of sperm plasma membrane for protons. Thus, the high extracellular pH helps us to achieve intracellular alkalinization sufficient for the activation of CatSper1 channel. Indeed, a very small H^+ conductance activated by membrane depolarization was detected in mouse spermatozoa (Lishko *et al.*, 2010).

Soon after the discovery of CatSper1, three other related sperm-specific 6TM proteins were identified based on their sequence homology to CatSper1: CatSper2, CatSper3 and CatSper4 (Quill *et al.*, 2001; Loblely *et al.*, 2003; Qi *et al.*, 2007). CatSper2-, CatSper3- and CatSper4-deficient mice showed the same phenotype as CatSper1(–/–) mice: the males were infertile and their spermatozoa could not develop hyperactivation (Quill *et al.*, 2003; Carlson *et al.*, 2005; Qi *et al.*, 2007). Interestingly, whole-cell patch-clamp recording from mouse spermatozoa demonstrated that CatSper2, 3 and 4 were required for the same flagellar pH-dependent Ca^{2+} channel as CatSper1 since this channel disappeared on spermatozoa deficient for any one of the CatSper proteins (Qi *et al.*, 2007). Moreover, CatSper2, CatSper3 and CatSper4 could be co-immunoprecipitated with CatSper1 from mouse testes lysate and after co-expression in a heterologous system, demonstrating that CatSper2, CatSper3 and CatSper4 form molecular complexes with CatSper1 (Qi *et al.*, 2007). From these experiments, and based on the classic architecture of other ion channels such as K_v and TRP (in which four 6TM subunits are required to form a single pore) (Yu *et al.*, 2005), it was concluded that the pore of CatSper channel is formed by four different CatSper subunits: CatSper1, 2, 3 and 4 (Qi *et al.*, 2007; Navarro *et al.*, 2008). In contrast to CatSper1, other pore-forming CatSper subunits have a lower number of positively charged residues in the S4 transmembrane domain, which probably explains the low voltage sensitivity of the CatSper channel (Qi *et al.*, 2007; Navarro *et al.*, 2008).

Although the CatSper channel has only weak voltage-dependence, it is still important for CatSper function and is directly connected to the channel's pH-sensitivity (Kirichok *et al.*, 2006). At low intracellular pH (~ 6.0–6.5), the CatSper voltage dependence is such that it holds the channel virtually closed at sperm physiological membrane potentials (from about –70 to 0 mV). However, an increase in intracellular pH significantly shifts CatSper voltage dependence toward the negative membrane potentials (Kirichok *et al.*, 2006). This new voltage dependence now allows the channel to be opened by lower potentials in the physiological range.

The discovery that the CatSper channel is activated by the elevation of intracellular pH helped us to explain how capacitation can cause hyperactivation. One of the hallmarks of capacitation is elevation of sperm pH_i (Parrish *et al.*, 1989; Zeng *et al.*, 1996; Suarez, 2008). This intracellular alkalinization should not only directly stimulate motility of the axoneme, but, by activating CatSper channel, will also provide the elevation of $[\text{Ca}^{2+}]_i$ required for hyperactivation. Indeed, it was later demonstrated that intracellular alkalinization

caused by extracellular application of NH_4Cl is sufficient for the induction of sperm hyperactivation (Marquez and Suarez, 2007).

Using optical methods for measuring sperm $[\text{Ca}^{2+}]_i$, it was found that in intact mouse spermatozoa membrane-permeable analogs of cAMP and cGMP activated CatSper-dependent Ca^{2+} entry (Ren *et al.*, 2001, Xia *et al.*, 2007). Thus, it was originally proposed that CatSper channel can be directly or indirectly activated by cyclic nucleotides (Ren *et al.*, 2001). However, other groups failed to demonstrate $[\text{Ca}^{2+}]_i$ elevation in response to membrane-permeable analogs of cAMP, to photolysis of caged cAMP or in response to bicarbonate-induced elevation of cAMP (Carlson *et al.*, 2003; Wennemuth *et al.*, 2003; Strünker *et al.*, 2011). It was, however, demonstrated that CatSper-dependent Ca^{2+} elevation induced by high K^+ /high-pH extracellular medium was strongly facilitated by bicarbonate that causes sACY-dependent elevation of intracellular cAMP (Carlson *et al.*, 2003; Wennemuth *et al.*, 2003; Xie *et al.*, 2006). It is important to stress out that in these experiments, the bicarbonate-induced elevation of sperm cAMP did not cause Ca^{2+} entry itself but simply facilitated the activation of CatSper by high K^+ /high-pH extracellular medium (Carlson *et al.*, 2003; Wennemuth *et al.*, 2003). In the patch-clamp experiments the CatSper current was not affected by cyclic nucleotides (Kirichok *et al.*, 2006). This demonstrated that the facilitation of CatSper channel by cyclic nucleotides is indirect and requires an intermediary signaling cascade that was obviously disrupted during the patch-clamp recording. In this respect, it has been proposed that cAMP facilitates CatSper-dependent Ca^{2+} entry via PKA-dependent phosphorylation (Wennemuth *et al.*, 2003; Nolan *et al.*, 2004).

Since in intact cells the activity of CatSper channel can be facilitated by cyclic nucleotides, the cyclic nucleotide-induced Ca^{2+} entry into mammalian spermatozoa that was originally assigned to the cyclic nucleotide gated channels (Wiesner *et al.*, 1998) can in fact be mediated by the CatSper channel. This hypothesis is supported by the fact that no ion channels directly gated by cyclic nucleotides have been detected with the patch-clamp technique in mouse or human spermatozoa (Kirichok *et al.*, 2006; Navarro *et al.*, 2007) (and unpublished observations in human spermatozoa). However, such channels are likely to be present in spermatozoa of marine invertebrate species (Darszon *et al.*, 1999, 2006a; Strünker *et al.*, 2006; Bonigk *et al.*, 2009).

Albumin, the main protein of the oviductal fluid and an important component of the *in vitro* capacitation media, has also been shown to cause CatSper-dependent Ca^{2+} influx into mouse spermatozoa (Xia and Ren, 2009a). Finally, it has been demonstrated that the Ca^{2+} influx into mouse spermatozoa induced by the glycoproteins of the egg's zona pellucida requires CatSper channel (Xia and Ren, 2009b). In the past, the zona-induced Ca^{2+} influx was assigned to the putative sperm Ca_v channels (Florman *et al.*, 1998; Darszon *et al.*, 1999). Although the mechanisms by which albumin and the glycoproteins of zona pellucida activate CatSper channel are yet to be established, these discoveries may lead to a new and improved understanding of fundamental aspects of sperm physiology such as capacitation and the acrosome reaction (Ren and Xia, 2010).

In addition to the four pore-forming subunits, three auxiliary subunits of the CatSper channel have been discovered: CatSper β , CatSper γ and CatSper δ (Liu *et al.*, 2007; Wang *et al.*, 2009; Chung *et al.*, 2011) (Fig. 3B). These are all sperm-specific proteins that

were identified as proteins that co-purify as a complex with CatSper1 from mouse testis lysate. CatSper β , CatSper γ and CatSper δ are located in the principal piece of the sperm flagellum, the region where the pore-forming CatSper subunits reside. While CatSper β has two predicted transmembrane helices with a large extracellular loop between them, CatSper γ and CatSper δ have just one predicted transmembrane helix and a large extracellular domain (Liu *et al.*, 2007; Wang *et al.*, 2009; Chung *et al.*, 2011) (Fig. 3B). Although the interaction of CatSper β and CatSper γ with the CatSper complex was clearly demonstrated biochemically (Liu *et al.*, 2007; Wang *et al.*, 2009; Ren and Xia, 2010;), it is not clear whether they are required for the functional CatSper channel. In contrast, CatSper δ does not only interact with the CatSper complex but has been shown to be essential for the functional CatSper channel: the whole-cell CatSper current disappears on CatSper δ ($-/-$) mouse spermatozoa (Chung *et al.*, 2011). CatSper δ ($-/-$) spermatozoa also have very low levels of CatSper1 protein compared with wild-type cells (Chung *et al.*, 2011). Since all three auxiliary subunits of the CatSper channel have large extracellular domains, any of them could potentially interact with extracellular ligands to modulate the activity of CatSper channel, similar to the auxiliary subunit of the voltage-gated Ca_v channels. However, the exact functions of CatSper β , CatSper γ and CatSper δ remain to be established.

CatSper β , CatSper γ , CatSper δ and CatSper2, are all undetectable on CatSper1 ($-/-$) sperm (Carlson *et al.*, 2005; Liu *et al.*, 2007; Wang *et al.*, 2009; Chung *et al.*, 2011). Conversely, CatSper1 protein disappears on CatSper2 ($-/-$) and CatSper δ ($-/-$) spermatozoa (Carlson *et al.*, 2005; Chung *et al.*, 2011). These observations suggest that if one of the proteins of the CatSper channel is lacking and the whole channel complex cannot be formed, the remaining CatSper proteins are degraded. At this time, the CatSper channel contains the highest number of independent subunits among all known ion channels, and it is quite possible that more CatSper subunits will be discovered in the future. It is likely that this complexity is the reason why all attempts to express CatSper channel in heterologous expression systems have failed. If, at some point, heterologous expression is achieved, it will make studies of function–structure relationships within CatSper channel possible and will further improve our understanding of how the regulation of sperm intracellular Ca^{2+} occurs at the molecular level.

The subunits of CatSper channel are present in the genome of all mammals and some invertebrate species, such as the sea urchin and sea squirt (Cai and Clapham, 2008). Since the discovery of CatSper channel, several mutations and deletions in CatSper1 and CatSper2 subunits have been shown to be associated with cases of male infertility in humans (Hildebrand *et al.*, 2010). Recent development of the whole-cell patch-clamp technique for human spermatozoa provided a tool for the identification and characterization of the current mediated by human CatSper channel (Lishko *et al.*, 2010). Under the recording conditions used to identify the mouse CatSper current and where it can be recorded in isolation from other sperm ion channels, we identified a highly Ca^{2+} -selective flagellar channel in human sperm (Lishko *et al.*, 2010, 2011). Similar to mouse CatSper, it was potently activated by intracellular alkalization and was only weakly voltage dependent. However, the voltage dependence of human CatSper was slightly steeper (slope factor approximately 20 compared with 30 in mice) and the half activation voltage was more positive

(+85 mV in human versus +11 mV in mice at $pH_i = 7.5$) than those of mouse CatSper (Kirichok *et al.*, 2006; Lishko *et al.*, 2011). Slope factors for strongly voltage-sensitive ion channels are ~ 4 .

Although in general, human CatSper current was similar to mouse CatSper current, the human CatSper had a steeper voltage dependence that was also shifted toward more positive potentials. This voltage dependence made human CatSper much less active at the negative physiological potentials across the sperm plasma membrane compared with mouse CatSper (Lishko *et al.*, 2011) (Fig. 5). Interestingly, we and others (Lishko *et al.*, 2011; Strünker *et al.*, 2011) were able to identify a potent extracellular physiological activator of human CatSper channel that, similar to intracellular pH, shifted the voltage dependence of human CatSper channel toward more negative, physiological potentials. This activator was progesterone, a major female steroid hormone released by the ovaries and the cumulus cells surrounding the egg in the Fallopian tubes (Lishko *et al.*, 2011; Strünker *et al.*, 2011) (Fig. 5). Interestingly, mouse CatSper was not activated by progesterone (Lishko *et al.*, 2011) (Fig. 5B). When combined with intracellular alkalinization, progesterone made human CatSper as active at negative physiological potentials as mouse CatSper (Lishko *et al.*, 2011).

Progesterone is probably the most potent activator of human spermatozoa (Publicover *et al.*, 2007). In nanomolar concentrations, it induces robust Ca^{2+} influx into human sperm cells (Thomas and Meizel, 1989; Blackmore *et al.*, 1990). Progesterone triggers sperm hyperactivation and the acrosome reaction, and is probably also the chemoattractant of human spermatozoa, although this role of progesterone is still debated (Uhler *et al.*, 1992; Roldan *et al.*, 1994; Revelli *et al.*, 1998; Eisenbach and Giojalas, 2006; Teves *et al.*, 2006). The progesterone receptor associated with human spermatozoa is probably the best-known example of a 'non-genomic progesterone receptor' whose action does not depend on the regulation of gene expression (Revelli *et al.*, 1998; Losel and Wehling, 2003; Luconi *et al.*, 2004), in contrast to the classic nuclear progesterone receptor (Evans, 1988). The effect of progesterone on human sperm cells was much faster than that mediated by the nuclear progesterone receptor and certainly did not depend on regulation of transcription since spermatozoa are transcriptionally silent. Although all physiological effects of progesterone on human spermatozoa depend on the progesterone-induced Ca^{2+} influx, the putative sperm progesterone receptor and the associated Ca^{2+} ion channel remained elusive for more than 20 years.

We established that progesterone activates human CatSper with an $EC_{50} \approx 7.7$ nM (Lishko *et al.*, 2011). The progesterone-binding site associated with CatSper channel is external and its pharmacology is different from that of the nuclear progesterone receptor (Lishko *et al.*, 2011; Strünker *et al.*, 2011). The effect of progesterone upon CatSper is enhanced by sperm capacitation (Lishko *et al.*, 2011; Strünker *et al.*, 2011). The action of progesterone is very rapid (latency < 36 ms) and does not depend on intracellular ATP, GDP, cyclic nucleotide, Ca^{2+} or other soluble intracellular messengers (Lishko *et al.*, 2011; Strünker *et al.*, 2011). The simplest explanation of these results is that receptors for progesterone are located within the CatSper channel complex consisting of CatSper1–4, CatSper β , CatSper γ or CatSper δ . However, a different, currently unidentified, protein associated with the CatSper channel may also serve as the CatSper-associated progesterone receptor.

As measured by optical methods in human sperm, prostaglandin E_1 (PGE_1) causes intracellular Ca^{2+} transients similar in amplitude and waveform to those induced by progesterone (Aitken *et al.*, 1986; Schaefer *et al.*, 1998; Shimizu *et al.*, 1998). Interestingly, patch-clamp experiments with human spermatozoa established that in addition to progesterone, human CatSper channel is activated by nanomolar concentrations of select PGs including PGE_1 (Lishko *et al.*, 2011; Strünker *et al.*, 2011). However, the effect of PGs is additive to the effect of progesterone and is likely mediated through a different receptor (Lishko *et al.*, 2011; Strünker *et al.*, 2011). The relative potency of the human CatSper activators identified in this work followed the sequence Progesterone $> PGF_{1\alpha} \approx PGE_1 > PGA_1 > PGE_2 \gg PGD_2$ (Lishko *et al.*, 2011). Although the physiological role of the effect of PGs on human spermatozoa remains unclear, PGs are present in large quantities in the seminal plasma (Mann and Lutwak-Mann, 1981) and are secreted by the oviduct and cumulus cells surrounding the oocyte (Espey and Lipner, 1994). Millimolar concentrations of Zn^{2+} present in the seminal plasma (Saaranen *et al.*, 1987) are likely to block CatSper channel and prevent massive elevation of $[Ca^{2+}]$ and hyperstimulation of spermatozoa in the seminal plasma. However, since free concentration of Zn^{2+} in the oviduct is very low (total concentration ≈ 20 μ M, nearly all of which is bound to albumin), CatSper channel could be potentiated by PGs as spermatozoa approach the site of fertilization (Schaefer *et al.*, 1998).

Considering all its direct (intracellular pH, progesterone and PGs) and indirect (cAMP, albumin and zona pellucida glycoproteins) activators, the CatSper channel appears to be a channel with true polymodal regulation. It should also be noted that CatSper channel is potentiated as a result of capacitation, presumably due to phosphorylation (Lishko *et al.*, 2011). The large number of subunits required to form CatSper channel may provide the molecular basis for such polymodal regulation. Since orthologs of CatSper subunits present in different species have low identity (50% or less) (Liu *et al.*, 2007; Ren and Xia, 2010), the regulation of main sperm Ca^{2+} entry pathway may differ significantly between species. Mouse CatSper channel, for example, is not sensitive to the activators of human CatSper, progesterone and PGs (Lishko *et al.*, 2011). This difference in CatSper regulation probably reflects different mechanisms of fertilization adopted by different species.

The last question that should be discussed in relation to the CatSper channel is the existence (or non-existence) of sperm Ca_v channels. The discovery that the putative voltage-gated Ca^{2+} influx disappeared on CatSper1 ($-/-$) and CatSper2 ($-/-$) knockout spermatozoa has shaken the very foundation of the hypothesis about the existence of sperm Ca_v channels (Carlson *et al.*, 2003, 2005). Currently, immunocytochemical reactivity for several different Ca_v subunits in mature spermatozoa remains the only evidence for the existence of sperm Ca_v in mature sperm cells (rather than spermatocytes) (Westenbroek and Babcock, 1999; Wennemuth *et al.*, 2000; Carlson *et al.*, 2003; Darszon *et al.*, 2006b). On the other hand, the lack of fertility phenotypes in Ca_v 3.1 and 3.2 knockout mice lacking T-type Ca_v channels do not support the existence of functional sperm Ca_v channels (Escoffier *et al.*, 2007). Finally, all attempts to identify voltage-gated Ca^{2+} currents electrophysiologically in mature mouse or human spermatozoa have failed. T-type Ca^{2+} channels could be recorded from mouse testicular sperm (Darszon *et al.*, 2006b), but they seemed to be silenced via an unknown mechanism and are

undetectable in the epididymal mouse sperm (Xia and Ren, 2009b). Ejaculated human spermatozoa do not possess functional Ca_v channels as recorded with the patch-clamp technique (Lishko and Kirichok, unpublished data). It is possible that the Ca_v channels identified in mature sperm cells by immunocytochemistry do exist, but remain silent during optical and patch-clamp experiments and can only be activated under certain conditions. However, this special type of activation would be very unusual for Ca_v channels, which are simply gated by membrane depolarization in somatic cells.

Voltage-gated proton channel H_v1 : the dominant proton conductance of human sperm

Since sperm pH_i is an important regulator of sperm motility and the acrosome reaction, together with Ca^{2+} it has been considered an important regulator of sperm function. However, when the discovery of pH-sensitive CatSper channel revealed that intracellular pH_i is also an important regulator of sperm intracellular Ca^{2+} signaling, it became obvious that pH_i is likely to be the principal regulator of sperm activity over Ca^{2+} . Intercellular alkalinization appears to be essential for the initiation of motility, capacitation, hyperactivation, chemotaxis and the acrosome reaction, and is likely to lie well upstream in the sequence of signaling events leading to sperm functional activation within the female reproductive tract. Despite the important role that intracellular alkalinization plays in sperm activation, the H^+ transport mechanisms of the sperm plasma membrane that extrude protons out of the sperm cell have long remained unknown.

Although intracellular pH-sensitive fluorescent probes were able to detect changes in sperm intracellular pH associated with activation of motility, capacitation, chemotaxis and the acrosome reaction, identification of the H^+ transport mechanisms involved using this method was very challenging. It was suggested that a Na^+/H^+ exchanger (NHE) (Bibring *et al.*, 1984; Lee and Garbers, 1986; Schackmann and Chock, 1986; Garcia and Meizel, 1999; Woo *et al.*, 2002) and a Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger are involved in sperm alkalinization at least in some species (Tajima and Okamura, 1990; Zeng *et al.*, 1996), but the actual molecules controlling sperm intracellular pH have never been identified.

The NHE seemed to be an especially attractive mechanism for elevation of sperm intracellular pH. At the very least, it seems to be ideally posed to cause the intracellular alkalinization required for initiation of sperm motility. Upon ejaculation, mammalian spermatozoa experience significant elevation of extracellular $[\text{Na}^+]$ from about 30 mM in cauda epididymis to 100–150 mM in the seminal plasma and 130–150 mM in the oviduct (Mann, 1964; Borland *et al.*, 1980; Owen and Katz, 2005). This increase in extracellular Na^+ could be used by the NHE to drive the export of protons and raise sperm pH_i . Therefore, the identification of a sperm-specific molecule that bore significant homology to known NHEs and was localized in the principal piece of sperm flagellum was especially exciting (Wang *et al.*, 2003). Male mice deficient in this putative NHE were completely infertile, and their spermatozoa had greatly impaired motility (Wang *et al.*, 2003). Although the newly identified molecule was dubbed sNHE (sperm NHE), it has been difficult to demonstrate that it actually functions as a NHE: similar to CatSper subunits, after

expression in heterologous systems, sNHE failed to be transported to the plasma membrane and exhibit functional activity (Wang *et al.*, 2003). Functional data from the native system (spermatozoa) were even less encouraging: sNHE seemed to have no effect on sperm pH_i since there was no significant difference in intracellular pH between wild type and sNHE ($-/-$) spermatozoa (Wang *et al.*, 2003).

Although it was later demonstrated that a chimera between sNHE and the ubiquitously expressed plasma membrane NHE isoform-1 (NHE1) is transported to the plasma membrane after heterologous expression, the chimeric protein showed very modest H^+ transport activity compared with NHE1 (Wang *et al.*, 2007). Finally, it was discovered that the expression level of the sACY is extremely low in sNHE ($-/-$) spermatozoa, and that the sperm motility defect can be rescued by the addition of membrane-permeable analogs of cAMP (Wang *et al.*, 2003, 2007). This certainly raised the question of whether the infertility phenotype of male sNHE-deficient mice is solely due to disruption of sACY, which has previously been shown to be required for sperm motility and male fertility (Esposito *et al.*, 2004; Hess *et al.*, 2005; Xie *et al.*, 2006). In conclusion, it is still not clear whether sNHE is a real NHE, and whether its putative NHE activity is required for male fertility. Further experiments are certainly needed to answer these important questions.

Development of the whole-cell patch-clamp technique for mouse and human spermatozoa provided a tool to directly address the problem of H^+ transport across the sperm plasma membrane. So far, patch-clamp experiments with mouse spermatozoa have not detected any significant proton currents (Lishko *et al.*, 2010). Certainly, this result does not mean that protons are not transported across the plasma membrane of mouse spermatozoa. Since the NHE is electroneutral (does not transport a net charge), its activity cannot be recorded with the patch-clamp technique. Furthermore, there may be other H^+ transport mechanisms that require specific intracellular or extracellular activators that were not present in our recording solutions.

In contrast to mouse spermatozoa, patch-clamping human spermatozoa revealed a very large voltage-gated H^+ current (Lishko *et al.*, 2010). We called this current and the underlying H^+ channel of the human sperm plasma membrane HSper (H^+ channel of sperm). The HSper current was one of the highest native H^+ currents ever recorded across the cell plasma membrane (Decoursey, 2003; 2008; Lishko *et al.*, 2010). Interestingly, HSper was a one-way channel: it only allowed outward transport of H^+ and thus was specifically designed for extrusion of protons, leading to intracellular alkalinization. HSper was activated by membrane depolarization, but the transmembrane H^+ concentration gradient favoring H^+ extrusion also activated HSper and helped the channel to open at much more negative membrane potentials. Thus, effectively, HSper was opened by the combined transmembrane electrochemical H^+ gradient favoring proton extrusion. A similar gradient favoring H^+ entry into the human spermatozoa failed to induce any inward HSper current. HSper current was potently inhibited by extracellular Zn^{2+} : nanomolar concentrations of this ion significantly reduced the amplitude and activation kinetics of HSper current. The combination of properties possessed by HSper (high selectivity for protons, conducting only the outward current, slow kinetics of activation and deactivation, gating by combined electrochemical H^+ gradient and high sensitivity to Zn^{2+})

(Lishko *et al.*, 2010) is shared by only one other channel, the voltage-gated proton channel H_vI (Ramsey *et al.*, 2006; Sasaki *et al.*, 2006; DeCoursey, 2010). Moreover, similar to H_vI (Alabi *et al.*, 2007), HSper was potentiated by fatty acids and inhibited by crude venom of Chilean rose tarantula *Grammostola rosea* that contained hanatoxin and other homologous toxins (Lishko *et al.*, 2010).

H_vI is a four-transmembrane domain protein homologous to the voltage-sensor domain of voltage-gated cation channels (Ramsey *et al.*, 2006; Sasaki *et al.*, 2006) (Fig. 3C). A functional H_vI channel is a dimer of two identical subunits, although each of the two subunits appears to function as an H^+ channel (Koch *et al.*, 2008; Lee *et al.*, 2008; Tombola *et al.*, 2008). H_vI is highly expressed in phagocytic immune cells, where it helps the enzyme NADPH oxidase generate high levels of reactive oxygen species required to kill bacteria (DeCoursey, 2010). More specifically, H_vI compensates for the membrane depolarization and intracellular acidification generated as the result of NADPH oxidase enzymatic activity and helps it produce reactive oxygen species at the highest possible rate (DeCoursey, 2010). Although this is currently the best-established physiological role of H_vI , the channel is also highly expressed in other cells such as basophils, B lymphocytes, microglia and alveolar epithelium and may have other functions independent of NADPH oxidase (Capasso *et al.*, 2011).

After demonstrating that the electrophysiological and pharmacological properties of HSper are identical to those of H_vI , we further established that human spermatozoa contain high amounts of H_vI protein and its corresponding mRNA (Lishko *et al.*, 2010). Interestingly, immunocytochemical analyses of human sperm revealed that H_vI was specifically localized within the principal piece of the sperm flagellum (Lishko *et al.*, 2010). This correlated with electrophysiological recordings from fractionated human spermatozoa showing that the HSper current originated from the sperm flagellum (Lishko *et al.*, 2010).

These experiments demonstrated that H_vI is the main H^+ conductance of the human spermatozoa, at least as recorded with the patch-clamp technique. Since sperm H_vI conducts H^+ only in the outward direction and due to the large amplitude of this current (Lishko *et al.*, 2010), sperm H_vI is likely to be the principal H^+ conductance responsible for intracellular alkalization and functional activation of human sperm. Specific localization of H_vI in the principal piece of the sperm flagellum makes it ideally positioned to activate pH-dependent proteins of the axoneme and the pH-dependent CatSper channel, and thus to control sperm motility.

Similar to CatSper, sperm H_vI appears to be controlled by multiple cues of the male and female reproductive tracts. First, the female reproductive tract has a higher pH (~7.4) than the acidic fluid of the cauda epididymis, which can have a pH as low as 6.5. The elevation of extracellular pH upon penetration of the human spermatozoa into the female reproductive tract should increase the transmembrane electrochemical driving force favoring H^+ extrusion and activate sperm H_vI (Lishko *et al.*, 2010). H_vI can also be activated by membrane depolarization (Lishko *et al.*, 2010), but currently it is not clear how such depolarization of the sperm plasma membrane can be achieved. Based on indirect studies, several ligand-gated ion channels were proposed to function in sperm cells, and some of these channels can potentially provide membrane depolarization to activate sperm H_vI (Meizel, 2004).

Second, sperm H_vI can be activated by removal of extracellular zinc (Lishko *et al.*, 2010). Human seminal plasma contains high levels of zinc (total concentration 2.2 ± 1.1 mM compared with 14 ± 3 μ M in serum) (Saaranen *et al.*, 1987). Upon ejaculation, seminal zinc diffuses into the female reproductive tract and in rats, has been shown to reach the Fallopian tubes (Gunn and Gould, 1958), where it is eventually absorbed by the oviductal epithelium and chelated by the dominant protein in the oviductal fluid, albumin (Ehrenwald *et al.*, 1990; Lu *et al.*, 2008). Thus, sperm H_vI should be inhibited by seminal zinc up to the moment when spermatozoa reach the Fallopian tubes, where the inhibition will be relieved to allow sperm intracellular alkalization, activation of Ca^{2+} influx through pH-sensitive CatSper channels and sperm hyperactivation. Regulation of sperm H_vI by zinc could explain why sperm hyperactivation occurs only in the Fallopian tubes close to the site of fertilization.

Third, low micromolar concentrations of endogenous cannabinoid anandamide strongly potentiate sperm H_vI (Lishko *et al.*, 2010). A related compound, arachidonic acid, required approximately 100 times higher concentration to achieve a similar potentiation (Lishko *et al.*, 2010). The effect of anandamide is not mediated by CB1 or CB2 cannabinoid receptors and is likely due to a direct interaction of anandamide with H_vI (Lishko *et al.*, 2010). Bulk concentrations of anandamide in the fluids of the male and female reproductive tracts are in the nanomolar range (Schuel and Burkman, 2005). However, it is possible that spermatozoa experience much higher concentrations in the direct proximity of sources of anandamide such as the cumulus cells that surround the egg in the Fallopian tubes (El-Talati *et al.*, 2009). If the concentration of anandamide in CO reaches low micromolar levels, anandamide can serve as an activator of sperm motility and help spermatozoa to penetrate the egg's protective vestments.

Fourth, sperm H_vI is potentiated during *in vitro* capacitation (Lishko *et al.*, 2010). The mechanism of this potentiation remains unknown, but since multiple proteins are phosphorylated during capacitation, we can hypothesize that H_vI also becomes phosphorylated at this time. Notably, phosphorylation is the primary mechanism of H_vI regulation in other tissues (Decoursey, 2003; Musset *et al.*, 2010). Although capacitation is a poorly understood process, intracellular alkalization is considered a key factor in this process (Parrish *et al.*, 1989; Zeng *et al.*, 1996; Darszon *et al.*, 1999; Suarez, 2008). The coincidence of capacitation and the enhancement of H_vI activity suggest a strong connection between these two events. Although it is possible that potentiation of H_vI is an important causative factor in capacitation, we cannot exclude the possibility that H_vI enhancement occurs late in the process of capacitation and is a mere consequence of it.

In conclusion, sperm H_vI appears to play an important role in the regulation of human sperm intracellular pH and the pH-dependent CatSper channel. By doing so, it can potentially influence almost every aspect of sperm behavior in the female reproductive tract, including initiation of motility, capacitation, hyperactivation, chemotaxis and the acrosome reaction. However, the exact physiological function of sperm H_vI remains to be established. Since no functional sperm H_vI channel has been identified in mice (Lishko *et al.*, 2010), this convenient genetic model cannot be used to identify the physiological role of human sperm H_vI . It is important to note that H_vI -deficient mice do not exhibit any fertility phenotype (Ramsey *et al.*, 2009); however, low levels of sperm *HVCN1* (H_vI) mRNA

are strongly correlated with male infertility in humans (Platts *et al.*, 2007). It is thus possible that studies of genetic infertility in humans will help us understand the exact role of H_vI in male fertility.

Slo3 (KSper): a K^+ channel that sets sperm resting membrane potential

Since the sperm H_vI channel and, to a lesser degree, CatSper channel depend on the membrane potential, it is important to identify the ion channels that control the sperm membrane potential. In many different cell types, the resting membrane potential is primarily set by K^+ channels, and is usually slightly more positive than the K^+ reversal potential ($E_K^+ \approx -80$ mV, depending on the cell type). Sperm cells are no exception to this rule.

Whole-cell patch-clamp recording from mouse spermatozoa isolated from corpus epididymis identified a constitutively active, weakly voltage-dependent K^+ channel that was potentiated by depolarization and was initially named KSper (K^+ channel of sperm) (Navarro *et al.*, 2007). Interestingly, similar to CatSper channel, mouse KSper was strongly potentiated by intracellular alkalinization (Navarro *et al.*, 2007). Patch-clamp recording from fractionated spermatozoa showed that the KSper channel originated from the principal piece of the sperm flagellum (Navarro *et al.*, 2007). Current-clamp recording in the whole-cell mode demonstrated that mouse sperm membrane potential is strongly dependent on intracellular pH (it becomes more negative at alkaline pH) and is affected by pharmacological modulators of KSper channel (Navarro *et al.*, 2007). Based on these experiments, it was concluded that KSper is the K^+ channel that sets the sperm resting membrane potential (Navarro *et al.*, 2007). It was also suggested that the membrane hyperpolarization observed as a result of sperm capacitation in mice is due to activation of KSper by the capacitation-dependent intracellular alkalinization. The most likely molecule mediating the KSper current was proposed to be a sperm-specific channel Slo3 (Navarro *et al.*, 2007) (Fig. 3D), which—after expression in a heterologous system—gave rise to K^+ and was activated by intracellular alkalinization (Schreiber *et al.*, 1998).

A few years later, the generation of Slo3-deficient mice confirmed that Slo3 is required for the mouse sperm K^+ channel potentiated by intracellular alkalinization (Santi *et al.*, 2010; Zeng *et al.*, 2011). Slo3-deficient male mice are infertile and their spermatozoa do not undergo membrane depolarization in response to intracellular alkalinization and do not exhibit the capacitation-dependent membrane hyperpolarization (Santi *et al.*, 2010; Zeng *et al.*, 2011). Slo3 null spermatozoa also show markedly deficient progressive motility and an impaired acrosome reaction induced by the calcium ionophore A23187 (Santi *et al.*, 2010; Zeng *et al.*, 2011).

It has recently been demonstrated that mouse KSper (Slo3) channel requires phosphatidylinositol 4,5-bisphosphate (PIP_2) for its activity and can be inhibited by sperm membrane receptors that cause PIP_2 hydrolysis such as putative sperm epidermal growth factor (EGF) receptor (Tang *et al.*, 2010). Since stimulation of spermatozoa with EGF triggers the acrosomal exocytosis, it was proposed that EGF may mediate its action at least partially through inhibition of KSper channel and membrane depolarization (Tang *et al.*, 2010).

These experiments seemed to identify Slo3 as the K^+ channel responsible for setting the resting membrane potential of mouse spermatozoa. However, the K^+ channel performing this function in human spermatozoa has not yet been identified. It is tempting to postulate that the same alkalinization-activated K^+ channel is present on human spermatozoa, but the significant differences in sperm ion channels between mice and humans, as well as an apparent lack of capacitation-induced hyperpolarization in human spermatozoa, suggest that such conclusions should be confirmed experimentally.

It is also important to mention that to understand the mechanism of regulation of sperm membrane potential, it is not sufficient to merely establish ion channels that control resting membrane potential (such as Slo3). In somatic cells, potassium channels set the resting potential, but the membrane depolarization required for the activation of voltage-gated ion channels is normally induced by Na^+ -permeable ion channels controlled by intracellular or extracellular cues such as cAMP or neuromediators. Similar Na^+ -permeable channels controlled by sperm intracellular second messengers or cues of the female reproductive tract remain to be identified in spermatozoa. Identification of these channels will help us to better understand the mechanisms of regulation of other sperm ion channels that depend on the membrane potential, such as H_vI and possibly CatSper.

Concluding remarks

The sperm whole-cell patch-clamp technique, combined with methods of molecular genetics and biochemistry, has resulted in a quantum leap in our understanding of sperm ion channels. Identification and functional characterization of important sperm ion channels such as CatSper, HSper (H_vI) and KSper (Slo3) have paved the way for a comprehensive and coherent model of electrical signaling in sperm.

Although characterization of sperm ion channels is not complete, it is very unlikely that the 40 or so different ion channel proteins supposedly expressed in spermatozoa are important for sperm physiology in any one particular species (Darszon *et al.*, 2006a). First, although many of these channels may be expressed as protein or as mRNA in spermatogenic cells or spermatozoa, they are not necessarily functionally active in the mature sperm cells. This seems to be the case, for example, with the voltage-gated Ca^{2+} channels that are functionally expressed in spermatogenic cells but become quiescent in mature spermatozoa (Ren and Xia, 2010; Xia and Ren, 2009b). Second, some of these channels may regulate sperm activity in one species but be functionally absent in another. The dramatic difference in functional expression of the H_vI channel between mouse and human spermatozoa should serve as a good example of closely related species with very different ion channel physiology (Lishko *et al.*, 2010). In any case, the patch-clamp technique should be used to prove that channels that were proposed to be functionally present on spermatozoa (Darszon *et al.*, 2006a) are indeed valid sperm channels. Three decades after its invention, the patch-clamp technique remains the most reliable method for functional identification and characterization of ion channels.

There is no doubt that functional identification of sperm ion channels using the whole-cell patch-clamp technique and optical methods for measuring intracellular Ca^{2+} and pH in intact sperm cells will eventually allow us to identify a relatively small number of ion channels that

control sperm activity and male fertility in any particular species. Since properties of sperm ion channels seem to differ significantly from species to species, successful completion of this work is especially important in humans to help understand numerous unexplained cases of male infertility and develop new non-hormonal contraceptives.

Authors' roles

Y.K. and P.V.L. conceived and wrote the manuscript.

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