Molecular physiology and pathology of Ca^{2+} -conducting channels in the plasma membrane of mammalian sperm

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Abstract

Current evidence indicates that mechanisms controlling the intracellular Ca^{2+} concentration play pivotal roles in determining sperm fertilizing ability. Multiple Ca^{2+} -permeable channels have been identified and characterized in the plasma membrane and in the acrosome membrane of mammalian sperm. This review summarizes the recent findings and assesses the evidence suggesting that these channels play roles in controlling a host of sperm functions ranging from motility to the acrosome reaction, and describes recent advances in the identification of the underlying gene defects of inherited sperm Ca^{2+} channelopathies.

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Introduction

Cytosolic Ca^{2+} signals can control several physiological processes in excitable and non-excitable cells. These signals are produced by opening channels permeable to Ca^{2+} either in the plasma membrane or in the membrane of intracellular organelles containing high Ca^{2+} concentrations. Ca^{2+} -permeable channels can catalyze the flow of millions of Ca^{2+} ions through non-conducting lipid bilayers and therefore a small number of channels can cause significant changes in a tiny cell such as the sperm within milliseconds. Over the last few years, a large repertoire of Ca^{2+} permeable channels has been detected in mammalian sperm; these channels respond to different stimuli and couple to different cellular responses. Changes in the intracellular concentration of Ca^{2+} due to the activation of such channels have been associated with different aspects of mammalian sperm function such as sperm motility, capacitation and the acrosome reaction.

Recently, molecular and genetic information has been reported on human and animal models which may shed new light on the causes of altered sperm function. A variety of Ca^{2+} channelopathies and targeted mutations have been described for sperm Ca^{2+} -permeable channels in the last few years. The continued integration of this data has proven to be decisive in our understanding of the normal functioning of sperm and will give insights into cellular processes and disease and might provide new opportunities for the development of diagnostics and therapeutics.

The main purpose of this review is to consider some recent contributions towards our understanding of the molecular physiology and pathophysiology of sperm Ca^{2+} -permeable channels and to discuss future avenues of research. Previous reviews by the research groups of Barratt, Catterall, Campbell, Darszon, de Lamirande, Florman, Kaupp, Miller, Patrat, Perez-Reyes, Primakoff, Putney, Publicover, Suarez and Visconti provide supplementary reading and complement the material of this review article.

Ca^{2+} -conducting channels in sperm

Voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} (Ca_v) channels are transmembrane proteins that open in response to membrane depolarization and allow Ca^{2+} ions to enter the cell from the extracellular space. Ca_v channels have been subdivided according to their electrophysiological and pharmacological properties into (i) low voltage-activated (LVA or T-type) channels, and (ii) high-voltage activated (HVA) channels, a class that includes the L-, N-, P/Q-, and R-types. The α_1 subunit protein is the permeation pathway of all Ca_v channels, and is also responsible for voltage sensing, and binding of channel-specific drugs and toxins. Molecular cloning has identified 7 different genes coding HVA Ca_v channel α_1 subunits ($\text{Ca}_v1.1$ to $\text{Ca}_v2.3$) and 3 genes encoding LVA channels ($\text{Ca}_v3.1$ to $\text{Ca}_v3.3$) (Catterall 2000, Catterall

et al. 2003). In contrast to the Ca_v3 channels that express by themselves as typical T-type Ca^{2+} channels in heterologous systems, HVA Ca_v channels function as oligomeric complexes comprising three auxiliary subunits (β , $\alpha_2\delta$, and γ) that modulate the properties of the Ca^{2+} currents (Arikkath & Campbell 2003, Kang & Campbell 2003). The sequence of the α_1 subunit exhibits repeats comprising four transmembrane modules or domains. Each module contains the canonical arrangement for voltage-gated ion channels, i.e. six transmembrane alpha helices (S1–S6) surrounding a central pore (Catterall 2000, Catterall *et al.* 2003). Modules are connected by linkers that are located in the intracellular milieu, as are both the N and C termini (Fig. 1).

Due to their small size, complex geometry and motile nature, sperm ion channels are very difficult to study by using conventional electrophysiological methods. In addition, molecular techniques that are of great value in associating gene products with specific ionic currents in many cell types cannot be applied directly to sperm, where transcription and translation have been terminated. Instead, sperm Ca^{2+} channels may be examined by applying planar bilayer techniques in combination with *in vivo* measurements using intracellular, ion selective and membrane potential-sensitive fluorescent probes. Likewise, immunocytochemical studies with specific antibodies have been performed to reveal the expression and localization of different Ca^{2+} channel isoforms.

However, the channels that are used by mature sperm must be synthesized by spermatogenic immature cells during spermatogenesis, where their structure and function can be readily examined using electrophysiology, as well as by molecular cloning (Felix *et al.* 2004). Notably, patch-clamp studies have revealed that T-type is the only Ca^{2+} current expressed in mouse spermatogenic cells (Arnoult *et al.* 1996, 1998, Lievano *et al.* 1996, Santi *et al.* 1996). This current shares many of the fundamental features of somatic cell LVA currents, including low voltage thresholds for activation and inactivation, about the same Ba^{2+} selectivity relative to Ca^{2+} , and inhibition by amiloride, pimozide, mibefradil and low concentrations of Ni^{2+} (Arnoult *et al.* 1996, 1998, Lievano *et al.* 1996, Santi *et al.* 1996, Perez-Reyes 2003). The channels that generate this current may be constructed from proteins of the Ca_v3 class, as expression of these genes has been reported in spermatogenic cells and sperm (Espinosa *et al.* 1999, Jagannathan *et al.* 2002a, Park *et al.* 2003, Trevino *et al.* 2004) (Table 1). In addition, the genes coding HVA $\text{Ca}_v1.2$, $\text{Ca}_v2.1$ and $\text{Ca}_v2.3$ channels have been identified in spermatogenic cells (Lievano *et al.* 1996), while $\text{Ca}_v1.2$, $\text{Ca}_v2.2$ and $\text{Ca}_v2.3$ genes have been reported in mature sperm (Park *et al.* 2003). Although the protein products of these genes seem to be expressed in both male germ cells and mature sperm (Serrano *et al.* 1999, Westenbroek & Babcock 1999) (Table 1), intriguingly, HVA currents are not detected in spermatogenic cells. It has been speculated that these channels might be inserted

in the spermatogenic cell membrane in a functionally inactive state and are activated, possibly by post-translational modifications, only in sperm (Serrano *et al.* 1999).

Store-operated Ca^{2+} channels

Ca^{2+} signaling in non-excitabile cells stimulated by agonists is divided into two phases: an initial release of Ca^{2+} from stores inside the cell, and a subsequent entry of Ca^{2+} from the extracellular space. Binding of an agonist to a G protein-coupled receptor activates phospholipase C, which breaks down phosphoinositides in the plasma membrane to form inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. The IP_3 triggers Ca^{2+} release from intracellular stores through IP_3 receptor channels (IP_3Rs), producing the initial transient increase in intracellular Ca^{2+} . After a short delay, Ca^{2+} also enters the cell from the extracellular space, producing a sustained elevation of Ca^{2+} which is called capacitative Ca^{2+} entry. The channels involved in this process are known as capacitative channels or store-operated channels (SOCs) (Putney 1986).

Three different hypotheses have been evoked to explain capacitative Ca^{2+} entry. Depletion of Ca^{2+} stores may trigger release or formation of a signaling factor that diffuses to the plasma membrane to activate the channels (Randriamampita & Tsien 1993). Alternatively, a conformational coupling mechanism has been suggested according to which proteins in the Ca^{2+} stores, specifically IP_3 receptors, directly interact with Ca^{2+} permeable channels in the plasma membrane via protein–protein interactions (Berridge 1995, Kiselyov *et al.* 1998). More recently, an exocytotic model, in which vesicles containing channels fuse with the plasma membrane upon an appropriate signal, has been proposed (Yao *et al.* 1999).

Recent evidence suggests that the protein encoded by the transient receptor potential (*trp*) gene expressed in *Drosophila* photoreceptors may be homologous with capacitative Ca^{2+} entry channels in mammals (Venkatachalam *et al.* 2002). The TRP proteins are six transmembrane-containing subunits that combine to form cation-selective ion channels. TRP proteins are a diverse group of proteins organized into six families: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), muclopin (TRPML), polycystin (TRPP), and ANKTM1 (TRPA). Mammals contain at least 22 distinct genes encoding these ion channels (Moran *et al.* 2004).

A total of seven closely related genes encoding TRPC channels have been identified in mammals (termed TRPC1–7). As mentioned above, C denotes canonical, as these genes are all closely related to the original *Drosophila* TRP channel. The mammalian TRPC channels consist of a group of six transmembrane-spanning segments and an additional pore-forming hydrophobic domain between segments 5 and 6. The topology of this pore-forming domain is similar to that of Ca_v channels (Fig. 1), although the voltage-sensing segment of the Ca_v channels is not conserved in TRP channels. Interestingly, TRPC1 seems

Figure 1 Topology of Ca²⁺-permeable channels in mammalian sperm. (A) The pore-forming subunit (α_1) of voltage-gated (Ca_v) channels has four repeats of the six transmembrane (S1–S6) spanning domain. The putative pore region is formed by a hydrophilic region between S5 and S6. The S4 domain has positively charged residues (lysine/arginine) that function as the voltage sensor of the channel. Key regions of Ca_v channel regulation include binding regions in the C-terminus for Ca²⁺ (EF hand) and calmodulin (CaM) (IQ domain) in most, but not all $\text{Ca}_v\alpha_1$ subunits. (B) Structural model of cation channels of the transient receptor potential canonical (TRPC) family. Six transmembrane spanning segments are linked by short extracellular or intracellular loops. The intracellular N-terminus contains four ankyrin-like repeats (Ank R) and a coiled-coil (CC) domain. Apparently, these are sites where TRPC channels interact with other proteins. CIRB is the putative CaM- and IP₃R-binding domain, and PDZ is the region that binds PDZ domains in other proteins. The TRP box in TRPC is EWKFAR. (C) Model of the architecture of a cyclic nucleotide-gated (CNG) channel. S1–S6 are the putative transmembrane domains. The cyclic nucleotide binding site has been defined by homology to the sequences of cAMP and cGMP binding proteins. A putative CaM binding domain in the N-terminus is labeled (IQ domain). (D) The sperm cation channel, CatSper, is also a six transmembrane spanning repeat protein. CatSper1 contains a remarkable abundance of histidine (His-Rich) residues in its N-terminus, which might be involved in the pH regulation of sperm motility. (E) The topology of the polycystin-2 channel is also determined by the paradigmatic six putative transmembrane domains of the Ca²⁺-permeable channels. The carboxyl terminal of polycystin-2 includes consensus binding regions for Ca²⁺ (EF hand), calmodulin and cytoskeletal proteins. (F and G) Except for the Ca_v channels where the four repeat protein is coded by the expression of one gene, the rest of the sperm functional Ca²⁺-permeable channels are formed by the expression of four separate subunits clustered around the central pore. Thus, the tetrameric channel complex can be formed by physical association of identical (homomers) (F) or different (heteromers) subunits (G).

Table 1 Expression of Ca²⁺ channels in the plasma membrane of mature mammalian sperm.

Channel type	Species	Experimental approach	Subunit detected	Reference
Voltage-gated	Mouse	ICC	Ca _v 1.2	Westenbroek & Babcock (1999)
	Mouse	ICC	Ca _v 1.2	Carlson <i>et al.</i> (2003)
	Rat	<i>In-situ</i> RT-PCR	Ca _v 1.2	Goodwin <i>et al.</i> (2000)
	Human	RT-PCR	Ca _v 1.2	Goodwin <i>et al.</i> (2000)
	Human	RT-PCR	Ca _v 1.2	Park <i>et al.</i> (2003)
	Human	ICC	Ca _v 1.2	Trevino <i>et al.</i> (2004)
	Mouse	ICC	Ca _v 2.1	Westenbroek & Babcock (1999)
	Human	RT-PCR	Ca _v 2.1	Park <i>et al.</i> (2003)
	Mouse	ICC	Ca _v 2.2	Wennemuth <i>et al.</i> (2000)
	Mouse	ICC	Ca _v 2.2	Carlson <i>et al.</i> (2003)
	Mouse	WB	Ca _v 2.2	Wennemuth <i>et al.</i> (2000)
	Mouse	ICC	Ca _v 2.3	Carlson <i>et al.</i> (2003)
	Human	RT-PCR	Ca _v 2.3	Park <i>et al.</i> (2003)
	Human	ICC	Ca _v 2.3	Westenbroek & Babcock (1999)
	Human	ICC	Ca _v 2.3	Trevino <i>et al.</i> (2004)
	Mouse	ICC	Ca _v 3.1	Trevino <i>et al.</i> (2004)
	Human	RT-PCR	Ca _v 3.1	Park <i>et al.</i> (2003)
	Human	ICC	Ca _v 3.1	Trevino <i>et al.</i> (2004)
	Mouse	ICC	Ca _v 3.2	Trevino <i>et al.</i> (2004)
	Human	RT-PCR	Ca _v 3.2	Park <i>et al.</i> (2003)
	Human	ICC	Ca _v 3.2	Trevino <i>et al.</i> (2004)
	Mouse	ICC	Ca _v 3.2	Trevino <i>et al.</i> (2004)
	Human	RT-PCR	Ca _v 3.2	Park <i>et al.</i> (2003)
	Human	ICC	Ca _v 3.2	Trevino <i>et al.</i> (2004)
TRPC	Mouse	ICC	TRPC1	Trevino <i>et al.</i> (2001)
	Human	ICC	TRPC1	Castellano <i>et al.</i> (2003)
	Mouse	ICC/WB	TRPC2	Jungnickel <i>et al.</i> (2001)
	Mouse	ICC	TRPC3	Trevino <i>et al.</i> (2001)
	Human	ICC	TRPC3	Castellano <i>et al.</i> (2003)
	Human	ICC	TRPC4	Castellano <i>et al.</i> (2003)
	Mouse	ICC	TRPC6	Trevino <i>et al.</i> (2001)
	Human	ICC	TRPC6	Castellano <i>et al.</i> (2003)
CNG	Bovine	ICC	CNGA3	Weyand <i>et al.</i> (1994)
	Bovine	ICC	CNGA3	Wiesner <i>et al.</i> (1998)
	Bovine	ICC/WB	CNGB1	Wiesner <i>et al.</i> (1998)
Catsper	Mouse	ICC/WB	Catsper1	Ren <i>et al.</i> (2001)
	Mouse	ICC/WB	Catsper2	Quill <i>et al.</i> (2001)

ICC, immunocytochemistry; RT-PCR, reverse-transcription polymerase chain reaction; WB, Western blot.

to be a key subunit of SOCs (Beech *et al.* 2003). Nevertheless, it seems not to act alone; there is evidence that it can heteromultimerise with the related proteins TRPC4, TRPC5 and polycystin-2 (Venkatachalam *et al.* 2002).

Interestingly, in mammalian sperm, activation of phospholipase C (PLC) generates IP₃, thereby mobilizing Ca²⁺ from the sperm's intracellular Ca²⁺ store, the acrosome (Fukami *et al.* 2003). These early responses appear to promote a subsequent sustained Ca²⁺ influx signal via SOCs that results in the acrosome reaction (see below) (Florman 1994, O'Toole *et al.* 2000, Breitbart 2002). Recent studies have provided evidence for the expression in sperm of TRPC1, 3, 4 and 6 (Trevino *et al.* 2001, Castellano *et al.* 2003) (Table 1), and TRPC2 has been described to play a role in the ZP3 (a glycoprotein constituent of the zona pelucida)-induced acrosome reaction in mouse sperm (Jungnickel *et al.* 2001). It is worth mentioning also that a sperm-enriched *Caenorhabditis elegans* TRPC homolog (TRPC-3) has been identified. Notably, *trp-3* mutant sperm are motile, but fail to fertilize oocytes after gamete contact. TRPC-3 is initially located in intracellular vesicles, and is translocated to the plasma membrane during sperm

activation. This event coincided with an increase in store-operated Ca²⁺ entry (Xu & Sternberg 2003).

Other relevant sperm Ca²⁺-permeable channels

Cyclic nucleotide-gated (CNG) channels belong to a heterogeneous gene superfamily of ion channels that share a common transmembrane topology and pore structure and that harbor in their COOH-terminal region a binding domain for nucleoside 3',5'-cyclic monophosphates (cNMPs). CNG channels are nonselective cation channels that allow the passage of divalent cations, in particular Ca²⁺. All known native CNG channels respond to both cAMP and cGMP, but lower concentrations of cGMP than cAMP are required to open the channels. The current model for the membrane topology of CNG channels is illustrated in Fig. 1. The core structural unit consists of six membrane-spanning segments (S1–S6), followed by a cNMP binding domain near the C terminus. A pore region is located between S5 and S6. The S4 segment in CNG channels resembles the voltage-sensor motif found in the Ca_v channels (Kaupp 1995, Kaupp & Seifert 2002).

The mammalian CNG channel genes fall into two different gene subfamilies (termed CNGB and CNGA). The testicular expression of several CNG channel subunits (A3, B1, and B3) has been suggested by cloning of cDNA from testis libraries and by Northern analysis (Kaupp & Seifert 2002). Antibodies specific for the A3 and B1 subunits labeled the flagellum of mature sperm and spermatogenic cells in cross-sections of seminiferous tubules (Wiesner *et al.* 1998) (Table 1). Heterologous expression of the A3 subunit cloned from testis produces channels that are ~200-fold more sensitive to cGMP than to cAMP (Weyand *et al.* 1994). Therefore, these channels might be involved in a cGMP-stimulated Ca²⁺ influx into intact sperm, and their localization on the flagellum suggests that Ca²⁺ entry through CNG channels controls sperm motility (Wiesner *et al.* 1998). Intriguingly, knockout mice lacking the CNG channel A3 subunit are fertile (Biel *et al.* 1999). Lastly, a Ca²⁺ permeable channel activated both by cNMPs and hyperpolarizing potentials (termed SPIH) was initially cloned from sea urchin testis and functionally expressed in HEK-293 cells (Gauss *et al.* 1998). A channel with similar characteristics named human hyperpolarization-activated and cyclic nucleotide-gated cation channel 4 (hHCN4) was then cloned from a human thalamus cDNA library and heterologously expressed (Seifert *et al.* 1999). Experimental evidence suggests that hHCN4 is also expressed in spermatogenic cells and sperm. Although the functional significance of pacemaker channels in sperm is not known, both the SPIH and hHCN4 may be involved in the generation of rhythmic activity that controls the waveform of flagellar beating (Seifert *et al.* 1999).

Likewise, two sperm-specific membrane proteins (CatSper 1 and 2) have been reported (Quill *et al.* 2001, Ren *et al.* 2001). These proteins are ion channel α subunits resembling the six-transmembrane one-repeat of Ca_v channels (Fig. 1). Interestingly, functional studies have shown that these channels may also be sensitive to cell membrane permeant cAMP and cGMP analogs. CatSper mRNA expression is restricted to the testis, and the subcellular localization of the protein seems to be confined to the sperm flagellum (Quill *et al.* 2001, Ren *et al.* 2001) (Table 1). More recently, by using *in silico* gene identification and prediction techniques, two novel members of the CatSper family (CatSper3 and 4) have been identified (Lobley *et al.* 2003). Each of the new CatSper genes are predicted to be expressed in the testis, and the corresponding proteins may share the characteristic molecular arrangement of the voltage gated channel ion transport α subunit found in voltage-gated Ca²⁺ channels. Interestingly, coiled-coil protein-protein interaction domains in the C-terminal tails of each of the CatSper channels have been identified, implying that all members of this family may interact directly or indirectly to form a functional tetramer (Lobley *et al.* 2003). However, neither CatSper1 nor CatSper2 has been shown to function as a cation channel when transfected into cells, singly or in conjunction (Quill *et al.* 2001, Ren *et al.* 2001). In addition, the

cloning of Catsper3 has been described (Arias *et al.* 2003). As predicted, RNA analysis indicates that the gene is predominantly expressed in testis; however, similar to other members of the CatSper family, expression in heterologous systems did not lead to the induction of identifiable currents (Arias *et al.* 2003).

Role of Ca²⁺ channels in sperm function

Sperm capacitation

Sperm capacitation is a highly complex physiological event occurring in the female genital tract, rendering the mammalian sperm capable of binding to and fusing with the oocyte. Although several hypotheses have been developed, the molecular mechanisms of capacitation are not well understood. Changes associated with this process include, among others, an increase in respiration and subsequent changes in the sperm motility pattern, removal of cholesterol from the plasma membrane, increases in pH_i and Ca_i²⁺, and activation of second-messenger cascades (de Lamirande *et al.* 1997, Purohit *et al.* 1999, Darszon *et al.* 2001). The most significant change in sperm after capacitation is its ability to undergo the acrosome reaction (AR).

Ion environment and ion fluxes through the sperm plasma membrane are highly important in capacitation. In particular, Ca_i²⁺ has been shown to be increased during capacitation. This may be the result of (i) reduced Ca²⁺ efflux due to inhibition of the Ca²⁺ ATPase pump, (ii) increased leakage of Ca²⁺ across the membrane due to instability caused by removal of cholesterol, and/or (iii) increased Ca²⁺ influx due to the activation of unidentified channels (Jagannathan *et al.* 2002b). However, regulation of Ca_v channels during sperm capacitation may also occur, although such a regulation has not been directly established. Within this context, there is evidence suggesting that T-type Ca_v channels through their window current might contribute to setting Ca_i²⁺ at the resting potential and therefore influence sperm capacitation. In spermatogenic cells, serum albumin induces an increase in Ca²⁺ window current by shifting the voltage dependence of both steady-state activation and inactivation of T-type Ca_v channels (Espinosa *et al.* 2000). As there is evidence that these channels are present in mature sperm (Darszon *et al.* 2001, Jagannathan *et al.* 2002b) serum albumin might facilitate an increase in Ca²⁺ entry, a prerequisite to capacitation. Another potential mechanism for regulation of Ca_v channels during sperm capacitation includes phosphorylation. The capacitation process has been correlated with increased tyrosine phosphorylation of a subset of sperm proteins (Visconti & Kopf 1998, Ficarro *et al.* 2003). Interestingly, T-type Ca²⁺ channel activity of mouse spermatogenic cells can be enhanced by tyrosine dephosphorylation (Arnoult *et al.* 1997), although no evidence for channel phosphorylation during capacitation has been documented.

Besides Ca_i^{2+} , an increase in pH_i has been reported in capacitation (de Lamirande *et al.* 1997, Purohit *et al.* 1999, Olds-Clarke 2003). Either or both Ca_i^{2+} and pH_i may regulate one or more types of K^+ channels in the sperm plasma membrane, causing the hyperpolarization of the membrane potential observed during capacitation. This hyperpolarization is thought to release the T-type Ca_v channels from inactivation such that they are competent to respond to a stimulus provided by the zona pellucida (ZP) and undergo the AR (Arnoult *et al.* 1999). Notably, a pH-regulated K^+ channel with strong inward rectification properties has been described in spermatogenic cells (Munoz-Garay *et al.* 2001). The increase in pH_i that occurs during capacitation may activate this channel, contributing, at least in part, to enhance the K^+ permeability that leads to hyperpolarization. A second putative mechanism for hyperpolarization during sperm capacitation is the opening of Ca^{2+} -activated K^+ channels. It has been shown that injection of RNAs of spermatogenic cells isolated from the rat testis into *Xenopus* oocytes resulted in the expression of currents that show similarity to maxi-K channels, the Ca^{2+} -activated K^+ channels of somatic cells (Chan *et al.* 1998, So *et al.* 1998, Wu *et al.* 1998a). Immunolocalization and RT-PCR assays showed these channels to be present in spermatogenic cells and sperm (Wu *et al.* 1998a). Such channels may be activated by the increase in Ca_i^{2+} upon capacitation.

The acrosome reaction

The sperm acrosome reaction (AR) is a fundamental reproductive strategy which is a prerequisite for successful fertilization. It involves exocytosis of the acrosomal vesicle contained in the head of the sperm. During this process, lytic enzymes and material required for sperm binding are released into the extracellular space leading to the fusion of the gametes. Ca^{2+} influx is an absolute requirement for the physiological AR in sperm from all species examined to date (Publicover & Barratt 1999, Darszon *et al.* 2001). In mammals, fertilization begins with the direct interaction of sperm and egg, a process mediated primarily by gamete surface proteins. To penetrate the cumulus cell barrier surrounding ovulated eggs, sperm use hyperactivated motility and a glycosylphosphatidylinositol (GPI) anchored surface hyaluronidase, named PH-20 (Primakoff & Myles 2002). Once the sperm has penetrated the cumulus cells and reaches the ZP, it undergoes exocytosis, releasing the acrosomal content. Sperm adhesion to the ZP is a carbohydrate-mediated event (Primakoff & Myles 2002).

Sperm-ZP adhesion activity has been confirmed by gene knockout of one sperm surface enzyme that putatively binds ZP3, β -1,4-galactosyl transferase I (GalT I). Compared with wild-type, GalT I-null sperm show substantially reduced binding of soluble ZP3 and no ZP3-induced acrosome reaction (Rodeheffer & Shur 2004a,b). ZP3-induced exocytosis of the acrosomal contents proceeds through two sperm signaling pathways. In the first,

ZP3 binding to GalT I and other potential receptors results in activation of a heterotrimeric GTP-binding protein and PLC, thus elevating the concentration of Ca_i^{2+} . In the second pathway, ZP3 binding to the same receptor(s) stimulates a transient influx of calcium through T-type channels. In a later phase of the signaling, these initial ZP3-induced events produce additional Ca^{2+} entry through TRPC family Ca^{2+} channels, resulting in a sustained increase in Ca_i^{2+} that triggers exocytosis (Darszon *et al.* 2001, Primakoff & Myles 2002) (Fig. 2).

The Ca_i^{2+} signal elicited by ZP is prolonged and the sperm AR occurs some minutes after the start of this signal. In contrast, the influx of Ca^{2+} through T-type Ca_v channels is transient (<500 ms) (Darszon *et al.* 2001, Jagannathan *et al.* 2002b). However, the comparison of the pharmacological profile of the T-type current of spermatogenic cells and the ZP-induced Ca_i^{2+} increase in mouse sperm shows great similarity (Arnoult *et al.* 1996, 1998). Furthermore, the initial response to ZP is a large, transient Ca_i^{2+} spike with kinetics comparable to those of T-currents (Arnoult *et al.* 1999). This initial Ca_i^{2+} entry induces a second, sustained Ca^{2+} influx. Recent evidence indicates that the sustained component of Ca^{2+} influx is mediated primarily by SOCs activated after depletion of a small Ca^{2+} store (O'Toole *et al.* 2000, Jungnickel *et al.* 2001), probably in the acrosome.

It has been proposed that TRPC2 participates in the sustained mouse sperm Ca^{2+} influx triggered by ZP3 (Jungnickel *et al.* 2001). However, other TRPCs or unknown subunits may substitute for TRPC2 since *TRPC2*^{-/-} mutant mice are fertile (Leypold *et al.* 2002). It is worth mentioning that the human TRPC2 gene appears to be a pseudogene considering that several independent expressed sequence tags (ESTs) show mutations introducing early stop codons (Zhu *et al.* 1996, Vannier *et al.* 1999). Lastly, as mentioned earlier, the sperm-enriched *C. elegans* TRPC homolog, *trp-3*, was shown to be required for fertilization (Xu & Sternberg 2003). As *C. elegans* oocytes lack egg coats, these data suggest that some TRPC channels might function to mediate Ca^{2+} influx at some stage in sperm-egg membrane interactions in addition to the AR. However, it should be noted that gamete interaction in nematodes is quite different from that in mammals. Actually, *C. elegans* sperm do not possess an acrosome, which makes the AR unnecessary for fertilization in this species.

Ca^{2+} channels and sperm motility

Although the external triggering mechanisms that initiate sperm motility are largely unknown, evidence supports a modification of the Ca^{2+} balance by several separate Ca^{2+} -dependent mechanisms. Elevation of Ca_i^{2+} can occur by entry of Ca^{2+} ions into cells through the plasma membrane or release of Ca^{2+} from internal stores. Therefore, the possibility that Ca_v channels are expressed in the sperm tail and may participate in the regulation of flagellar

Figure 2 Diagrammatic representation of the mouse sperm AR. Depicted are the binding of an acrosome-intact sperm to the egg's ZP (left), and the initiation of the AR (right). In the sperm head, the activation of the ZP3 receptor on the plasma membrane results in a transient Ca²⁺ influx through Ca_v T-type channels, and the activation of the phospholipase C (PLC) through a G protein-mediated signaling pathway. PLC activation results in the production of IP₃ and the subsequent opening of Ca²⁺ permeable channels that promotes the release of Ca²⁺ from an intracellular storage compartment, possibly the acrosome. T Ca_v-channel opening and depletion of Ca²⁺ from internal stores act in concert to activate store-operated channels (SOCs) and produce a persistent Ca²⁺ influx that directly drives acrosomal exocytosis.

motility has been investigated. Immunolocalization studies showed that at least two Ca_v3 channels ($\alpha_{13.2}$ and $\alpha_{13.3}$) are heterogeneously distributed in mouse and human sperm. These channels are present in the sperm flagella and compounds known to inhibit them induce a small decrease in human sperm motility, indicating they might participate in regulating this function (Trevino *et al.* 2004). In addition, confocal immunofluorescence data have shown that at least four distinct types of capacitative Ca²⁺ channels (TRPC1, 3, 4 and 6) are expressed and differentially localized in the human sperm. By analyzing the effects of distinct TRPC channel antagonists using a computer-assisted assay, evidence has been provided that these proteins may play an important role in controlling human sperm flagellar movement (Castellano *et al.* 2003).

Likewise, at some time before fertilization, mammalian sperm undergo a change in movement pattern, named

hyperactivation, which is critical to the success of fertilization because it enhances the ability of sperm to penetrate the egg's ZP (Ho & Suarez 2001*a,b*). Recent experimental evidence suggests that hyperactivated motility may be regulated by an IP₃R-gated intracellular Ca²⁺ store in the neck region of mammalian sperm (Ho & Suarez 2001*b*). This is supported by the fact that thapsigargin induced an increase in Ca_i²⁺ sufficient to switch on hyperactivation in the absence of external Ca²⁺ (Ho & Suarez 2001*b*). In addition, as mentioned earlier, the unique sperm cation channel, CatSper, is expressed by meiotic and postmeiotic spermatogenic cells but not by other cells, and is present in the sperm flagellum, suggesting a role in the regulation of sperm motility. In line with this, targeted disruption of mouse CatSper gene results in male sterility, due mainly to the inability of sperm to maintain normal patterns of motility and their inability to penetrate the egg's ZP (Ren *et al.* 2001).

Knockout models for studying sperm function and inherited sperm Ca^{2+} channelopathies

The genes for CatSper1 and CatSper2 have been disrupted (Ren *et al.* 2001, Carlson *et al.* 2003, Quill *et al.* 2003) and, as expected, male mice but not females are infertile. Interestingly, CatSper1 seems to be essential for depolarization-evoked Ca^{2+} entry. Carlson *et al.* (2003) have shown that when sperm of a wild-type mouse received brief applications of a depolarizing medium (high K^+), Ca^{2+} channels are opened causing Ca_i^{2+} to increase slightly. After recovery and a brief conditioning exposure to bicarbonate (which initiates several cellular responses that result in Ca^{2+} channel facilitation), a third stimulus evokes a much faster and larger Ca^{2+} increase. In sharp contrast, sperm of a CatSper1 null mouse showed little or no depolarization-evoked Ca^{2+} increase. These data support the idea that CatSper1 forms functional Ca^{2+} channels in the membrane of the sperm flagellum, alone or with unidentified partners that perhaps include other members of the CatSper family (Carlson *et al.* 2003, Lobley *et al.* 2003). In addition, sperm from CatSper1 null mice did not develop the large amplitude, asymmetric waveform found for hyperactivated wild-type sperm. This requirement of CatSper1 for hyperactivated motility is consistent with the Ca^{2+} dependence of hyperactivation (Ho & Suarez 2001a), as well as with the presence of CatSper1 in flagellar membranes, and with the ability of CatSper1 null sperm to penetrate ZP-free, but not ZP-intact, eggs (Ren *et al.* 2001). Similarly, it has been shown that CatSper2 is essential for the generation of the hyperactivated form of sperm motility. Hence, although CatSper2 $-/-$ cells can undergo capacitation and the AR, the time-dependent appearance of hyperactivated movement characteristics (high track velocity and nonlinear trajectory) is not present in the CatSper2 null sperm (Carlson *et al.* 2003). This may explain why CatSper2 knockout sperm fail to penetrate the ZP of intact eggs (Carlson *et al.* 2003).

Interestingly, recent genetic studies have linked male human infertility to a mutation in the CatSper2 gene (Avidan *et al.* 2003). In these studies, a 106 kb tandem duplication and a large genomic deletion on chromosome 15q15 were identified in three members of a family suffering from asthenoteratozoospermia and nonsyndromic deafness in addition to congenital dyserythropoietic anemia type I (CDAI). Besides the CDAI mutation, these patients carried a deletion causing the inactivation of four genes. CatSper2, partially removed by the deletion, appears to be the best candidate for the etiology of the observed male infertility. The implication of CatSper2 in asthenoteratozoospermia is the first description of an autosomal gene associated with nonsyndromic male infertility in humans (Avidan *et al.* 2003).

In addition, a member of the TRPP class of Ca^{2+} channels, called polycystin-2, is encoded by the *PKD2* gene. This polypeptide is an integral membrane glycoprotein with similarity to the Ca_v1 channel α_1 subunits

(Mochizuki *et al.* 1996), and indeed seems to behave as a non-selective Ca^{2+} -permeable channel (Hanaoka *et al.* 2000, Gonzalez-Perrett *et al.* 2001). Mutations in *PKD2* cause autosomal dominant polycystic kidney disease (ADPKD) (Wu *et al.* 1998b, 2000), a genetic disorder in which the renal parenchyma is progressively substituted by fluid-filled cysts (Peters & Breuning 2001). Interestingly, a mutant mouse with ADPKD (Tg737) displays abnormal ciliary structure and function. Tg737 mutant mice have defects in bronchial and photoreceptor cilia, implying a relationship between ADPKD with primary ciliary defects (Yoder *et al.* 1996, Kierszenbaum 2004). The Tg737 mouse lacks a protein called polaris which co-localizes with polycystin-2 in cilia (Yoder *et al.* 2002). Notably, sperm tail development is abortive in the Tg737 mutant.

Moreover, molecular studies have indicated that the developing mouse heart, kidney and pancreas are particularly susceptible to *Pkd2* gene disruption, with the ensuing phenotypes often resulting in mid-gestational embryonic lethality (Wu *et al.* 2000). It has not yet been determined whether the *Pkd2* null embryos have alterations in germ cell differentiation. However, recent cellular and molecular analysis indicates that *Drosophila Pkd2* is expressed in the tail and the head of sperm, and that targeted disruption of *Pkd2* results in male infertility without affecting spermatogenesis (Gao *et al.* 2003). The mutant sperm are motile but fail to swim into the storage organs in the female (seminal receptacles and spermathecae), suggesting that the *Drosophila PKD2* Ca^{2+} permeable channel operates in sperm for directional movement inside the female reproductive tract (Gao *et al.* 2003). Supporting a role of polycystin-2 in *Drosophila* fertilization is the finding that a *Drosophila* flagellar polycystin-2 homolog specifically expressed in the male germ cells (called *amo*) is localized in the sperm flagellum (Watnick *et al.* 2003). A targeted disruption of the *amo* gene causes sterility. Both the structure of the testis and the characteristics of sperm motility in the *amo* mutant were normal. However, sperm of the mutant deposited in the uterus were unable to enter the female sperm storage organs (Watnick *et al.* 2003).

This is interesting because sea urchin and human sperm, which also express sperm-specific members of the PKD1 family, also move directionally to meet the egg in the oviduct. The finding in human, sea urchin and *Drosophila* sperm of polycystin gene homologs provided support for evolutionary conservation and unveiled genetically defined components required for fertilization (Kierszenbaum 2004). In line with this, it is worth mentioning that a small group of patients whose sperm lacked the central microtubules (the axonemal 9 + 0 defect) of the flagellum were diagnosed ADPKD (Okada *et al.* 1999). However, there have been no genetic studies of the defect in the central microtubules in infertile men, and therefore the genetic linkage between ADPKD and this defect in sperm remains to be determined.

Likewise, in the Ca_v channel field exciting new insights into the function of these proteins have been obtained by examining the phenotypes of animal models in which Ca_v genes have been deleted. The knockout approach has been applied to almost all Ca_v channel α_1 subunit genes, and has also been used to try to elucidate the actions of the various auxiliary subunits (Miller 2001, Muth *et al.* 2001). Although the information regarding the function of sperm Ca_v channels has appeared slowly, it would be anticipated that this strategy would be very useful in allowing the understanding of many aspects of sperm Ca_v channel physiology.

Knockout mice of obvious interest are those for the Ca_v3 channels. Mice lacking Ca_v3.1 channels show thalamocortical relay neurons lacking the burst mode firing of action potentials (Kim *et al.* 2001), while mice deficient in Ca_v3.2 channels have constitutively constricted coronary arterioles and focal myocardial fibrosis (Chen *et al.* 2003). Very recently, by using mice deficient for Ca_v3.1 channels, it has been reported that the T-type current activity in spermatogenic cells is not reduced in the knockout mice (Stambouliau *et al.* 2004). In addition, the biophysical and pharmacological properties of the T-type current from the Ca_v3.1 channel-deficient mice suggest that Ca_v3.3 may not contribute to the whole-cell Ca²⁺ current in spermatogenic cells. Together, these data suggest that (i) T-type Ca²⁺ current in mouse spermatogenic cells is mainly carried through Ca_v3.2 channels, (ii) Ca_v3.1 channels may contribute to a minor extent, and (iii) Ca_v3.3 channels are unlikely to contribute significantly to the T-type Ca²⁺ current recorded in spermatogenic cells (Stambouliau *et al.* 2004). However, it is interesting that although the impact of the gene disruption on the male gamete physiology has not been studied in detail, Ca_v3.2 null mice show apparently normal reproduction (Chen *et al.* 2003). The preservation of the male reproductive function in these animals might be the result of compensatory changes in the expression of other Ca_v proteins.

Moreover, it has been suggested that several Ca_v α_1 subunits of the HVA class exist in mammalian sperm, including Ca_v2.3 (Westenbroek & Babcock 1999, Wenne-muth *et al.* 2000, Trevino *et al.* 2004) which encodes the R-type Ca²⁺ currents (Smith *et al.* 1999, Tottene *et al.* 2000). The presence of this type of current in mature sperm has been suggested pharmacologically (Wenne-muth *et al.* 2000). Initial studies proposed the Ca_v2.3 channel as a candidate for the LVA Ca²⁺ currents observed in spermatogenic cells (Lievano *et al.* 1996); however, more recently, it has been reported that these channels may not contribute to these Ca²⁺ currents (Sakata *et al.* 2001). Instead, Ca_v2.3 channels are expected to play roles in the control of capacitation, the AR and/or the flagellar movement, although no definite functions have been defined as yet. In order to try to elucidate the functions of these channels, a mouse model lacking Ca_v2.3 was developed (Sakata *et al.* 2002). Although male mice lacking Ca_v2.3 were found to be fertile,

the Ca²⁺ transients induced by mannose-bovine serum albumin (BSA) were significantly lower than that of wild-type sperm. Mannose-BSA has been shown to increase Ca_i²⁺ in human sperm, which accounts for its ability to induce the AR (Blackmore & Eisoldt 1999). Previous observations indicated that Ca_v channels may be involved in the actions of mannose-BSA (Blackmore & Eisoldt 1999, Son *et al.* 2000), although a potential mannose-BSA-induced internal Ca²⁺ rise by a different activation pathway cannot be ruled out. Lastly, the linearity of movement was apparently increased in Ca_v2.3 null sperm (Sakata *et al.* 2002), suggesting that these channels may be functional in sperm and may play roles in Ca_i²⁺ signals and the control of flagellar motility.

As described earlier, the mammalian sperm AR is initiated by binding to the ZP. This event is thought to induce a transient Ca²⁺ influx through Ca_v channels (Darszon *et al.* 2001, Jagannathan *et al.* 2002b). A tyrosine kinase-regulated PLC may also be activated during ZP binding (Patrat *et al.* 2000), whose activation generates IP₃, mobilizing Ca²⁺ from an intracellular Ca²⁺ store, presumably the acrosome. The initial Ca²⁺ response appears to promote a subsequent sustained Ca²⁺ influx via SOCs that results in the AR (Florman 1994, O'Toole *et al.* 2000). Experimental evidence for the expression of TRP protein channels in sperm, putative SOCs, has been provided (Jungnickel *et al.* 2001, Trevino *et al.* 2001). Notably, in PLC δ 4 $-/-$ mice there is an alteration in the release of Ca²⁺ induced by ZP, as well as a reduction in Ca²⁺ influx through SOCs (Fukami *et al.* 2003). Nevertheless, given that some of the sperm from PLC δ 4 null mice undergo the AR, the possibility that other PLC isoforms might be involved in the AR cannot be excluded (Fukami *et al.* 2003).

Challenges and prospects

In numerous research fields, including male gamete biology, molecular research is now focusing on the identification and characterization of genes and proteins. Numerous Ca²⁺ channels responsible for sperm Ca_i²⁺ signals have been cloned, and the consequence is the generation of cDNA and antibody probes which have been used to investigate channel organization, localization and function at the molecular level. However, sperm physiological genomics is considered a starting point for the more challenging aim of understanding how a cell actually functions at a molecular level during health and disease. To achieve this goal, new technologies are emerging. For instance, proteomics can be applied to study a host of aspects of male gamete physiology such as following the expression of particular proteins (including Ca²⁺-permeable channels) in germ cells during spermatogenesis (Cossio *et al.* 1997, Guillaume *et al.* 2001), as well as proteins putatively involved in post-testicular sperm maturation (Dacheux *et al.* 1998, Starita-Geribaldi *et al.* 2001). In addition, proteomics may allow the mapping and

characterizing of sperm surface proteins that (i) are phosphorylated during capacitation (Naaby-Hansen *et al.* 2002, Ficarro *et al.* 2003), (ii) are responsible for gamete recognition during interaction with the ZP (Peterson *et al.* 1991), and (iii) may activate the signaling cascades that trigger the sperm AR and may sustain sperm motility (Ostrowski *et al.* 2002). In the future, it will be possible to navigate throughout sperm databases containing complementary information on nucleic acid and protein sequences, genome mapping, diseases, protein structure, post-translational modifications, antibodies and cellular localization of antigens, signaling pathways, etc. (Celis *et al.* 1998). Hence, proteomics and other molecular tools may provide us with a better mechanistic understanding of male gamete function.

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