Sperm channel diversity and functional multiplicity

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Abstract

Ion channels are extraordinarily efficient machines that move ions in diversely controlled manners, allowing cells to rapidly exchange information with the outside world and with other cells. Communication is the currency of fertilization, as it is of most fundamental cell signaling events. Ion channels are deeply involved in the dialogue between sperm, its surroundings, and the egg. How sperm swim, find the egg and fertilize it depend on ion permeability changes modulated by environmental cues and components of the egg outer layer. Different ion channels distinctly localized in these tiny, amazing cells perform specific decoding functions that shape the sophisticated behavior of sperm. It is not surprising that certain sperm ion channels are turning out to be unique. New strategies to characterize sperm ion transport have opened exciting possibilities to dissect sperm–egg signaling and unveil novel contraception targets.

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Introduction

Ion channels are amazing membrane proteins that efficiently catalyze ion transport through the non-conducting lipid bilayer. A single ion channel, one single protein, allows millions of ions per second to flow across the membrane. Only a few of these channels are necessary to trigger, in milliseconds, substantial electric and concentration changes in a small cell like sperm. It is not surprising that such remarkable transducers were evolutionarily selected to perform fast information exchanges between the external world and cells or within them. Ionic gradients across cells not only determine membrane potential (E_m) through ion-selective channels, permeant ions can modulate enzymes and further influence channel activity causing additional E_m changes and ion flow. E_m governs the rates and direction of ion-flow through channels and exchangers – its fluctuations modulate intracellular pH (pH_i), intracellular Ca^{2+} ($[Ca^{2+}]_i$) and other second messengers that influence sperm maturation, motility and the acrosome reaction (AR).

Mature, competent male and female gametes produced in the testis or ovaries are needed to achieve fusion and fertilization. Identifying the key mechanisms and molecules involved in the sperm–egg dialog remains

incomplete. Sea urchins spawn tens of billions of sperm into the sea. Upon release, motility ensues, powered by a microscopic flagellar engine that is exquisitely regulated by chemical signals from the environment and the egg. On the other hand, the potential for motility in sperm from internal fertilizers (reptiles, birds and mammals) develops during their journey through the vas deferens (a duct from the epididymis to the urethra) and the epididymis (Morisawa 1994, Yanagimachi 1994). Activation proceeds when sperm are ejaculated into the female reproductive tract.

Environmental cues activate motility through transduction events involving sperm ion channels (Darszon *et al.* 2005, Quill *et al.* 2006). Mammalian sperm have to further mature within the female reproductive tract through a process named capacitation (Visconti *et al.* 2002). Though millions of sperm are released, only a few reach their goal (Suarez & Ho 2003). The mechanisms of sperm chemotaxis and its importance for some species still possess intriguing questions (Eisenbach 1999).

Motile mature sperm from all species possessing an acrosome – a large, Golgi-derived secretory vesicle located in the anterior region of the sperm – must undergo the AR to fertilize the homologous egg (Yanagimachi 1994, Neill &

Vacquier 2004), during which the acrosomal and plasma membranes fuse at multiple sites. This reaction releases certain components needed for the sperm to penetrate the egg coat and subsequently fuse with the egg plasma membrane. External ions, particularly Ca^{2+} , are remarkably influential on sperm maturation, motility and the AR (Yanagimachi 1994, Jungnickel *et al.* 2003). These features of sperm function emphasize the essential role ion channels play in the physiology of this important cell.

In this brief review we have attempted to highlight sperm functional aspects where ion channels play a key role (motility, capacitation and AR), and used working models to illustrate testable hypotheses that can contribute to a better understanding of how sperm fertilize eggs. We regret having to be so concise and have had to leave out some important contributions. Excellent recent reviews may make up for these limitations (Baldi *et al.* 2002, Visconti *et al.* 2002, Jungnickel *et al.* 2003, Neill & Vacquier 2004, Zhang & Gopalakrishnan 2005, Jimenez-Gonzalez *et al.* 2006).

Motility

Motility is perhaps the most easily observed function of sperm, and is yet arguably the least well understood. Ion channels have long been suspected to critically influence sperm motility. However, until recently, direct evidence of involvement in this process has been lacking due to the difficulties in performing experiments with individual motile sperm.

An early hint that ion channels regulate motility came from observations that the composition of the external milieu may have profound effects on this process. Often sperm are maintained immotile in the testes prior to release, in for example, sea urchins, this immotile state is principally maintained by a high extracellular K^+ ($[\text{K}^+]_e$) (Darszon *et al.* 2005). Activation of sperm motility occurs upon spawning, owing to the reduction of $[\text{K}^+]_e$. Manipulating the $[\text{Ca}^{2+}]$ to which permeabilized or demembrated invertebrate or mammalian sperm are exposed alters flagellar bending asymmetry, and high $[\text{Ca}^{2+}]$ inhibits flagellar motility (Brokaw 1979, Tash & Means 1982). Ca^{2+} also plays a critical role in chemotaxis, whereby a sperm responds to a gradient of chemoattractant surrounding the egg, and reorients its trajectory to swim up the gradient (see Fig. 1). This process has been observed most frequently in the sperm of invertebrate marine animals, but exists also in mammalian and plant (bracken) sperm. In all studied cases, chemotaxis is dependent on the presence of external Ca^{2+} , suggesting that Ca^{2+} -permeant pathways participate in this process.

Mammalian sperm manifest two general forms of motility, activated and hyperactivated. Activated motility, as observed in freshly ejaculated sperm, displays a relatively low-amplitude flagellar beat that propels the sperm through the initial stages of its journey through the female reproductive tract. It is stimulated by Ser/Thr and Tyr

phosphorylation of flagellar proteins (reviewed in Turner 2006). The phosphorylation cascade is regulated in part by the action of cAMP on protein kinase A (PKA), which in turn may be activated by stimulation of soluble adenylyl cyclase (sAC). This enzyme is regulated by bicarbonate ions (HCO_3^-) and Ca^{2+} (Litvin *et al.* 2003, Esposito *et al.* 2004). Although the source of the Ca^{2+} that might up-regulate sAC activity is unknown, Ca^{2+} channels in the plasma membrane are a strong possibility.

Hyperactivated motility is observed in sperm isolated from the upper regions of the female reproductive tract. Initiation of hyperactivated motility often coincides with the onset of capacitation, although the two processes are independent (Ho & Suarez 2003). It consists of exaggerated, large amplitude flagellar beats that cause the sperm to swim in characteristic 'figure-of-eight' formation in low-viscosity medium. In high-viscosity medium, hyperactivated motility is more progressive (Suarez & Ho 2003). Hyperactivation is thus thought important for sperm progression through the higher-viscosity environment of the oviduct and possibly for penetration of the egg cumulus.

Ca^{2+} is required for the initiation and maintenance of hyperactivated motility by directly regulating components of the axonemal machinery. In hyperactivated hamster sperm, transitory increases in $[\text{Ca}^{2+}]_i$ were recorded that temporally correlated with the flagellar beat frequency (Suarez *et al.* 1993). The source for the Ca^{2+} appears to be the redundant nuclear envelope (RNE), a reticular structure at the flagellum neck. In bull sperm, agonists of the inositol 1,4,5-trisphosphate (IP_3) receptor induced hyperactivation, and IP_3 receptors (IP_3R) were located on the RNE (Ho & Suarez 2003). In immobilized human sperm, a gradient of progesterone stimulated long-lasting oscillations in $[\text{Ca}^{2+}]_i$ that originated from a sperm head region containing the RNE (Harper *et al.* 2004). The oscillations correlated with increases in flagellar activity that are comparable to those occurring during hyperactivation. The Ca^{2+} oscillations were dependent on ryanodine receptor (RyR) activity rather than that of IP_3Rs , in contrast to bull sperm in which RyRs were not detected (Suarez & Ho 2003, Jimenez-Gonzalez *et al.* 2006). In both bull sperm and human sperm, the increases and oscillations in $[\text{Ca}^{2+}]_i$ and the motility changes were dependent on external Ca^{2+} , but not immediately, suggesting that this effect is due to an eventual failure to replenish intracellular stores with external Ca^{2+} .

A number of voltage-gated Ca^{2+} (Ca_v) channel subunits have been detected in mammalian sperm (Fig. 2), and Ca_v channel activity has also been measured in spermatogenic cells and sperm (Arnoult *et al.* 1996, 1999, Wennemuth *et al.* 2000). $\text{Ca}_v1.2$, 2.1 and 2.3 subunits were localized to the proximal piece of mouse sperm flagella (Westenbroek & Babcock 1999), as was the auxiliary β_3 subunit (Serrano *et al.* 1999). Sperm from $\text{Ca}_v2.3$ knockout mice displayed increased linearity in their swimming behavior, although mice were normally fertile (Sakata *et al.* 2002). Additionally, all three T-type Ca_v3 channel subunits have

Figure 1 Summary of the different ion channels and their spatial distribution in sperm. The figure includes channels that have been immunolocalized to the region indicated. Additional evidence for the presence of many of these channels such as RT-PCR, *in situ* hybridization and western blots is available (Darszon *et al.* 2005). Sea urchin flagella do not have principal and mid pieces, channels found in the flagella of this species are indicated as present in the principal piece. Channels that participate in functions such as volume regulation (not described in the text) have also been included (Yeung *et al.* 2006).

also been localized to the flagellum of mouse and human sperm (Trevino *et al.* 2004). Other channels possibly involved in motility belong to the cyclic nucleotide-gated channel family (CNG). The first sperm ion channel cloned and heterologously expressed was from this family (Weyand *et al.* 1994). These channels were immunolocalized along the length of the flagellum (CNGA3) or confined to the principal piece alone (CNGB1) of bovine sperm (Wiesner *et al.* 1998) however CNGA3 knockout mice are normally fertile (Turner 2006). Furthermore, members of the transient receptor potential (TRP) channel family have been localized to the flagella of both human

(transient receptor potential canonical; TRPC1, C3 and C6) and mouse sperm (TRPC1, C3, C4 and C6), inhibitors of TRP channels blocked human sperm motility (Castellano *et al.* 2003). The most convincing evidence for the role of ion channels in sperm motility comes from a novel class of Ca^{2+} channels, named CatSpers, whose expression is confined to the testes. Four members of this family have been described so far, and two of them, CatSpers 1 & 2, localize to the flagella (Quill *et al.* 2001, Ren *et al.* 2001, Lobley *et al.* 2003, Jin *et al.* 2005). Knockout mice for *catsper1* are infertile, due to a specific inability to undergo hyperactivation (Ren *et al.* 2001, Carlson *et al.*

Figure 2 (A) The normal circular trajectory of a sea urchin sperm is interrupted by a sharp turn, caused by a Ca^{2+} -dependent increase in flagellar asymmetry. (B) Uncaging of cGMP induces an increase in intracellular Ca^{2+} and a transient increase in flagellar asymmetry (0–50 ms) that rapidly recedes (75–100 ms). For experimental details, please see (Wood *et al.* 2005). Sea urchin sperm swim in a circular pattern because their flagella beat with an inherently asymmetrical sinusoidal waveform (see A). As the plane of the flagellar waveform is slightly skewed, the sperm swim in a helical path in ‘open water’ that converts to a two-dimensional circular pattern once the sperm encounter a surface, such as the base of a microscopic imaging chamber. This fortuitous detail permits extended observation of motility, as the sperm swim in circles within the microscope field-of-view for prolonged periods. In many species of urchin, egg-derived peptides stimulate the sperm to alter its trajectory by undergoing a transient sharp turn – red trajectory in (A). These turns are necessary, but not sufficient, for chemotaxis, as they are generated in response to egg peptides derived from chemotactic (*Arbacia punctulata*; Bohmer *et al.* 2005) and non-chemotactic (*S. purpuratus*; C Wood unpublished data) species alike. The turn itself results from a transient increase in flagellar asymmetry, which depends on the presence of Ca^{2+} in the extracellular medium. Recently it was shown that these turns occur in response to a transient increase in intracellular Ca^{2+} in the flagellum (Bohmer *et al.* 2005, Wood *et al.* 2005), although as yet it is not known how such increases are generated. Upon binding to sperm, egg peptides activate a guanylate cyclase, and cGMP levels rise rapidly. Indeed, in the absence of egg peptides, uncaging cGMP inside a swimming sperm is sufficient to stimulate sperm to undergo sharp turns which depend on a rapid increase in intracellular Ca^{2+} (see (B) and Bohmer *et al.* 2005, Wood *et al.* 2005). The link between the increase in cGMP and flagellar Ca^{2+} is unknown, but one suggestion is that cGMP acts indirectly to increase intracellular Ca^{2+} by altering another intracellular parameter such as pH or membrane potential (Darszon *et al.* 2005, Hildebrand & Kaupp 2005). Reports of the pH changes occurring after egg peptide binding or release of cGMP are contradictory, with results from *S. purpuratus* showing that a pH increase precedes the increase in Ca^{2+} , while data from *A. punctulata* indicate that the pH increase occurs later (see Darszon *et al.* 2005, Hildebrand & Kaupp 2005). Measurements of membrane potential changes in *S. purpuratus* show a rapid hyperpolarization event occurring before a larger depolarization, which has been proposed to activate a hyperpolarization-sensitive Na^+/H^+ exchanger to lead to the increase in pH, and remove inactivation from voltage-sensitive Ca^{2+} channels which open upon depolarization (see Darszon *et al.* 2005, Hildebrand & Kaupp 2005). Antibodies to Ca_v channels label the flagella of sea urchin sperm, and inhibitors of Ca_v channels such as nimodipine and Ni^{2+} are able to block both the rapid increase in Ca^{2+} and the turning event upon liberation of cGMP. The main evidence pointing to the importance of the hyperpolarization event is the effect of increasing the K^+ concentration of the extracellular medium; under such conditions, binding of egg peptides increases the concentration of cGMP, but blocks all downstream changes in pH, membrane potential, Ca^{2+} and cAMP. Uncaging of cGMP under these conditions produces similar results, unlike uncaging of cAMP which results in increases in intracellular Ca^{2+} (see Darszon *et al.* 2005, Hildebrand & Kaupp 2005). This evidence has been taken to propose a model whereby increases in cGMP activate K^+ -permeant channels in the membrane by an unknown mechanism, leading to hyperpolarization of the membrane.

2003). These knockout mice also show defects in cAMP and depolarization-induced Ca^{2+} entry (Ren *et al.* 2001, Carlson *et al.* 2003). *catsper1* and *catsper2* knockout mice have identical phenotypes because neither protein alone localizes to the flagellum without co-expression of the other (Quill *et al.* 2003, Carlson *et al.* 2005). Whole-cell patch-clamp recordings sealing on cytoplasmic droplets of sperm revealed a constitutively active, weakly voltage-dependent Ca^{2+} current that is strongly potentiated by intracellular alkalinization (Kirichok *et al.* 2006). This cur-

rent is absent in *catsper1* knockout mice, and is localized to the flagella principal piece, demonstrating that CatSper1 is a component of a flagellar Ca^{2+} channel. Interestingly, simultaneous depolarization and internal alkalinization induced by external K^+ and NH_4Cl addition trigger a Ni^{2+} sensitive $[\text{Ca}^{2+}]_i$ elevation. This voltage dependent channel could contribute to the increase in $[\text{Ca}^{2+}]_i$ that occurs during capacitation (Fraire-Zamora & Gonzalez-Martinez 2004) and possibly during hyperactivation. The channel is permeable to Na^+ in the absence

of external Ca^{2+} (Espinosa & Darszon 1995, Gonzalez-Martinez 2003).

In invertebrate sperm very little is known of the role of ion channels in regulating motility. The sperm of many marine species demonstrate chemotaxis, and in all known cases this process is dependent on the extracellular $[\text{Ca}^{2+}]$. In many such situations the chemotactic behavior manifests as a series of turns interspersed with periods of straighter swimming, it is these turns that are inhibited in the absence of external Ca^{2+} . In sea urchin, it was recently shown that cGMP, the first signaling intermediate generated upon binding of egg-derived peptides, may stimulate sperm to undergo chemotaxis-like motility changes that require Ca^{2+} entry into the flagellum through a specific pathway that is sensitive to inhibitors of Ca_v channels (see Fig. 2, Wood *et al.* 2005). Furthermore $\text{Ca}_v1.2$ and 2.3 have been immunolocalized to the sea urchin sperm flagella (Granados-Gonzalez *et al.* 2005), hinting at a role for these channels in regulating chemotaxis. The opening of a rapid flagellar Ca^{2+} entry pathway has also been shown to generate chemotactic turns in a response to a native chemoattractant in a different species of sea urchin (Bohmer *et al.* 2005). Another Ca^{2+} channel localized to the flagella of sea urchin sperm is the IP_3 receptor (Zapata *et al.* 1997), a protein usually associated with liberation of Ca^{2+} from internal Ca^{2+} stores, of which there are none in the flagella of sea urchin sperm.

Additionally, in sea urchin sperm, two hyperpolarization-activated and cyclic nucleotide-gated channels (HCN) have been immunolocalized to the flagella compartment, namely SpHCN1 (Gauss *et al.* 1998) and SpHCN2 (Galindo *et al.* 2005). These channels could open in response to the hyperpolarization event(s) and/or increases in cyclic nucleotides that follow binding of egg peptides, and an inhibitor of HCN channels alters the kinetics of the early $[\text{Ca}^{2+}]_i$ changes induced by such peptides (Nishigaki *et al.* 2004).

In ascidian sperm, an egg-derived factor both activates sperm motility and stimulates chemotaxis. Sperm activation is sensitive to inhibitors of Ca_v channels, and chemotaxis is blocked by an inhibitor of store-operated Ca^{2+} (SOC) channels (Yoshida *et al.* 2003) indicating a role for these channel families in differing motility phenomena.

Sperm capacitation

Mature epididymal sperm acquire competence to fertilize during the sperm transit through the female reproductive tract. This complex process, called capacitation, involves several changes in sperm. These include reorganizing the plasma membrane as a consequence of cholesterol removal, extensive protein tyrosine phosphorylation, hyperpolarization of the E_m and increases in pH_i and $[\text{Ca}^{2+}]_i$ (reviewed by Visconti *et al.* 2002, Darszon *et al.* 2005). Capacitation is also associated with the appearance

of hyperactivated motility, which some authors still consider as part of capacitation (Visconti *et al.* 2002, Suarez & Ho 2003). All these changes prime sperm to respond to the zona pellucida (ZP), the extracellular egg coat that induces the AR. Although the molecular mechanisms and the signal transduction events are only partially defined, capacitation and tyrosine-phosphorylation have been shown to be regulated by a cAMP-dependent pathway involving PKA (Visconti *et al.* 2002; Fig. 3).

Recently, it was proposed that the changes in membrane fluidity and organization that occur during capacitation are in part a consequence of HCO_3^- activation of scramblases that move phospholipids in both directions across the plasma membrane (Harrison & Gadella 2005). These changes can alter ion transport for instance of Ca^{2+} and HCO_3^- itself, thus activating sAC. It has been proposed that sAC is mainly responsible for the cAMP changes during capacitation (Esposito *et al.* 2004, Hess *et al.* 2005), although a transmembrane AC may also contribute (Fraser *et al.* 2005; Fig. 3).

Numerous studies have demonstrated that capacitation is dependent on different ions present in the capacitation medium, such as Ca^{2+} , K^+ , HCO_3^- and Na^+ (Visconti *et al.* 2002, Darszon *et al.* 2005). *In vitro* capacitation requires the presence of three key components: Ca^{2+} , HCO_3^- and bovine serum albumin (BSA). Several types of ion channels and transporters have been reported in mammalian sperm (Darszon *et al.* 2005; Fig. 2), but how they participate in the regulation of E_m , pH_i and $[\text{Ca}^{2+}]_i$ during capacitation is not well established. Non-capacitated sperm maintain a relatively depolarized E_m (~ -35 to -45 mV) that hyperpolarizes (-80 mV) during capacitation (Arnoult *et al.* 1999, Munoz-Garay *et al.* 2001). This has been proposed to remove inactivation from T-type Ca_v channels and recruit them to a closed state, from which they are available for opening during the induction of the AR by ZP3 (Zona Pellucida Protein 3) (discussed further below in the mammalian acrosome reaction section; Arnoult *et al.* 1999). The identity of the ion transport system(s) responsible for the $[\text{Ca}^{2+}]_i$ increase observed during capacitation is not known. Ca_v channels other than T-type as well as several members of the TRP family have also been identified in sperm (Fig. 2) and their precise role during capacitation, if any, remains to be established.

Since external K^+ and K^+ -channel blockers influence the hyperpolarization that accompanies mouse sperm capacitation, it is thought that the K^+ permeability contributes to this process (Arnoult *et al.* 1999, Munoz-Garay *et al.* 2001, Acevedo *et al.* 2006). Molecular and functional evidence indicate the presence of voltage-gated K^+ channels (Salvatore *et al.* 1999), Ca^{2+} -activated K^+ channels (Chan *et al.* 1998) and inwardly rectifying K^+ (K_{ir}) channels (Munoz-Garay *et al.* 2001, Felix *et al.* 2002, Acevedo *et al.* 2006) in spermatogenic and sperm cells. Before capacitation, sperm pH_i is relatively acidic and may down-regulate sperm K_{ir} channels, thereby maintaining E_m depolarized and indirectly preventing unregulated

oxide synthase activities (Neill & Vacquier 2004, Darszon *et al.* 2005).

The fusion of plasma and acrosomal membrane during the AR produces hybrid vesicles (HVs) that contain at least three types of ionic channels. A cAMP activated cationic channel sensitive to Ba^{2+} was most frequently encountered in black lipid membrane (BLM) recordings (Schulz *et al.* 2004). Ionic channels from flagellar membranes with similar characteristics have been also identified in BLMs (Labarca *et al.* 1996) and patch-clamp techniques in sea urchin sperm (Sanchez *et al.* 2001). The two other channels were also cationic and had larger conductances (Schulz *et al.* 2004).

Besides suREJ1, sea urchin sperm contain suREJ2 and suREJ3 that have been cloned and sequenced. The three suREJ proteins possess a REJ module, shared by the human autosomal dominant polycystic kidney disease (ADPKD) protein, polycystin-1 (PC1), and by PCREJ, a testis-specific protein in mammals of unknown function. ADPKD is caused by mutations in the *PKD1* and *PKD2* genes, encoding the transmembrane proteins PC1 and PC2 respectively (Neill & Vacquier 2004). In mammals PC1 and PC2 associate to form non-selective cation channels (Xu *et al.* 2003). PC2 or TRPP2, a member of the TRP superfamily of proteins, has amino acid sequence similarity to Ca_v and other cation channels (Nilius & Voets 2005). This protein itself forms Ca^{2+} -permeable nonselective channels that may be involved in ligand binding (Ikeda & Guggino 2002).

suREJ3 includes a C-terminal transmembrane region that is homologous to Ca_v channels which has been implicated in associations with PC2 (Xu *et al.* 2003). Indeed, sea urchin sperm possess a PC2 homolog (suPC2) associated with suREJ3 in the sperm plasma membrane in a thin band overlying the acrosomal vesicle (Mengerink *et al.* 2002, Neill & Vacquier 2004). Notably, a high-conductance, Ca^{2+} -permeable channel, whose properties resemble some of those displayed by the PC1–PC2 channels, has been recorded in BLMs containing sea urchin or mouse sperm plasma membranes (Lievano *et al.* 1990, Beltran *et al.* 1994). The sea urchin sperm channel discriminates poorly between monovalent and divalent cations ($\text{PCa}/\text{PNa} \sim 5$; Lievano *et al.* 1990). This evidence suggests that REJ and PC proteins may be involved in the ion fluxes that follow binding of FSP and lead to the AR. As described later, TRP channels are present in mouse sperm where they seem to participate in the AR (Jungnickel *et al.* 2001, Trevino *et al.* 2001).

Ca^{2+} uptake during the sea urchin sperm AR precedes the changes in pH_i and $[\text{Na}^+]_i$ and is mediated at least by two different Ca^{2+} channels (Rodriguez & Darszon 2003). FSP triggers a fast transient increase in $[\text{Ca}^{2+}]_i$ that is sensitive to verapamil and dihydropyridines (DHPs; Ca_v channel blockers that inhibit the AR). Five seconds later, a second Ca^{2+} channel insensitive to the latter blockers, permeable to Mn^{2+} and Na^+ , and pH_i dependent, opens for minutes and leads to the sperm AR. Interestingly, one of the first two Ca_v channel orthologs (Ca_v 2.3) recently

identified in *Strongylocentrotus purpuratus* sea urchin sperm is preferentially localized in the acrosome area (Granados-Gonzalez *et al.* 2005). On the other hand, as in the mammalian sperm AR (Santi *et al.* 1998, O'Toole *et al.* 2000, Jungnickel *et al.* 2001), the second channel appears to be a SOC channel (Gonzalez-Martinez *et al.* 2001). Indeed, IP_3 receptors have been detected in sea urchin sperm, possibly in the acrosome (Zapata *et al.* 1997). It is known that IP_3 -mediated release of Ca^{2+} from intracellular stores activate SOCs (Nilius & Voets 2005). Though not a physiological event, the second channel can be opened by a lower molecular weight (~ 60 kDa) hydrolyzed form of FSP, which can increase $[\text{Ca}^{2+}]_i$ but does not induce AR by itself (Hirohashi & Vacquier 2002). Namely, activation of this second channel alone causes acrosomal exocytosis but not acrosomal actin polymerization (Hirohashi & Vacquier 2003).

Elevation of the $[\text{K}^+]_e$ to 30–50 mM or addition of tetraethylammonium (TEA), a K^+ channel blocker, inhibits the AR and the increases in $[\text{Ca}^{2+}]_i$ and pH_i associated to this reaction in *S. purpuratus* sperm (Darszon *et al.* 2005). These results suggest the participation of K^+ channels in the AR. In *L. pictus* sperm, binding of FSP induces a fast K^+ -dependent hyperpolarization (Gonzalez-Martinez & Darszon 1987) which precedes and probably leads to the activation of a Ca^{2+} -dependent Na^+/H^+ exchange that in turn increases pH_i (Gonzalez-Martinez *et al.* 1992). This hyperpolarization could remove inactivation from Ca_v channels (Gonzalez-Martinez & Darszon 1987, Lievano *et al.* 1990).

Besides Ca^{2+} channels, TEA^+ -sensitive K^+ (Lievano *et al.* 1985, Gonzalez-Martinez *et al.* 1992, Labarca *et al.* 1996) and Cl^- channels have been recorded in planar bilayers with incorporated *S. purpuratus* sperm plasma membranes. The sea urchin AR is inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), an anion channel and transporter blocker (Morales *et al.* 1993). The sensitivity of some of the ion channels recorded in planar bilayers to inhibitors of the AR is consistent with their involvement in this fundamental event (Darszon *et al.* 2005).

Acrosome reaction in mammalian sperm

After traveling to find the egg, sperm must recognize its extracellular coat, the zona pellucida (ZP) and bind to it. The AR allows sperm to penetrate this layer and fuse with the egg. This process is accompanied by the release of lytic enzymes and the fusion of the acrosome and plasma membranes in the sperm head. Among the components of ZP, ZP3 is recognized as the natural AR inducer. The ZP3 receptor has not been unequivocally identified in the sperm plasma membrane though several candidates exist (Jovine *et al.* 2005). The signaling cascade that culminates with the AR is not fully understood but intensive research has shed light on this crucial phenomenon (Fig. 4). ZP and other agents like progesterone trigger AR by influencing

Figure 4 (A) Intracellular changes elicited upon ligand binding to sperm receptors during the acrosome reaction (AR). Initially there are increases in E_m , pH_i and a Ca^{2+} transient. Production of IP_3 causes Ca^{2+} store depletion that results in SOC channel opening responsible for the sustained Ca^{2+} increase that precedes the AR. (B) Channels and molecules involved in the changes that lead to the mammalian sperm AR. ZP3 binding to its receptor: activates a G_i protein involved in the pH_i change possibly mediated by Na^+/H^+ transporters; causes membrane depolarization (possibly by GABA, ACh and Gly receptors or an unidentified cation channel) that opens Ca_v channels which cause the initial Ca^{2+} transient influx; Ca^{2+} activation of $PLC\delta$ generates diacylglycerol (DAG) and IP_3 consuming its substrate, PIP_2 . IP_3 thereafter binds to its receptor and causes Ca^{2+} -store depletion which in turn (possibly in conjunction with DAG) activates SOC channels at the plasma membrane. Ca^{2+} , cAMP and small G proteins act together to activate the SNARE machinery required for membrane fusion. SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; PTx, pertussis toxin; EPAC, exchange protein directly activated by; Cyt, Synaptotagmin.

ion fluxes, phospholipid metabolism, cAMP levels, and protein phosphorylation. During the AR there are increases in $[Ca^{2+}]_i$ and pH_i , the latter being sensitive to pertussis toxin (PTx) that inactivates G_i proteins. The current model proposes that ZP3 binding generates a biphasic Ca^{2+} response involving at least three separate yet sequentially-linked Ca^{2+} channels. In mouse sperm, initially $[Ca^{2+}]_i$ elevates to micromolar levels within 50 ms and relaxes back to resting values within 200 ms. The pharmacology and kinetics of this excursion are consistent with opening of T-type Ca_v channels (Ca_v3 ; Arnoult *et al.* 1999). Furthermore, the AR can be inhibited by several Ca_v3 channel antagonists such as DHPs, pimozide and Ni^{2+} . Molecules such as urocortin, fenvalerate and gossypol have been shown to inhibit T-type currents in spermatogenic cells and may have contraceptive applications (Bai & Shi 2002, Tao *et al.* 2005, Xiao *et al.* 2006). This family of Ca_v s has three members, all of them have been immunolocalized in mammalian sperm. $Ca_v3.1$ and $Ca_v3.2$ localize to the acrosomal region, however, knockout mice for these two Ca_v isoforms are fertile (Chen *et al.* 2003, Stambouljian *et al.* 2004), and it could be that double Ca_v channel knockouts would help clarify the role of these channels. The participation of Ca_v3 in the human sperm AR is much less clear. Additionally, other members of the Ca_v superfamily and CatSper (Lobley *et al.* 2003) have been identified in mammalian sperm (Fig. 2) but

their function remains unknown (Guzman-Grenfell & Gonzalez-Martinez 2004).

The second $[Ca^{2+}]_i$ rise is slower and sustained, lasting several minutes and is necessary for AR completion. ZP3 binding causes an IP_3 increase which in turn releases Ca^{2+} from the acrosome – the sperm Ca^{2+} store (Walensky & Snyder 1995, De Blas *et al.* 2002). The evidence supporting this model includes the identification of the Ca^{2+} -sensitive $PLC\delta4$ in sperm, with the $PLC\delta4$ null mouse being infertile. Furthermore, IP_3 receptors have been immunolocalized to the acrosome and also to the RNE. This generation of IP_3 and emptying of intracellular Ca^{2+} stores, as in somatic cells, leads to a further $[Ca^{2+}]_i$ increase due to opening of SOCs at the plasma membrane. It is likely that the SOC is a member of the TRP family. TRPC2 has been implicated in the ZP3-induced mouse AR (Jungnickel *et al.* 2001) and the acrosome region of these sperm contains at least TRPC1, 2 and 5 (Fig. 2). Maitotoxin, which induces Ca^{2+} uptake through its action on TRPs, is the most potent AR inducer aside from ZP (Trevino *et al.* 2006). The possible participation of more than one TRP cannot be discarded due to the fact that TRPC2 null mice are fertile (Stowers *et al.* 2002) and that TRPC channels can form heteromultimers (Nilius & Voets 2005).

An unresolved question is how the TRP channels involved in the AR are activated? There are several hypotheses and the diversity of this family means it is unlikely that

a single mechanism exists. Recently several TRP regulating molecules have been identified and it is worthwhile exploring their participation in the AR signaling cascade. STIM (Stromal Interaction Molecule) is a TRP-interacting protein that translocates from the ER to the plasma membrane to function either as the communication factor between the Ca^{2+} -store and the plasma membrane or forming part of the channel itself (Zhang *et al.* 2005). TRP channels can also be regulated by phosphatidylinositol biphosphate (PIP_2 ; Suh & Hille 2005) and by newly discovered regulatory proteins such as junctate (Stambouliau *et al.* 2005) and enkurin (Sutton *et al.* 2004) that bind specifically to certain TRP isoforms modulating their activity.

There are other agonists such as progesterone, gamma-aminobutyric acid (GABA), acetylcholine (ACh) and glycine that can induce the AR but their physiological significance remains to be established. The presence of ionotropic receptors for GABA (GABA_AR) (Wistrom & Meizel 1993) ACh (Kumar & Meizel 2005) and glycine (GlyR) (Sato *et al.* 2000) in sperm has been documented and mutant mice for nAChR (null) and GlyR (point mutation) showed reduced ZP-induced AR and *in vitro* fertility (Meizel & Son 2005). Considering that intracellular Cl^- was estimated to be 40 mM in capacitated human sperm, opening of Cl^- channels, such as the GABA_AR receptor or the GlyR, should result in Cl^- efflux, leading to depolarization of the E_m (Garcia & Meizel 1999). The proposal is that these neurotransmitter receptors are responsible for the initial depolarization required to activate the Ca_v channels involved in the initial Ca^{2+} rise.

In human sperm, progesterone also induces a biphasic Ca^{2+} response similar to that of ZP3. However, when applied as a gradient (simulating the physiological conditions), the initial Ca^{2+} rise is replaced by oscillations proposed to be mediated by RyR-like receptors (Harper *et al.* 2004). These results suggest progesterone may help sperm to penetrate through the egg coat by inducing hyperactivation-like flagellar movement as well as the AR.

Concluding remarks

Recent developments in the tools available to study ion channels are improving our understanding of how these integral membrane proteins operate and are regulated in the sperm. Voltage and ion-sensitive fluorescent dye imaging, novel patch-clamp approaches, bilayer reconstitution, molecular biology and the growing number of available genomes are unveiling the fascinating and often unique properties of sperm ion channels. Correlating ion channel location and function will enable us to understand how these proteins participate in the finely orchestrated sperm functions involved in generating a new individual.

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