Int. J. Dev. Biol. 52: 595-606 (2008)
doi: 10.1387/ijdb.072550ad

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Sperm-activating peptides in the regulation of ion fluxes, signal transduction and motility

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ABSTRACT Echinoderm sperm use cyclic nucleotides (CNs) as essential second messengers to locate and swim towards the egg. Sea urchin sperm constitute a rich source of membrane-bound guanylyl cyclase (mGC), which was first cloned from sea urchin testis by the group of David Garbers. His group also identified speract, the first sperm-activating peptide (SAP) to be isolated from the egg investment (or egg jelly). This decapeptide stimulates sperm mGC causing a fast transient increase in cGMP that triggers an orchestrated set of physiological responses including: changes in: membrane potential, intracellular pH (pHi), intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and cAMP levels. Evidence from several groups indicated that cGMP activation of a K^+ selective channel was the first ion permeability change in the signaling cascade induced by SAPs, and recently the candidate gene was finally identified. Each of the 4 repeated, 6 trans-membrane segments of this channel contains a cyclic nucleotide binding domain. Together they comprise a single polypeptide chain like voltage-gated Na^+ or Ca^{2+} channels. This new type of channel, named tetraKCNG, appears to belong to the exclusive club of novel protein families expressed only in sperm and its progenitors. SAPs also induce fluctuations in flagellar $[Ca^{2+}]i$ that correlate with changes in flagellar form and regulate sperm trajectory. The motility changes depend on $[Ca^{2+}]i$ influx through specific Ca^{2+} channels and not on the overall $[Ca^{2+}]i$ in the sperm flagellum. All cilia and flagella have a conserved axonemal structure and thus understanding how Ca^{2+} regulates cilia and flagella beating is a fundamental question.

KEY WORDS: *sea urchin, calcium, potassium channel, cyclic nucleotides, flagella*

Introduction

Marine animals produce vast numbers of gametes to contend with the enormous dilution that occurs during external fertilization in the sea. A larger egg or oocyte also increases the probability of fertilization by sperm under these conditions (Podolsky, 2001). However, the size of the female gamete is inversely proportional to fecundity due to a number of physico-chemical parameters that limit maximum egg dimensions (Levitan, 1996), a dilemma for any species. Many marine species have evolved matrix-like and/or gelatinous structures that physically increase the size of the egg, thus enhancing the probability of gamete interaction (Podolsky, 2001; Podolsky, 2002). Some species have further increased the apparent size of their eggs by producing a diffusible chemoattractant for their homologous sperm (Miller, 1985; Podolsky, 2002; Riffell and Zimmer, 2007).

The regulation of sperm motility and chemotaxis are fascinating biological phenomena involving a navigational system finely tuned to enhance the probability that sperm find the egg. Chemotaxis requires that the tiny sperm must be able to detect rapidly and precisely the chemoattractant concentration, controlling its flagellar form to regulate its swimming pattern.

In this review we describe historical aspects as well as current information about the signaling processes triggered by sperm-activating peptides (SAPs) that lead to a working model of sea urchin sperm chemotaxis. We focus particularly on the ion trans-

Abbreviations used in this paper: CNs, cyclic nucleotides; mGC, membrane bound guanylyl cyclase; SAP, sperm-activating peptide.

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Published online: 4 July 2008

0214-6282/2008/$35.00

port systems involved and comment on future perspectives in this important and fast developing field.

Sperm-activating peptides

SAPs are diffusible peptide components of the egg outer investment (or egg jelly) in echinoderms such as sea urchins and starfish (Nishigaki *et al.*, 1996; Suzuki, 1995). The activity of these peptides is easily detected in slightly acidified seawater (pH 6.2-6.8) in which suppressed sperm motility and respiration (oxygen consumption) are restored by addition of ethanolic extracts of egg jelly (Ohtake, 1976). This recovery in motility and respiration depends on the ability of SAPs to increase sperm intracellular pH (pHi), and both NH_4Cl and monensin, an ionophore which exchanges Na^+/H^+, can mimic the activity of SAPs (Hansbrough and Garbers, 1981a). This property was used to screen components of the egg investments, and led to the sequencing of the first SAP which was purified from *Hemicentrotus pulcherrimus* by the group of Dr. Norio Suzuki (Suzuki *et al.*, 1981). On the other hand, the group of Dr. David Garbers explored components of the egg jelly that transiently increase sperm cGMP in *Strongylocentrotus purpuratus* and purified speract (Garbers *et al.*, 1982; Hansbrough and Garbers, 1981b), the identical molecule that Suzuki's group had obtained. Since then, hundreds of SAPs have been identified by Suzuki's group from numerous species of sea urchin (Suzuki, 1995). These peptides are classified into 5 (or 6) groups depending on sequence similarity and cross-reactivity. SAPs function as moderate, though not complete, species-specific molecules since species that belong to the same taxonomic order share the same class of SAPs (Suzuki, 1995). One sea urchin species can produce different SAP isoforms; one gene encodes multiple SAPs, which are connected in tandem with a spacer amino acid (or several amino acids) forming a large precursor protein (Matsumoto *et al.*, 1999; Ramarao *et al.*, 1990). Interestingly, speract and its isoforms are not synthesized in the oocyte but in the accessory cells that surround the oocyte (Suzuki, 1995), in contrast to the starfish *Asterias amurensis* where SAPs, called asterosaps, are synthesized by the oocyte itself (Matsumoto *et al.*, 1999).

Since sperm display near maximum motility in normal seawater (pH 8.0) (Ohtake, 1976), the physiological function of SAPs had not been wholly recognized until resact, a SAP purified from *Arbacia punctulata* (Suzuki *et al.*, 1984), was demonstrated to have chemotactic activity towards its homologous sperm (Ward *et al.*, 1985a). However, the full physiological relevance of SAPs is still not completely understood as *S. purpuratus* sperm do not show chemotactic responses to speract. In addition, SAPs probably facilitate the penetration of sperm through the egg jelly (Suzuki and Garbers, 1984) and/or promote the acrosome reaction in some species (Nishigaki *et al.*, 1996).

SAP Receptors and the Membrane-Associated Guanylate Cyclase

Assisted by their high abundance, chemical cross-linking was successfully employed to identify the sea urchin sperm SAP receptors. In *S. purpuratus* sperm, speract was found cross-linked to a 77 kDa membrane protein whose amino acid sequence indicated a single transmembrane segment close to its C terminus, and many cysteines in the extracellular speract-binding domain (Dangott and Garbers, 1984; Dangott *et al.*, 1989). The 77 kDa protein activates detergent-solubilized mGC (Bentley *et al.*, 1988), and is homologous to a scavenger receptor domain found in macrophages and endothelial cells (Kodama *et al.*, 1990; Rohrer *et al.*, 1990). In the case of resact, a SAP isolated from *A. punctulata* egg jelly, cross-linking occurred directly with the sperm mGC (Shimomura *et al.*, 1986). It is still not understood why the SAPs from these two sea urchin species bind to such functionally divergent receptors, although it is worth remembering that *S. purpuratus* and *A. punctulata* share a relatively distant (~160 MYA) common ancestor (De Giorgi *et al.*, 1996; Littlewood and Smith, 1995).

Analysis of the cDNA encoding mGC from *S. purpuratus* and *A. punctulata* sea urchin sperm indicates they encode similar polypeptides with a single transmembrane domain (Dangott *et al.*, 1989; Thorpe and Garbers, 1989). The intracellular (catalytic) domains are highly conserved across the species, whereas the extracellular (putative binding) domains show greater diversity, as would be expected for receptors that interact with distinctly different peptides. The *A. punctulata* mGC was reported to have 989 amino acids with an amino-terminal signal sequence. The probable 28 amino acid single transmembrane domain divides the protein into a 478-residue amino-terminal domain and a 459 residue carboxyl-terminal domain. The transmembrane region is immediately followed by three basic residues, Arg-Lys-Arg, a primary sequence feature of a cytoplasmic region common to many membrane proteins that represents the stop transference signal anchoring proteins to the membrane. The extracellular domain has four potential N-linked glycosylation sites. The cytoplasmic C-terminal 95 amino acids contain 20% serine, the likely regulatory sites for phosphorylation, that can alter its apparent size. Recently, it was reported that the intracellular region of mGC harbors a conserved cyclase-homology domain that had not been reported in the published sequences of *A. punctulata* mGC, but present in the *S. purpuratus* mGC sequence (Dangott *et al.*, 1989; Kaupp *et al.*, 2007; Singh *et al.*, 1988; Thorpe and Garbers, 1989). The intracellular region of mGC also contains a domain with homology to protein kinases that is also found in the mammalian natriuretic peptide receptor (NPR) (Singh *et al.*, 1988). Its function is incompletely understood and has been postulated to operate as a negative regulatory element (Chinkers and Garbers, 1989; Koller *et al.*, 1992). When cultured cells expressing recombinant NPR were treated with geldanamycin, an inhibitor of hsp90 ATPase function, NPR-stimulated production of cGMP was inhibited (Kumar *et al.*, 2001). This suggested that hsp90 was required for NPR processing and/or stability. This NPR kinase domain specifically binds a protein-serine/threonine phosphatase 5 that may mediate NPR desensitization (Chinkers, 1994). Moreover, NPR has 5-6 phosphorylated residues in this kinase-domain depending on the isoform, which influence its guanylate cyclase activity (Potter and Hunter, 1998a; Potter and Hunter, 1998b). Six phosphorylated serine residues of sea urchin mGC were identified; five of them are located in the kinase-homology region and four of them undergo dephosphorylation on stimulation by resact (Kaupp *et al.*, 2008). It remains to be seen if the kinase-like domain of the sea urchin sperm mGC has a regulatory role.

Kinetics and regulation of membrane bound guanylyl cyclase

The binding of SAP transiently increases mGC activity, with the subsequent decrease correlated to dephosphorylation of serine residues and a decrease in apparent molecular weight (MW)

(Bentley *et al.*, 1986; Ramarao and Garbers, 1985; Suzuki *et al.*, 1984; Ward *et al.*, 1985b). Evidence indicates that pHi regulates the phosphorylation state of mGC and thus its activity (Shimomura *et al.*, 1986). Artificially increasing sperm pHi with monesin or NH_4Cl decreases the apparent MW of mGC (Ramarao and Garbers, 1985), suggesting that pHi could be the primary signal for enzyme dephosphorylation and concomitant inactivation, however it remains to be established whether pHi-sensitive phosphatases play a role in this process. As the enzyme is dephosphorylated, its activity returns to basal levels, thereby desensitizing the system to the presence of the egg peptide (Bentley *et al.*, 1986). It appears that the rates of inactivation and dephosphorylation of mGC are similar (Kaupp *et al.*, 2007). Desensitization could allow the enzyme to adapt to SAP binding of its receptor since their interaction is practically irreversible (Kon >> Koff) (Nishigaki and Darszon, 2000; Nishigaki *et al.*, 2001). Sperm flagella have a large number of high affinity SAP receptors (10^4-10^6 receptors, depending on the species) (Kaupp *et al.*, 2007; Nishigaki and Darszon, 2000; Shimomura and Garbers, 1986; Smith and Garbers, 1983), and it has been calculated that *A. punctulata* sperm can detect a single resact molecule (Kaupp *et al.*, 2003). Furthermore, it has been reported that a pHi increase enhances the receptor's affinity for the peptide and that as binding increases, mGC responsiveness to the peptide decreases (Bentley *et al.*, 1986; Nishigaki and Darszon, 2000; Nishigaki *et al.*, 2001; Shimomura *et al.*, 1986).

The transient nature of the cGMP increase upon SAP binding to its receptor is ensured also by the rapid degradation of cGMP as well as desensitization of mGC. Several studies using relevant inhibitors indicate that phosphodiesterases (PDEs) strongly influence the rate of cGMP decrease (Kaupp *et al.*, 2003; Schulz *et al.*, 1989). Recently, sea urchin sperm phosphoproteomics led to the discovery of PDE5 and PDE11A as substrates of PKA (Su *et al.*, 2005). Among them, PDE5 (suPDE5) was first cloned and characterized (Su and Vacquier, 2006). This enzyme hydrolyzes only cGMP and its activity is enhanced by pH increase. Treating sperm with egg jelly leads to suPDE5 phosphorylaton increasing its enzymatic activity. Furthermore, the phosphorylated form of this enzyme is mainly localized in sperm flagella. These findings suggest that suPDE5 is involved in SAP signaling cascade rapidly degrading cGMP. The combination of high sensitivity to SAPs and adaptation to their continuous presence could be the key to controlling the timing of the turns as sperm swim towards the egg in a chemoattractant concentration gradient.

Sea urchin sperm-activating peptides induce a K^+ dependent hyperpolarization mediated by a novel tetrameric, K^+ selective, cyclic nucleotide gated channel (tetraKCNG)

Lee and Garbers in 1986 discovered that speract activated a K^+-dependent Na^+/H^+ exchange by hyperpolarizing *S. purpuratus* sperm flagella and flagellar plasma membrane vesicles. Their evidence suggested that the decapeptide was opening K^+ channels (Garbers, 1989; Lee and Garbers, 1986). Thereafter, Babcock *et al.* (1992) showed that in swollen sea urchin sperm speract causes a transient, K^+-selective, permeability increase mediated by K^+ channels, consistent with patch-clamp experiments. This and later work also described the speract-induced elevation of $[Ca^{2+}]i$ in swollen sperm (Babcock *et al.*, 1992; Cook and Babcock, 1993b). Galindo *et al.* (2000) then established, in flagellar plasma membrane vesicles isolated from *S. purpuratus* sperm, that a K^+ selective channel was activated by speract and directly by cGMP. The carbocyanine dye employed in these experiments is positively charged and distributes between the inside and outside of the cell in ~ seconds, according to the membrane potential (Waggoner, 1979). Because of its positive charge, it also partitions into internal organelles with negative potential such as the mitochondria, therefore it is necessary to collapse their potential using an uncoupler. Under these conditions the cyanine dye undergoes a large fluorescence change per mV of plasma membrane potential displacement that can be calibrated using valinomycin (Darszon *et al.*, 1994). Though the ionic gradients in swollen sperm and in flagellar membranes are different from those in normal sperm, the speract-induced hyperpolarization was shown to be due to a cGMP dependent and K^+ selective permeability increase. These responses are only weakly dependent on external Ca^{2+} and other ions, and are mainly determined by the K^+ gradient (Babcock *et al.*, 1992; Beltran *et al.*, 1996; Cook and Babcock, 1993b; Galindo *et al.*, 2000). Later experiments confirmed that in normal sea urchin sperm speract induced a K^+-dependent hyperpolarization followed by a depolarization which was partly due to Ca^{2+} influx (Beltran *et al.*, 1996; Reynaud *et al.*, 1993). The overall results also suggested that this decapeptide opened Ca^{2+} permeable channels (Cook and Babcock, 1993a; Schackmann and Chock, 1986).

The findings described above taken together led to the proposal of a working model of the signaling cascade triggered by speract. This model indicated that the first ion permeability change triggered by the increase in cGMP was due to the activation of a K^+ selective channel possibly directly modulated by cGMP. The model also included a Ca^{2+} channel activated by depolarization and cAMP ((Darszon *et al.*, 1999); page 492). The limitations in temporal resolution of the early reported changes in sea urchin sperm cGMP, cAMP, pHi, $[Ca^{2+}]i$ and membrane potential precluded establishing their sequence and eventually understanding the mechanisms involved in the regulation of motility by SAPs. At around the same time the groups of Kaupp and Darszon begun independent efforts to implement fast measurements to kinetically resolve the responses induced by SAPs. Both groups utilized fast mixing experiments and developed caged SAPs and cyclic nucleotides. Caged compounds are useful tools to elucidate signaling processes with high spatial and temporal resolution in single cells and cell populations (Bohmer *et al.*, 2005; Kaupp *et al.*, 2003; Nishigaki *et al.*, 2004; Nishigaki *et al.*, 2001; Strunker *et al.*, 2006; Tatsu *et al.*, 2002; Wood *et al.*, 2005; Wood *et al.*, 2007).

In what follows information will be described considering the basic sequence of the proposed model and not the chronology of the findings. Fast mixing experiments from Kaupp's group demonstrated that the first event after resact binding to *A. punctulata* sperm mGC was a large cGMP increase which at nM concentrations reached a peak at ~200 ms and decayed to a plateau higher than the baseline in by ~5-10 s. cAMP did not change appreciably in the initial 200 ms after resact addition, and its eventual increase was smaller and slower, reaching a peak at ~1-2 s and also decaying to a plateau significantly higher than the starting level (Kaupp *et al.*, 2003).

Later, fast membrane potential recordings in *A. punctulata* sperm using the electrochromic voltage-sensitive dye di-8-ANEPPS, revealed that indeed, the first ionic permeability change triggered

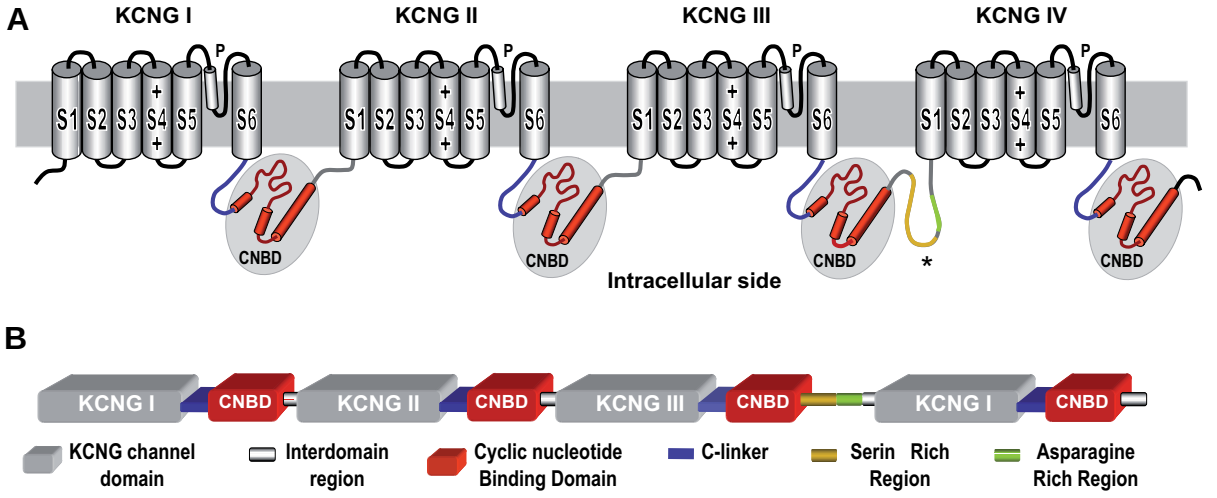

Fig. 1. Membrane topology of tetrameric K⁺selective, cyclic nucleotide gated channel (Sp-tetraKCNG). (A) *The transmembrane segments (S1–S6) for the four KCNG domains (I-IV), the pore region (P) and the four cyclic nucleotide binding domains (CNBD) are shown. The voltage sensor (S4) for each KCNG domain is indicated by positive charges. The CNBD in* **(B)** *is represented in (A) with the three α-helices drawn as tubes. A C-linker* **(B)** *connects each CNBD to the end of S6. The interdomain region between KCNG II and III (denoted by an asterisk in A) contains a serine rich region and an asparagine rich profile (shown in B); these regions could therefore function in protein-protein interactions.*

by uncaging resact is a transient hyperpolarization (Strunker *et al.*, 2006). Uncaging cGMP also triggered the transient hyperpolarization which was followed by a depolarization. As anticipated, the cGMP induced hyperpolarization had a shorter delay (~ 6 ms) than that triggered by resact. These results and the K^+ dependence of the hyperpolarization are consistent with the activation of a cGMP-gated, K^+ selective channel. Interestingly, Strunker *et al.* (2006) found that *A. punctulata* express a protein that has the characteristics of a K^+-selective, cGMP gated channel. This work provided the sequence of one subunit and suggested that the channel was probably made of several subunit repeats. Thereafter, work by Galindo *et al.* (2007) in *S. purpuratus*, reported the complete sequence of a novel sperm cyclic cGMP gated channel that is K^+ selective and constructed from four linked repeat modules (Sp-tetraKCNG) with an apparent MW of ~250 kD. Most voltage-gated K^+ channels have four separate, six transmembrane helices (SI-S6), and they require tetramerization to constitute a functional channel. However, as in Ca_V or Na_V channels, Sp-tetraKCNG has the four repeat modules connected in one protein chain eliminating the need for tetramerization (see Fig. 1). Each Sp-tetraKCNG repeat module has the GYGD sequence, distinctive of the selectivity filter for K^+ selective channels, and the cyclic nucleotide binding domain (CNBD) linked to S6. The channel is the first of its category and is evolutionarily situated between K^+ selective and voltage-dependent EAG channels, and the cationic and voltage-independent CNG channels, both families having a CNBD (Galindo *et al.*, 2007). The amino acids recognized to be essential for cyclic nucleotide binding to the four Sp-tetraKCNG CNBDs were compared with those of PKA, PKG, and CNG, EAG-like, HCN and KAT family channels. All four tetraKCNG CNBDs domains have a Thr residue that forms a hydrogen bond with the guanine 2-amino group of cGMP, but not with cAMP. Additionally, this Thr shares the same position as in CNG channels and PKGs indicating that the four tetraKCNG CNBDs display a sequence similarity to the cGMP-binding region of CNG channels and of PKGs (Galindo *et al.*, 2007).

The activity of CN regulated channels has been examined by fusing *S. purpuratus* purified flagellar plasma membranes with planar lipid bilayers (Galindo *et al.*, 2007; Labarca *et al.*, 1996). Some time ago cAMP-gated cationic single channels were documented using this strategy (Labarca *et al.*, 1996). In 2007, single channel recordings from a K^+-selective channel activated by cGMP and inhibited by DCB, a CNG channel blocker (Hofmann *et al.*, 2005), but not by ZD7288 which blocks HCN channels (Shin *et al.*, 2001), were described. This K^+ selective cGMP regulated channel opens in the absence of cGMP, therefore, it could influence the sperm resting membrane potential. A similar channel was recorded after incorporating *L. pictus* sperm flagellar membranes into planar bilayers (Galindo *et al.*, 2007). A recent review by the Kaupp group (Kaupp *et al.,* 2008) indicates that the expressed *A. punctulata* tetraKCNG channel is very sensitive and selective to cGMP (ED_{50} ~ 50 nM), while cAMP is 1000 times less potent.

Events occurring after membrane hyperpolarization

Nishigaki *et al.* (2001) reported fast mixing experiments with saturating speract concentrations documenting that pHi starts increasing with a delay of ~ 70 ms while the $[Ca^{2+}]i$ elevation occurs with a delay of ~ 200 ms. In addition, they found using fluorescent speract that the speract receptor in sperm underwent a pHi dependent increase in affinity at ~130 msec.

To avoid potential problems created by mechanical and hydrodynamic damage and disruption of cells during fast mixing experiments, a backbone-caged speract was synthesized that contains a 2-nitrobenzyl group at a backbone amide. This caged speract derivative has a considerably reduced affinity for its receptor (IC_{50} = 950 nM) (Tatsu *et al.*, 2002). UV irradiation photocleaves the 2-nitrobenzyl group ($\tau = 26\ \mu s$), and the liberated speract adduct has near identical affinity for its receptor (IC_{50} = 0.67 nM) and is indistinguishable from native speract in its ability to increase sperm pHi and $[Ca^{2+}]i$. Experiments using this caged speract

confirmed that the SAP-induced pHi increase precedes any changes in $[Ca^{2+}]i$ at all SAP concentrations tested. In addition, these fast measurements revealed a decrease in $[Ca^{2+}]i$ prior to its elevation (Nishigaki *et al.*, 2004). Both directions of the $[Ca^{2+}]i$ change were completely blocked in high K^+ seawater. The use of membrane-permeant caged cyclic nucleotides was also implemented. Like speract, uncaging cGMP (see Fig. 2) induced a fast pHi increase that preceded the $[Ca^{2+}]i$ increase. Although uncaging of both cGMP and cAMP increased $[Ca^{2+}]i$, the preceding decrease in $[Ca^{2+}]i$ was only observed following release of cGMP. This cGMP-stimulated $[Ca^{2+}]i$ decrease was more apparent following a second photolytic event, once $[Ca^{2+}]i$ had been elevated by an initial flash. The same pattern of $[Ca^{2+}]i$ changes was observed after uncaging of cGMP in individual sperm. The functional and pharmacological profiles of the initial $[Ca^{2+}]i$ decrease suggest that it is due to the activity of a Na^+/Ca^{2+} exchanger which is itself stimulated by the Sp-tetraKCNG-induced hyperpolarization. Notably, Su and Vacquier (2002) cloned a Na^+/K^+-Ca^{2+} exchanger present in *S. purpuratus* sperm. The set of findings just described led to a more detailed model of the signaling events triggered by speract where the hyperpolarization created by the opening of Sp-tetraKCNG results in the activation of two voltage-dependent exchangers, one Na^+/H^+ and one Na^+/K^+-Ca^{2+} (see Fig. 3). It is worth noting that Garbers' group cloned a novel Na^+/H^+ exchanger (NHE) present in mammalian sperm. This exchanger possesses a longer C-terminus compared to other somatic NHEs which surprisingly encodes a voltage-sensor domain and a CN binding domain (Wang *et al.*, 2003). Heterologous expression indicated that this sperm NHE physically interacts with soluble adenylyl cyclase (sAC) (Wang *et al.*, 2007). These data suggest that sperm NHE is up regulated by membrane potential and cAMP in mammalian sperm. Interestingly, the sea urchin homologue of sperm NHE has been identified from a signaling complex of sAC in *S. purpuratus* (Nomura and Vacquier, 2006).

The Kaupp group has reported that in *A. punctulata* and *S. purpuratus* the pHi increase at low SAP concentrations occurs after, not before, the $[Ca^{2+}]i$ elevation and that the intracellular alkalinization is not due to activation of Na^+/H^+ exchange induced by the hyperpolarization but probably caused by cytosolic proton consumption during GTP and ATP replenishment (Kaupp *et al.*, 2006; Solzin *et al.*, 2004; Strunker *et al.*, 2006). These results are in conflict with those obtained by Nishigaki *et al.* (2001, 2004). Furthermore, Soltzin *et al.* (2004) concluded that in *A. punctulata* sperm, uncaging cGMP either does not hyperpolarize the sperm, or that the hyperpolarization does not increase pHi, as it does in *S. purpuratus* sperm. In Fig. 2 we show our experiments with *A. punctulata* sperm where uncaging cGMP clearly induced an increase in pHi. It must be noted that these new findings are easier to reconcile with the previous observation that a high external K^+ concentration inhibits all of the speract-induced changes in sperm barring the increase in cGMP (Harumi *et al.*, 1992); that is to say, the membrane hyperpolarization and the increase in pHi are equally sensitive to such inhibition. Though particularities of the experimental conditions may explain these discrepancies, it is difficult to understand why if both SAPs and cGMP activate tetraKCNG and induce hyperpolarization, the first induces a pHi change but the second does not.

Sperm hyperpolarization has other important consequences. What is the link between the SAP induced hyperpolarization and the sea urchin sperm $[Ca^{2+}]i$ increase? Some time ago it was found that hyperpolarizing sea urchin sperm with valinomycin enhanced the $[Ca^{2+}]i$ increase caused by depolarizing with a subsequent K^+ addition. Though originally interpreted in the context of the acrosome reaction, these experiments suggested that hyperpolarization could remove inactivation from Ca_v channels in sea urchin sperm, and thus allow them to open upon depolarization (Gonzalez-Martinez *et al.*, 1992). Transcripts for two Ca^{2+} channel $\alpha 1$ subunits similar in sequence to $Ca_v1.2$ and $Ca_v2.3$ were identified in sea urchin testis (Granados-Gonzalez *et al.*, 2005). In addition, antibodies against rat $Ca_v1.2$ and $Ca_v2.3$ channels differentially labeled proteins in the flagella and acrosome of mature *S. purpuratus* sea urchin sperm, and Ca_v channel antagonists like nimodipine and Ni^{2+} diminished the valinomycin-enhanced, K^+-induced $[Ca^{2+}]i$ elevation (Granados-Gonzalez *et al.*, 2005). However, the definitive explanation for the link between the fast SAP or cGMP induced transient hyperpolarization and the $[Ca^{2+}]i$ increase was provided by their kinetic relationship (Strunker *et al.*, 2006). Removal of inactivation from a T-type Ca^{2+} channel by the cGMP-induced transient opening of tetraKCNG was consistent with the temporal relation between these changes and with their K^+ dependence. Furthermore, a T-type Ca^{2+} channel (Cav3) sequence was identified in the *S. purpuratus* genome (Kaupp *et al.*, 2007; Strunker *et al.*, 2006).

Sea urchin sperm express two hyperpolarization-activated and cyclic nucleotide-gated channels (HCN), SpHCN1 (Gauss *et al.*, 1998) and SpHCN2 (Galindo *et al.*, 2005). SpHCN1 was the

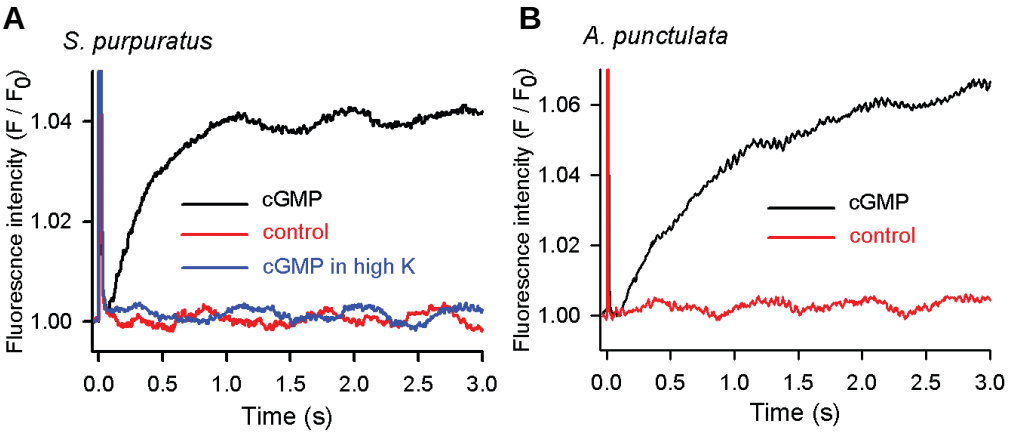

Fig. 2. Changes in sperm pHi stimulated by uncaging a cGMP analogue (membrane permeant Bhc-cGMP). S. purpuratus **(A)** *and* A. punctulata **(B)** *sperm were loaded both with the pH indicator BCECF and Bhc-cGMP. The fluorescence intensity (ex 500 nm, em > 515 nm) of a sperm suspension was recorded every 5 ms in a spectrofluorometer. At time 0, cGMP was photo-released by a UV pulse (1-2 ms) using a Xenon flash lamp. Control experiments were performed without Bhc-cGMP and with Bhc-cGMP in 50 mM K^+ seawater (cGMP in high K). Representative results from more than 3 experiments are shown.*

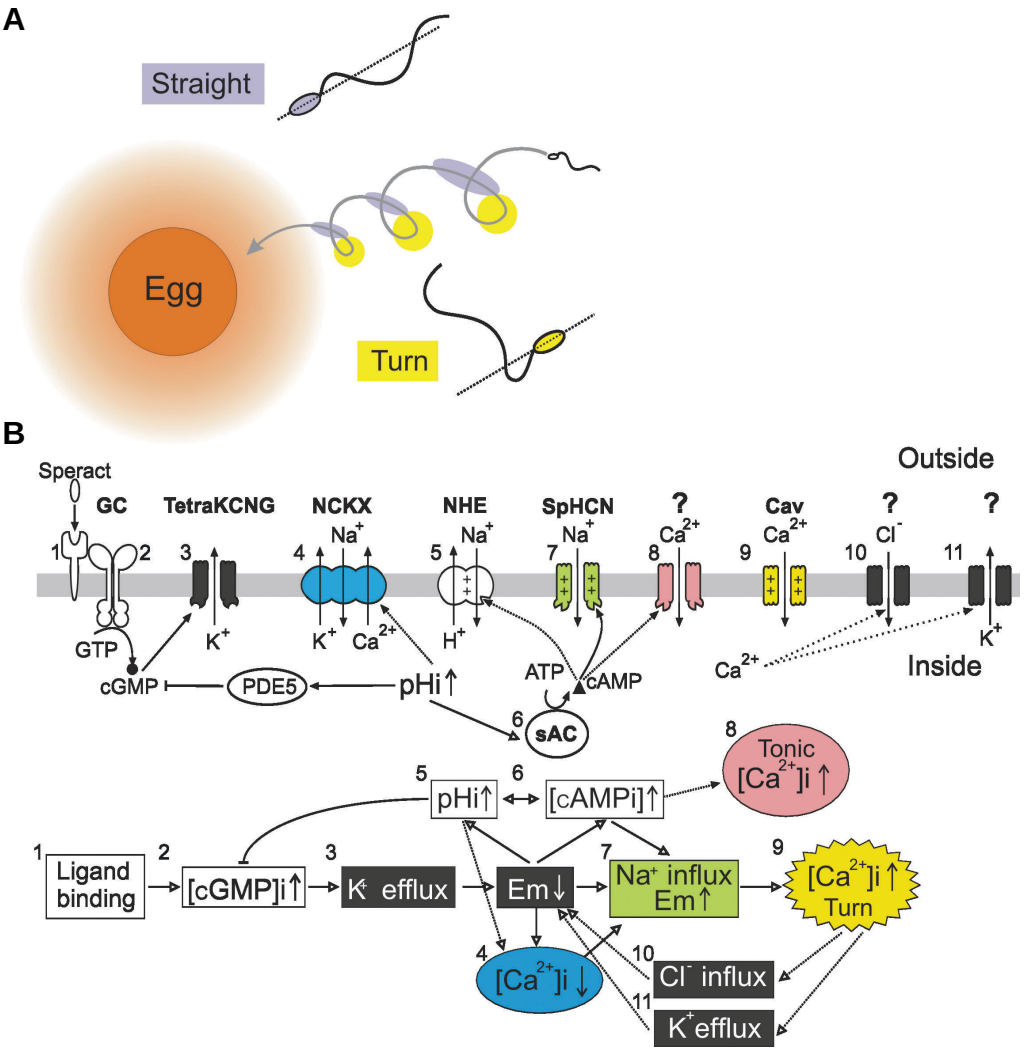

Fig. 3. Speract signalling model. **(A)** *Sperm activating peptide (SAP) diffusion away from the egg establishes a concentration gradient that sperm recognize and change their motility behavior in order to increase the probability of sperm/egg encounter. In the presence of a SAP gradient, sperm undergo a series of turns that are generated by increases in flagellar bend asymmetry intercalated with periods of straighter swimming that serve to direct the sperm towards the egg.* **(B)** *Proteins involved in speract signaling and their relationship: 1, speract receptor; 2, guanylate cyclase (GC); 3, cGMP-regulated K^+ channel (TetraKCNG); 4, K^+-dependent Na^+/Ca^{2+} exchanger (NCKX); 5, Na^+/H^+ exchanger (NHE); 6, soluble adenylate cyclase (sAC); 7, sperm hyperpolarization-activated and cyclic nucleotide-gated channel (SpHCN); 8, cAMP-regulated Ca^{2+} channel; 9, voltage-gated Ca^{2+} channel(Ca_v); 10, Ca^{2+}-regulated Cl^--channel (CaCC); Ca^{2+}-regulated K^+-channel (CaKC). After receptor binding, speract induces synthesis of cGMP that binds and opens TetraKCNG channels leading to hyperpolarization of the membrane potential (Em). The hyperpolarization activates spHCN channels, removes inactivation from Ca_v channels, facilitates the Ca^{2+} extrusion activity of NCKX and the activation of SpNHE and AC. HCN channels open when Em hyperpolarizes and intracellular cAMP elevates, which contributes to Em depolarization by Na^+ influx. Increases in $[Ca^{2+}]i$ and $[Na^+]i$ lead to further depolarization. The increase in Ca^{2+} levels in the flagellum enhances flagellar bending and leads the sperm to turn via an unknown mechanism operating in the axoneme. It is possible that increased Ca^{2+} levels open CaCC and/or CaKC channels, and that Cl^- influx, with a possible contribution from K^+ efflux, hyperpolarizes the membrane potential, removing inactivation from Ca_v channels and opening spHCN channels. The previous mechanism is then cyclically repeated to generate a train of Ca^{2+} increases that produce a repetitive sequence of sperm turns. The sequence continues until one or more of the molecular components in the pathway are downregulated. Cyclic AMP activates a poorly characterized Ca^{2+} influx pathway, which may contribute to the $[Ca^{2+}]i$ increase in the head.*

first sea urchin sperm channel cloned and heterologously expressed (Gauss *et al.*, 1998). These channels could open in response to the hyperpolarization event(s) and/or increases in cAMP that follow SAP binding. ZD7288, an inhibitor of HCN channels, alters the kinetics of the early $[Ca^{2+}]i$ changes induced by such peptides (Nishigaki *et al.*, 2004). Since SpHCN channels are present mainly in the flagellum, in principle, they could be involved in chemotaxis (Kaupp and Seifert, 2001). SpHCN1 displays a poor K^+ selectivity (P_{K^+}/P_{Na^+} ~5), therefore allowing Na^+ influx under physiological conditions and possibly contributing to the re- or depolarization of sperm. Channels with similar characteristics have been described in planar bilayers containing sperm flagellar plasma membranes (Labarca *et al.*, 1996) and by patch-clamp techniques in swollen sea urchin sperm (Sanchez *et al.*, 2001).

As previously indicated, the pHi increase induced by SAPs contributes to dephosphorylate and inhibit mGC (Garbers, 1989; Kaupp *et al.*, 2007). It is possible that the decrease in cGMP levels could lower K^+ permeability and contribute to repolarize sperm (Cook and Babcock, 1993b). The hyperpolarization and other resulting permeability changes lead also to the stimulation of the sperm adenylate cyclase (AC) activity (Beltran *et al.*, 2007; Beltran *et al.*, 1996; Garbers, 1989). Though it was once thought that sperm, including sea urchin sperm, had only one type of AC and no G proteins, this is currently subject to considerable debate. Recently an ortholog of the mammalian sperm soluble AC (sAC) was cloned and sequenced from a sea urchin *S. purpuratus* testis cDNA library (Nomura *et al.*, 2005). Its activity is higher in the presence of Mn^{2+} than Mg^{2+}, it is pHi and Ca^{2+} dependent (Nomura *et al.*, 2005) and indirectly activated by a membrane

potential hyperpolarization (Beltran *et al.*, 1996). Beltrán *et al.* (2007) provided evidence that the sea urchin sAC (susAC) is stimulated by HCO_3^-, as is the mammalian sperm sAC. Possibly the indirect activation of susAC by a hyperpolarization could be related to a membrane potential dependent HCO_3^- transport. In addition, Beltrán *et al.* (2007) showed that *S. purpuratus* sperm express orthologs of several isoforms of transmembrane ACs (tmACs) and selective inhibitors for sAC and tmAC indicated that both enzyme types participate in the sperm acrosome reaction. Furthermore, G proteins are expressed in sea urchin sperm (Capasso *et al.*, 1990; Cuellar-Mata *et al.*, 1995; Ohta *et al.*, 2000).

Ca^{2+} and the regulation of motility

Although Ca^{2+} plays an important role in a variety of sperm functions, being central to such critical processes as the acrosome reaction, in this section we will focus on the function of Ca^{2+} as an intermediate in the SAP signaling cascade and its effect on sperm motility, in which the central role of Ca^{2+} has long been generally recognized (Brokaw, 1974; Brokaw *et al.*, 1974).

The first demonstration that speract could alter intracellular $[Ca^{2+}]i$ was by Schackmann and Chock (1986). They loaded *S. purpuratus* sperm with the Ca^{2+} sensitive dyes quin2 and indo-1 and reported that exposure to speract triggered an increase in the average $[Ca^{2+}]i$ in a cell population that returned to near baseline levels after ~40 seconds. Later studies showed that resact, which can stimulate chemotaxis in the homologous sperm, also increases $[Ca^{2+}]i$ (Cook *et al.*, 1994; Kaupp *et al.*, 2003). It is important to emphasize that in both cases the increase in $[Ca^{2+}]i$ entirely depends on the presence of extracellular Ca^{2+}, implying that Ca^{2+} enters the sperm from the external medium, and is not released from an intracellular source. The full details of the signaling pathway between speract binding and Ca^{2+} influx remain to be determined, as previously indicated.

Recapitulating, the first event upon speract or resact binding to their respective receptors is a transient upregulation of mGC activity (Ramarao and Garbers, 1985), with a resulting increase of cGMP (Hansbrough and Garbers, 1981b). Thereafter cGMP directly opens the SptetraKCNG channel, leading to a membrane potential hyperpolarization (Babcock *et al.*, 1992; Galindo *et al.*, 2007; Lee and Garbers, 1986; Strunker *et al.*, 2006). It has been proposed that this hyperpolarization removes inactivation from Ca_v channels (Granados-Gonzalez *et al.*, 2005; Strunker *et al.*, 2006), which then subsequently open following repolarization of the membrane potential. This repolarization event may be enhanced by the activation of SpHCN channels that could open in response to the combination of hyperpolarization and increased cyclic nucleotide concentrations (see Fig. 3).

Another important element that is probably involved in the Ca^{2+} entry pathway is the increased levels of cAMP generated by speract binding. Although there are currently no strong candidate mechanisms for a cAMP-regulated Ca^{2+} entry pathway in sperm, evidence for a link between cAMP and Ca^{2+} entry has accumulated (Cook and Babcock, 1993a; Nishigaki *et al.*, 2004; Wood *et al.*, 2003). Stopped-flow fluorimetry and the use of caged CN-derivatives indicated that in *A. punctulata* sperm cGMP stimulates Ca^{2+} entry through an early, transient pathway and cAMP contributes to Ca^{2+} entry via activation of late, more sustained pathway (Kaupp *et al.*, 2003).

Further evidence for the presence of more than one SAP-stimulated Ca^{2+} entry pathway in sea urchin sperm comes from Ca^{2+} measurements in single sperm. Imaging Ca^{2+} in individual sperm adhered to a coverslip revealed the Ca^{2+} increase as biphasic in nature, consisting of transient and discrete phasic fluctuations superimposed on a more sustained tonic increase (Wood *et al.*, 2003). Furthermore, the increase in the flagella, where the phasic fluctuations were more pronounced, displayed differing kinetics to the Ca^{2+} increase in the head, where the tonic increase was more evident. This suggested not only the existence of multiple Ca^{2+} entry pathways, but that the molecular components of each pathway are unevenly distributed, or activated, in different subcompartments of the sperm. The components of the Ca^{2+} entry pathway(s) await precise identification, but as described in previous sections Ca_v channels play a key role. Inhibitors of Ca_v channels such as nimodipine and Ni^{2+} were shown to inhibit the fast, phasic Ca^{2+} increase in flagella without significantly inhibiting the tonic Ca^{2+} increase. Indeed the authors tentatively ascribed the train of phasic Ca^{2+} increases to the repetitive opening of LVA (or T-type) Ca_v channels, due to the relatively high concentration of nimodipine required for inhibition, and the fact that Ni^{2+} is less effective in blocking HVA channels. As mentioned previously, the kinetic relationship between the SAP-stimulated hyperpolarization and the subsequent Ca^{2+} increase in *A. punctulata* sperm have again implicated LVA channels as the most likely candidates for generating the earliest phase of the rise in $[Ca^{2+}]i$ (Strunker *et al.*, 2006).

The molecular components comprising the prolonged, tonic phase of Ca^{2+} entry (as characterized in single sperm) are also yet to be identified, but cAMP is a good candidate activator of such Ca^{2+} entry. Indeed when the external K^+ concentration is elevated to 20 – 50mM, a condition that inhibits the cGMP-induced hyperpolarization and Ca_v channel-mediated Ca^{2+} entry, uncaging of cAMP leads to elevated $[Ca^{2+}]i$, suggesting that a cAMP-sensitive Ca^{2+} entry pathway is present (Nishigaki *et al.*, 2004). Another target for cAMP is the SpHCN channel; Nishigaki *et al.* (2004) showed that an inhibitor of this channel (ZD7822) augments the $[Ca^{2+}]i$ decrease that precedes the cGMP and speract induced $[Ca^{2+}]i$ elevation. In addition, since it has been reported that 2 CatSper homologues are found in the *S. purpuratus* genome (Xia *et al.*, 2007), this channel would certainly be a candidate to contribute to the sustained Ca^{2+} uptake triggered by SAPs. A link between cAMP and the pHi sensitive-CatSper could involve cAMP regulation of a voltage dependent Na^+/H^+ exchanger, or cAMP-dependent regulation of the CatSper phosphorylation state. It has also been suggested that HVA channels contribute to the SAP-stimulated rise in $[Ca^{2+}]i$, and that their opening is delayed relative to the LVA channels (Strunker *et al.*, 2006).

An important component of the regulation of sperm $[Ca^{2+}]i$ are the mechanisms for Ca^{2+} clearance. Both Ca^{2+}-ATPases and a $Na^+/Ca^{2+}/K^+$ (NCKX) exchanger have been identified in sea urchin sperm (Jayantha Gunaratne and Vacquier, 2007; Su and Vacquier, 2002). The former appears localized to the sperm head (Gunaratne *et al.*, 2006), and it is uncertain whether it plays a significant role in regulating the Ca^{2+} response to speract, although Ca-ATPase inhibitors increase $[Ca^{2+}]i$ and inhibit flagellar motility. The NCKX is localized to the flagellum (Su and Vacquier, 2002), and is probably involved in mediating a slight decrease in

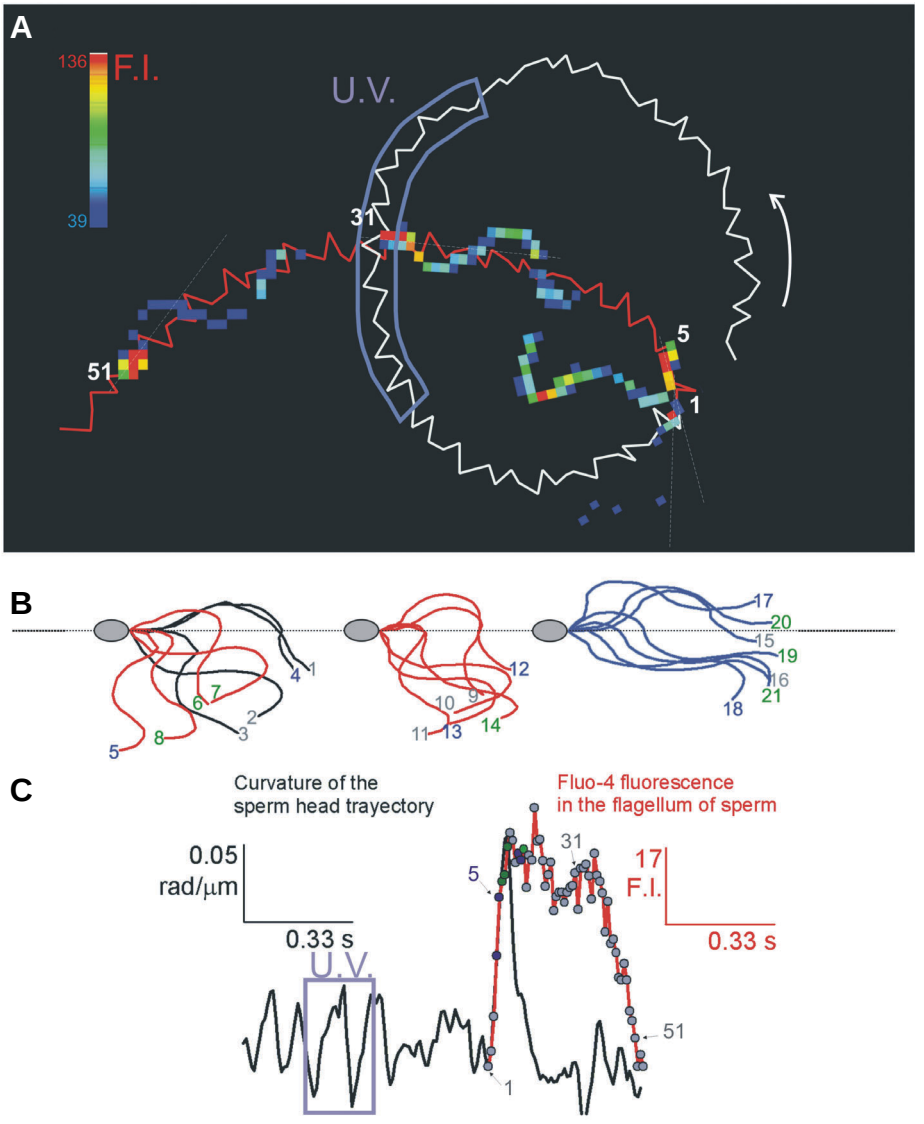

Fig. 4. Intracellular Ca^{2+} changes control the swimming behavior of sea urchin sperm. (A) *Images of a typical transient increase in fluo-4 fluorescence of a motile sperm. Interval between successive images ~8 ms. The trace shows the sperm swimming trajectory throughout a single speract-induced Ca^{2+} fluctuation. The red line is the sperm trajectory during the phase of Ca^{2+} increase, and white lines are the sperm trajectory before speract uncaging. Purple box indicates UV exposure (200 ms). Curved arrow shows sperm swimming direction. Color bar shows fluorescence intensity after background subtraction. The numbers (1, 5, 31, and 51) indicate selected sperm images through the experiment undergoing a speract-induced Ca^{2+} fluctuation. A straight line shows the head axis.* **(B)** *Position of the flagellum from a single sperm in relation to a normalized head axis. Black traces are the position of the flagellum from first four visible images of the flagellum after speract uncaging. Red traces are the position of the flagellum in moment of maximum Ca^{2+} increase induced-flagella curvature changes. Blue traces are the position of the flagellum in final seven images presented. Dashed line indicates long axis of head. Colored numbers indicates successive beats (gray represents 1st, 4rt, 7th; green 2nd, 5th, 8th; blue 3rd, 6th, 9th flagella beats).* **(C)** *Change in curvature of sperm trajectory measured from a rolling average curvature from 3 frames (black trace). Red trace shows the fluo-4 fluorescence (average along length) in the flagellum of sperm shown before and during the first Ca^{2+} fluctuation (1.4 s). Purple box indicates UV exposure (200 ms). Points corresponding to each of the flagella images are ~8 ms apart (see B). Numbers indicate selected images shown in (A).*

$[Ca^{2+}]i$ that occurs just prior to the SAP-stimulated Ca^{2+} increase (Nishigaki *et al.*, 2004). This decrease could possibly be involved in 'fine-tuning' the timing of the fast Ca^{2+} increase to subsequently spatially coordinate a motility response. The NCKX may also contribute to the fast Ca^{2+} decrease necessary to form each rapid Ca^{2+} increase into a Ca^{2+} fluctuation, and which permits, as a consequence, the generation of trains of Ca^{2+} fluctuations as observed in single cells.

Having reviewed the potential SAP-activated mechanisms for regulating $[Ca^{2+}]i$ we now turn to their function in the context of sperm biology. The role of SAPs is to establish a long-range dialogue between the egg and the sperm, such that the probabilities of sperm-egg union are maximized. Diffusion of the SAP resact away from the *A. punctulata* egg establishes a chemical gradient of the peptide that homologous sperm recognize and swim upwards in an attempt to localize the egg (Ward *et al.*, 1985a). The process, termed chemotaxis, is widespread among marine organisms of different phyla (Miller, 1985). Not all SAPs are chemoattractants, however, with speract having no reported chemoattractant effects on *S. purpuratus* sperm. Nonetheless, speract is still a potent modulator of sperm motility (Cook *et al.*, 1994) that, through mechanisms apart from chemotaxis, likely raises the probabilities of a sperm-egg encounter.

Ca^{2+} has also long been recognized as a regulator of sperm motility, and it was shown that extracellular Ca^{2+} is an absolute requirement for chemotaxis (Brokaw, 1974), an observation that has held for every single instance of sperm chemotaxis studied. A crucial breakthrough came once techniques were developed for reconstituting motile sperm after their flagella had been demembranated by mild detergent treatment. In such axonemes it was discovered that the concentration of Ca^{2+} regulates the asymmetry between the principal and reverse flagella bends, with higher concentrations of Ca^{2+} increasing the degree of asymmetry (Brokaw, 1974). It had been previously established that increases in flagellar bend asymmetry were an essential component of chemotactic motility changes, as in the presence of chemoattractant, sperm undergo one or a series of turns that are generated by increases in bend asymmetry that serve to direct the sperm towards the chemoattractant source (Miller and Brokaw, 1970). It is precisely these bend asymmetry-determined chemo-

tactic turns that are inhibited in the absence of extracellular Ca^{2+}.

Thus a welter of data has accumulated that indicates a link between increases in $[Ca^{2+}]i$ and chemotaxis through regulated increases in flagellar bend asymmetry. It is also well established that SAPs both increase $[Ca^{2+}]i$ and induce motility changes in homologous sperm that in the case of *A. punctulata* lead to chemotaxis. Yet evidence that the complex multiphasic increases in $[Ca^{2+}]i$ stimulated by SAP binding were directly regulating sperm motility was for many years lacking, until techniques were developed for measuring Ca^{2+} increases in motile flagella of swimming sperm (see Fig. 4). The first such report in sea urchin sperm used uncaging of cGMP to stimulate an increase in $[Ca^{2+}]i$ (Wood *et al.*, 2005). This revealed a biphasic increase $[Ca^{2+}]i$ in the flagella, comprised of a fast and transient initial rise followed by a more sustained plateau phase. In the head, a slower increase to the plateau phase was recorded. The fast Ca^{2+} increase in the flagellum was temporally correlated with a transient turning event created by an increase in the degree of flagellar bending. Nimodipine and Ni^{2+} treatment abolished both the fast and transient Ca^{2+} increase in the flagella (without significantly inhibiting the slower phase of the Ca^{2+} increase in both flagellar and head regions) and the transient turns. Importantly, this demonstrated that turning events and the degree of flagellar bending depend not on an increase in flagellar $[Ca^{2+}]i$ *per se*, but were mediated by Ca^{2+} entry through a specific 'fast' pathway localized to or activated in the flagellar compartment alone. Crucially, however, the pattern of Ca^{2+} increase observed following cGMP uncaging differed from that seen with speract treatment of single immobilized sperm, in that trains of Ca^{2+} fluctuations were not observed.

The question as to what role SAP-stimulated Ca^{2+} increases play in regulating sperm motility thus remained open until caged versions of the peptides resact and speract (Kaupp *et al.*, 2003; Tatsu *et al.*, 2002) were used to stimulate swimming sperm, with the responses of individual sperm recorded. These allowed step increases in the concentration of resact and speract to be generated in the vicinity of swimming sperm without perturbing their basal motility, as would occur through turbulent mixing by addition of native resact or speract. More so, by introducing inhomogeneities in the pattern of the uncaging UV light, such that the intensity is greatest in the centre of the field of view and falls away towards the edges, gradients of SAP concentration were created to try and simulate the pattern of SAP dispersal around an egg (Bohmer *et al.*, 2005). Uncaging of resact in the vicinity of *A. punctulata* sperm stimulated a train of Ca^{2+} increases, each of which was accompanied by a turning event (Bohmer *et al.*, 2005). Interspersed between each turn was a period of straighter swimming, a sperm motility pattern characteristic of chemotaxis, and uncaging of resact in a gradient pattern promoted the accumulation of sperm at the centre of the gradient. The repetitive Ca^{2+} fluctuations, the sperm turns and the accumulation of sperm were all dependent on the presence of extracellular Ca^{2+}.

In the case of *S. purpuratus* sperm stimulated by uncaging speract, ostensibly similar results were obtained (see Fig. 4) that vary in one crucial detail – sperm swimming in a gradient of speract do not, under laboratory conditions, undergo chemotaxis (Wood *et al.*, 2007). This result was not unexpected, as *S. purpuratus* sperm do not display chemotaxis under a number of alternative assays that look for directed motility towards a source of speract. Nevertheless, the sequence of events triggered by speract uncaging were notably similar to those seen in the chemotactic species *A. punctulata*, namely a series of Ca^{2+} fluctuations that were individually correlated with a turning event, and that were interspersed by periods of straighter swimming. Strikingly, altering the kinetics of the Ca^{2+} fluctuations with niflumic acid, an inhibitor of Ca^{2+}-activated Cl^- channels (CaCC), led to gross changes in sperm motility patterns (Wood *et al.*, 2007). This not only emphasizes the importance of coordinating the detailed characteristics of the Ca^{2+} fluctuations and the sperm turns for regulating sperm motile behavior, but suggests that other channels, possibly CaCC, finely tune the SAP signaling pathway to enhance reaching the egg. The likely function of CaCC in this pathway also suggests a mechanism for generating trains of Ca^{2+} fluctuations, as their opening would lead to hyperpolarization of the membrane potential and thus remove inactivation from Ca_v channels. Successive cycles of hyperpolarization and depolarization could thus generate the observed trains of Ca^{2+} fluctuations through repeated opening of Ca_v channels (see Fig. 4).

Perspectives

It is remarkable that two sea urchin sperm species display such similar Ca^{2+} responses when stimulated by SAPs, and which result in similar modifications to sperm motility, yet only sperm from *A. punctulata* display chemotaxis. Clearly the difference lies in subtle details of the relationship between these components (SAPs, Ca^{2+} and the flagella) that are yet to be revealed. The detailed choreography of these components determines the navigational dexterity sperm have to follow the cues of the egg to find it. The stroboscopic fluorescence imaging techniques employed in studying Ca^{2+} changes in motile flagella are not yet fast enough to reveal changes in flagellar conformation at the level of a single beat cycle. Since improvements in sensitivity and speed of fluorescence detection systems are constant and ongoing we can anticipate advances in our understanding in this direction.

The currently published single-cell fluorescence microscopy studies report motility changes in sperm swimming in a reduced field of only 2 dimensions, whereas maximizing the probability of a sperm-egg encounter *in vivo* is a three-dimensional problem. Unpicking all of the differences between chemotactic and non-chemotactic SAP-stimulated motility changes and their relationship to Ca^{2+} will doubtless require the development of supra-video rate 3D fluorescence microscopy techniques.

Another question worth considering, apart from *how* the chemotactic and non-chemotactic responses differ, is to *why* two such differing reproductive strategies exist. It is worth remembering that the sperm and egg of sea urchin normally encounter one another in the turbulent open ocean, and the reproductive mechanisms of individual species were evolutionarily honed by their particular marine environment (Riffell *et al.*, 2004). The fertilization efficiency of marine animals varies according to the hydrodynamic forces experienced by gametes; in the case of the red abalone (*Haliotis rufescens*) the efficiency peaked sharply at levels of laminar shear similar to those found in its natural environment (Riffell and Zimmer, 2007). The fertilization success of *S. purpuratus* gametes also varies according the degree of shear experienced (Mead and Denny, 1995). It has been predicted that chemotaxis functions efficiently within a particular range of laminar shear values, and at higher values the plume of

chemoattractant shrinks in size and fragments to the extent that it no longer confers useful long-range information to the sperm (Riffell and Zimmer, 2007). It remains to be seen whether environmental factors such as turbulence and shear account for the presence or absence of chemotactic mechanisms in individual species of sea urchin.

Finally, it is important to remind ourselves that cilia have a conserved axonemal structure throughout the animal kingdom. They have been found to be present in almost all cell types and to participate in fundamental biological processes aside from motility as revealed by the variety of diseases associated to mutations in ciliar and flagellar proteins such as: polycystic kidney disease, *situs inversus*, primary cilia dyskinesia, retinal degenerative disease, planar cell polarity, and developmental diseases grouped within Bardet-Biedl syndrome, whose characteristic phenotypes include obesity and diabetes (Sloboda and Rosenbaum, 2007). Therefore, understanding how Ca^{2+} regulates cilia and flagella beating has a much wider impact than was earlier realized.

Acknowledgements

The authors would like to thank Professor Michael Whitaker for continuous encouragement. The work was supported by CONACyT, UC-MEXUS, NIH, DGAPA (UNAM) and The Wellcome Trust.

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