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Control of hyperactivation in sperm

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BACKGROUND: Sperm hyperactivation is critical to fertilization, because it is required for penetration of the zona pellucida. Hyperactivation may also facilitate release of sperm from the oviductal storage reservoir and may propel sperm through mucus in the oviductal lumen and the matrix of the cumulus oophorus. Hyperactivation is characterized by high amplitude, asymmetrical flagellar bending. METHODS: This is a review of the original literature on the mechanisms that regulate hyperactivation, including physiological factors and signaling pathways. RESULTS: Computer-assisted semen analysis systems can be used to identify hyperactivated sperm by setting minimum thresholds for curvilinear velocity (VSL) and lateral head movement and a maximum threshold for path linearity. Hyperactivation is triggered by a rise in flagellar Ca^{2+} resulting from influx primarily through plasma membrane CatSper channels and possibly also by release of Ca^{2+} from a store in the redundant nuclear envelope. It requires increased pH and ATP production. The physiological signals that trigger the rise in Ca^{2+} remain elusive, but there is evidence that the increased Ca^{2+} acts through a calmodulin/calmodulin kinase pathway. Hyperactivation is considered part of the capacitation process; however, the regulatory pathway that triggers hyperactivation can operate independently from that which prepares sperm to undergo the acrosome reaction. Hyperactivation may be modulated by chemotactic signals to turn sperm toward the oocyte. CONCLUSIONS: Little is known about exactly what triggers hyperactivation in human sperm. This information could enable clinicians to develop reliable fertility assays to assess normal hyperactivation in human sperm samples.

Keywords: sperm hyperactivation; intracellular calcium; CatSper; signaling pathways; human sperm

Hyperactivation is required for fertility

In 1970, Yanagimachi reported that hamster sperm became extremely active as they gained the ability to fertilize oocytes *in vitro*. He observed that sperm swimming in this manner could be seen through the walls of the oviductal ampulla and proposed that this 'hyper-active' movement provides sperm with greater propulsion for reaching the oocyte or for passing through the cumulus and zona pellucida (Yanagimachi, 1970).

Years later, Yanagimachi's proposals were confirmed by various observations and experiments. Upon entering the oviduct, sperm encounter a mucous secretion that is quite thick in some species (Jansen, 1978; Jansen, 1980; Jansen and Bajpai, 1982; Suarez *et al*., 1997). In the human oviduct, thick mucus secretions were found in isthmic segments taken from women in the late follicular phase (Jansen, 1980). *In vitro*, boar sperm that were not hyperactivated were observed to stick to pig oviductal mucus and fail to penetrate it (Suarez *et al*., 1992). Mucus increases the viscosity and elasticity of the aqueous milieu in which sperm swim. When mouse and hamster sperm are placed in medium made highly viscoelastic by addition of methyl cellulose or long-chain polyacrylamide, sperm that are hyperactivated move through it more effectively than those that are not (Suarez *et al*., 1991; Suarez and Dai, 1992). Sperm from CatSper null mutant mice, which are unable to hyperactivate, do not penetrate artificial mucus as well as hyperactivating wild-type sperm (Quill *et al*., 2003). Thus, hyperactivated sperm are likely to be more effective at swimming through oviductal mucus *in vivo*.

In addition to viscoelastic mucus secreted into the oviduct, sperm also encounter a highly viscoelastic environment when they enter the matrix of the oocyte's cumulus oophorus. The high viscoelasticity of the cumulus matrix is primarily attributed to hyaluronan (Dandekar *et al*., 1992). Although penetration of the matrix may be assisted by hyaluronidase expressed on the surface of the sperm head (Kim *et al*., 2005), hyperactivation would undoubtedly assist sperm as well.

There is strong evidence that hyperactivation is required for penetrating the zona pellucida. Hamster sperm were incubated under capacitating conditions until they hyperactivated and then were added to oocytes. After the sperm bound to the zonae, Ca^{2+} channel blockers were added to inhibit hyperactivation. Although the sperm remained motile and underwent acrosome reactions, most failed to penetrate the zonae (Stauss *et al*., 1995). Years after those experiments, mice that were null mutants for CatSper proteins were developed. The sperm from the nulls were progressively motile and could undergo acrosome reactions; however, they could not hyperactivate and failed to

penetrate the zonae pellucidae of oocytes *in vitro*. If the zonae were removed, the sperm were able to fertilize normally (Ren *et al*., 2001; Quill *et al*., 2003).

The behavior of hamster and mouse sperm swimming within transilluminated oviducts suggested to observers that hyperactivation also enables sperm to move about effectively in the oviductal lumen. While hyperactivated sperm placed on a microscope slide in simple aqueous medium often spend much of their time swimming in circles, those observed swimming within the oviduct cover space rapidly in a manner that should increase chances of encountering the cumulus–oocyte complex. This is at least partly because the surfaces sperm encounter in the oviduct are not hard and flat like those of microscope slides. Hyperactivated hamster sperm were seen to glide rapidly over the mucosal surface of the ampulla (Katz and Yanagimachi, 1980). Mouse sperm were observed to use the deep flagellar bends characteristic of hyperactivation to turn around within pockets of mucosa and escape out into the central lumen (Suarez and Osman, 1987). It has not been possible to observe the behavior of sperm within the oviducts of humans; however, it is known that they encounter a similar environment, consisting of a highly folded mucosa and complex narrow passageways.

In many mammalian species, a reservoir of sperm forms within the isthmus of the oviduct when sperm become attached to the mucosal epithelium (reviewed by Suarez and Pacey, 2006). There is evidence that the attachment of sperm to epithelium prolongs their motile lives (Pollard *et al*., 1991; Gwathmey *et al*., 2006). In several species, this interaction is known to involve carbohydrate recognition; that is, protein on the surface of sperm binds specifically to a glycosylated receptor on the epithelium (Suarez and Pacey, 2006). In cattle, the proteins on sperm that bind them to the epithelium have been identified as members of the BSP (bovine seminal plasma) family (Gwathmey *et al*., 2003, 2006). The oviductal receptors for the BSP proteins contain fucose and have been identified as members of the annexin family of proteins (Ignotz *et al*., 2007). As bull sperm become capacitated, they shed at least one of the BSP proteins (Gwathmey *et al*., 2003). In the oviduct, capacitation would thus reduce binding affinity of sperm for the oviductal epithelium. As sperm lose binding affinity for epithelial receptors, hyperactivation could assist them in pulling off of the epithelium. In the mouse, only sperm exhibiting hyperactivated movement in the oviduct were observed to detach from the epithelium (DeMott and Suarez, 1992).

Human sperm observed detaching from oviductal epithelium *in vitro* showed a greater incidence of hyperactivation than sperm that had not yet attached to epithelium (Pacey *et al*., 1995), which indicates that hyperactivation may be required to pull human sperm from the epithelium and/or that binding to the epithelium hyperactivates human sperm.

Altogether, the evidence indicates that all of Yanagimachi's proposals were correct. After sperm enter the oviduct, hyperactivation enables them to reach oocytes and penetrate their vestments. Whereas hyperactivation may serve all of these functions in human sperm, this should be verified by direct investigation. Hyperactivation likely assists human sperm in penetrating viscoelastic materials, because human sperm do encounter mucus in the oviduct (Jansen, 1980), and must also penetrate a viscoelastic cumulus matrix and the zona pellucida. However, hyperactivation has not been observed directly within the human oviduct and detailed reports on the swimming patterns of sperm recovered from the human oviduct are lacking.

The objective of this review was to synthesize published findings on the mechanisms that regulate hyperactivation, including physiological factors and signaling pathways that trigger hyperactivation in motile sperm. Sperm develop the ability to swim as they pass through the epididymis and rapidly begin to swim (activate) as they are released from the epididymis. For reviews on maturation of the motile apparatus and activation of motility, please see Eddy (2006) or Turner (2003, 2006).

The flagella of hyperactivated sperm beat asymmetrically

In most species, mature sperm are held immotile within the epididymis until they are released, whereupon they quickly begin to swim. This process is known as activation of motility. Activated sperm generate nearly symmetrical flagellar beats (Fig. 1), which propel them in nearly linear trajectories (Suarez and Dai, 1992; Mortimer and Swan, 1995a; Ho *et al*., 2002). When sperm become hyperactivated, the amplitude of the flagellar bend increases, usually only on one side of the flagellum. This produces a beat pattern that is highly asymmetrical, often causing hyperactivated sperm to swim in circles on glass slides. Extremely asymmetrical bends produce figure-of-eight movement patterns. During asymmetrical flagellar beating, steady rolling of the head can result in helical tracks, which have been described for hyperactivated human sperm (Morales *et al*., 1988). In some species, particularly mice, hyperactivated sperm trace erratic paths due to intermittent production of deep bends. One could argue that this represents switching back and forth between the activated and hyperactivated state, but only instantaneous assessment of signaling, which has not been done, can address the issue.

CASA can assess hyperactivation, although imperfectly

Computer-assisted semen analysis (CASA) has been used to detect hyperactivation and to identify the percentage of sperm in a sample that are hyperactivated. An accepted procedure for

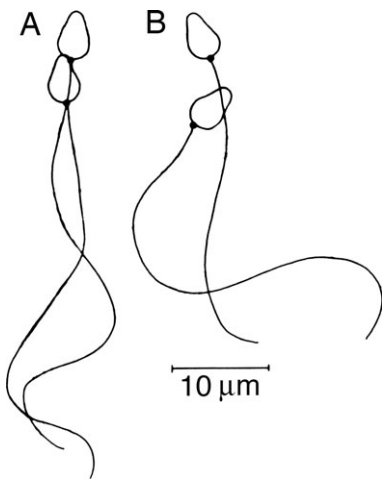

Figure 1: Flagellar bending patterns of activated (**A**) and hyperactivated (**B**) sperm. Adapted from Morales *et al*., (1988).

defining hyperactivation by a particular CASA system is to incubate sperm under conditions that support *in vitro* fertilization (that is, 'capacitating' conditions) and compare swimming patterns of those sperm with control sperm that have been incubated an equal amount of time under noncapacitating conditions. The investigator then identifies measurements that are affected by the capacitating conditions and uses the information to set threshold values for identifying hyperactivated sperm (Mortimer and Mortimer, 1990; Murad *et al*., 1992; Mortimer, 1997; Kay and Robertson, 1998). This has been the approach used to identify hyperactivation in human sperm, because of the difficulties involved in observing sperm in the Fallopian tubes of women or recovering tubal sperm. Incubation under capacitating conditions, however, does not work for some species, such as bull sperm, which do not show significant changes in movement patterns when incubated in capacitation medium (Marquez and Suarez, 2004).

To have the CASA system identify hyperactivated sperm, thresholds are usually set for a minimum curvilinear velocity and a maximum path linearity (that is, a minimum curvature of path trajectory). In addition, a minimum threshold is often set for the distance that the sperm head moves side-to-side as the sperm advances, which is known as the amplitude of lateral head displacement (ALH). This is an indirect measure of flagellar bend amplitude, because the head is wagged from side-to-side by the developing principal and reverse bends and it gets wagged farther from side-to-side by deeper flagellar bends. Because ALH is not a direct measurement of the flagellar bend, it is imperfect; however, CASA systems compensate considerably for the lack of accuracy with reasonable precision and acquisition of data from hundreds of sperm in less than a minute. CASA systems can miss hyperactivated sperm that swim in very tight circles, because they cannot track them properly. Image or frame collection rate can also affect the accuracy of the system (Mortimer and Swan, 1995a; Mortimer, 1997). Playback features should always be used to check the accuracy of the system and re-adjust thresholds if necessary. For human sperm, threshold values have been established for some CASA systems; however, there are no universally accepted criteria. For more detailed discussions of the use of CASA systems to measure hyperactivation, see (Mortimer, 1997; Kay and Robertson, 1998).

Because most CASA systems can only track and analyze head movements, investigators who study the regulation of flagellar movement use other methods to characterize the flagellar wave. In most mammalian sperm, irregular flagellar waveforms do not allow one to bisect waves in order to get accurate measures of amplitude and wavelength. Instead, other methods are used to assess waves. The most common is to measure curvature at points along the flagellum and to then plot curvature against distance from the base of the flagellum (Carlson *et al*., 2005; Ishijima *et al*., 2006). However, even with assistance from image analysis software, this process is extremely tedious and time-consuming. First, digital videos of the moving sperm must be obtained and then individual frames must be selected for analysis of the flagellar bends. It is only practical to make these measures of curvature on 10–20 sperm per treatment sample. Such low sample numbers can only provide representative measures of the population if the sperm behave fairly uniformly or if a uniform subgroup is identified for sampling. To say the least, it is difficult to make this type of analysis of human semen samples, which are notoriously heterogeneous.

CASA and curvature values are affected by viscosity of the medium, because high viscosity dampens flagellar waves. Also, sticking of sperm heads to surfaces in the slide chamber affect curvature. Acrosome-reacted sperm are especially sticky. Due to the large size of the acrosome in hamster sperm, acrosomal status cannot be ascertained on hyperactivated sperm without special staining (Suarez and Dai, 1995). The reacted sperm can be seen to stick to glass, even in the presence of albumin, and sticking results in an increase in the amplitude of the reverse bend, which often switches the bending pattern from asymmetrical to symmetrical (Suarez, personal observations). Sticking can be minimized by coating surfaces with nonstick agents such as agar (Suarez *et al*., 1991) or agarose (Ignotz and Suarez, 2005).

Ca^{2+} signaling triggers hyperactivation

Ca^{2+} is the primary second messenger that triggers hyperactivated motility. Treating sperm with Ca^{2+} ionophores A23187 or ionomycin induces hyperactivation (Suarez *et al*., 1987, 1992; Marquez and Suarez, 2006; Xia *et al*., 2007). Using the fluorescent Ca^{2+} indicator indo-1, cytoplasmic Ca^{2+} levels were measured in the flagella of hyperactivated hamster sperm and found to be higher than in activated sperm (Suarez *et al*., 1993). Other fluorescent Ca^{2+} indicators have also detected intracellular Ca^{2+} increases during stimulation of hyperactivation (Ho and Suarez, 2001; Xia *et al*., 2007).

Demembranated sperm models have been used to study the regulation of hyperactivated motility. Sperm are demembranated by treatment with Triton X-100 to disrupt the plasma membrane, mitochondrial membranes and acrosomal membranes—leaving intact the nucleus, cytoskeletal elements and proteins anchored to these structures. In the flagellum, the intact cytoskeleton includes the axoneme, dense outer fibers and fibrous sheath (Ho *et al*., 2002). After demembranation, sperm are immotile; however, they can be reactivated by transfer to an 'intracellular medium' (low in Na^+ and high in K^+) and adding ATP. Demembranated bull sperm reactivate in intracellular medium containing $\sim$50 nM of Ca^{2+}. When the Ca^{2+} is raised to $\sim$100 nM, some sperm begin to hyperactivate, and most hyperactivate when Ca^{2+} reaches $\sim$400 nM (Ho *et al*., 2002). The responses of demembranated sperm indicate that Ca^{2+} acts directly upon cytoskeletal elements to regulate motility, because membranes and soluble cytoplasmic elements are gone. Similar results are obtained with demembranated/reactivated sea urchin sperm, which lack dense outer fibers and the fibrous sheath (Brokaw and Nagayama, 1985), indicating that the Ca^{2+} affects the axoneme directly, rather than through the other skeletal structures. Selective regulation of the activity of a group of dynein arms by Ca^{2+} was demonstrated to be the basis for the changes in flagellar bending patterns brought about by high Ca^{2+} concentrations in demembranated sea urchin sperm (Bannai *et al*., 2000).

Demembranation studies have not been conducted on human sperm to study Ca^{2+} regulation of hyperactivation, although human sperm have been successfully reactivated after demembranation (Murad *et al*., 1992). Sperm from cynomolgus monkeys have been demembranated and reactivated; they show similar

responses to raised Ca^{2+} in the reactivation medium as those reported for bull sperm (Ishijima *et al*., 2006).

Demembranated sperm have been used further to identify the target of the Ca^{2+} signal. When calmodulin (CaM) was extracted from bull sperm during demembranation, motility was not reactivated unless exogenous CaM was added back. Adding 1 μm Ca^{2+} with CaM hyperactivated the reactivated demembranated sperm. When peptide inhibitors of calmodulin kinase II (CaMKII) were added with the CaM, hyperactivation was reduced by 75%; whereas, W-7 or a peptide inhibitor of myosin light chain kinase added with the CaM did not inhibit hyperactivation. Furthermore, when intact motile sperm were treated with KN-93, a membrane-permeant inhibitor of CaMKII, caffeine-induced hyperactivation was inhibited without impairing normal motility. The inactive analog KN-92 had no effect (Ignotz and Suarez, 2005). CaM and CaMKII were immunolocalized to the flagellum in bull and mouse sperm (Ignotz and Suarez 2005; Schlingmann *et al*., 2007). Altogether, these results indicate that hyperactivation is triggered by Ca^{2+}/CaM activation of CaMKII.

In human sperm, motility declined over time in the presence of CaM kinase inhibitors KN-93 and KN-62, but no specific inhibition of hyperactivation was observed. The inhibitors caused a reduction in ATP content, which could account for the decreased motility. CaMKIV was detected in the flagellum using a monoclonal antibody, but a monoclonal antibody against CaMKII did not detect its presence (Marin-Briggiler *et al*., 2005). Thus, additional investigations are needed regarding the role of CaM kinases in regulation of human sperm motility and hyperactivation.

Plasma membrane channels and intracellular stores can provide Ca^{2+} for hyperactivation

Second messenger Ca^{2+} can come from two sources: extracellular Ca^{2+} brought in through plasma membrane channels and/or Ca^{2+} stored in organelles (Fig. 2). The predominant source of Ca^{2+} for hyperactivation is extracellular Ca^{2+} brought in through plasma membrane Ca^{2+} channels formed by proteins in the CatSper family (Kirichok *et al*., 2006). CatSper proteins are only expressed in male germ cells and localize to the principal piece of the flagellum in mature sperm (Carlson *et al*., 2005; Jin *et al*., 2007). The channels can be activated by raising intracellular pH (Kirichok *et al*., 2006). Male mice that are null mutants for CatSper-1, -2, -3 or -4 are infertile and the infertility has been correlated to a failure to hyperactivate (Ren *et al*., 2001; Quill *et al*., 2003; Jin *et al*., 2007; Qi *et al*., 2007). As described above, CatSper null sperm fail to penetrate the zona pellucida, but can fertilize oocytes from which the zona has been removed (Ren *et al*., 2001; Quill *et al*., 2003). While CatSper null sperm can swim progressively, the pattern of flagellar bending in the activated sperm is actually slightly abnormal and resembles that of wild-type sperm treated with a cell-permeant form of the Ca^{2+} chelator BAPTA to lower intracellular Ca^{2+} (Marquez *et al*., 2006). This indicates that the sperm cannot maintain even the low Ca^{2+} levels required to support normal activated motility and this deficit could also contribute to infertility.

Homologs to murine CatSper genes have been identified in the human genome. Transcripts of CatSper genes were detected in higher amounts in human semen samples with high sperm motility than those with low sperm motility (Li *et al*., 2007); however, the functional significance of mRNA in samples of mature sperm is unclear. Three brothers who were homozygous for a mutation followed by a deletion on chromosome 15 that encompassed the last two exons of CatSper2 were infertile. Sperm numbers were normal, but sperm motility and morphology were abnormal, with >90% of sperm showing short, coiled flagella (Avidan *et al*., 2003). In a limited study, the group mean expression of CatSper was lower in subfertile patients whose sperm showed motility deficits than in subfertile patients whose sperm showed normal motility (Nikpoor *et al*., 2004).

Other types of plasma membrane Ca^{2+} channels have been detected in mammalian sperm. Various voltage-gated Ca^{2+} channels have been detected in mouse and human sperm (Wennemuth *et al*., 2000; Trevino *et al*., 2004; Carlson *et al*., 2005). Transient receptor potential (TRPC-1, -3, -4, -6) channels (Castellano *et al*., 2003) have been identified in the principal pieces of human sperm flagella by antibody labeling. Cyclic nucleotide-gated Ca^{2+} channels have been localized to bull sperm flagella (Wiesner *et al*., 1998).

Null mutant mice have been developed for some of these voltage-gated channels (Platzer *et al*., 2000; Saegusa *et al*., 2000; Ino *et al*., 2001), TRPC4 channels (Freichel *et al*., 2001) and cyclic nucleotide-gated channel protein CNGA3 (Biel *et al*., 1999), but the null mutant males were not infertile. Nevertheless, homozygous null pups were underrepresented in heterozygotes crossings of voltage-gated $Ca_v1.3$ null mutants (Platzer *et al*., 2000) and $Ca_v2.3$ null mutant sperm showed greater straight-line velocity (VSL) and linearity (LIN) by CASA measurements in some media, indicating a reduction in the development of hyperactivation (Sakata *et al*., 2002). Null mutants of some types of Ca^{2+} channels were embryonic lethals (Seisenberger *et al*., 2000) or died shortly after birth (Jun *et al*., 1999) and thus could not be tested for effects on fertility. Furthermore, null mutants have not been produced against all members of these families of channels that might be present in sperm. Finally, although there is electrophysiological evidence that CatSper proteins are the only source of Ca^{2+} current in the flagella of mature sperm (Kirichok *et al*., 2006), other types of Ca^{2+} channels may have been present during the electrophysiological testing but inactived, e.g. by phosphorylation. Thus, other types of channels could contribute Ca^{2+} for hyperactivation without being absolutely essential to the process. For reviews of Ca^{2+} and other ion channels found in sperm, see (Darszon *et al*., 2006, 2007; Benoff *et al*., 2007; Publicover *et al*., 2007).

In addition to extracellular sources, Ca^{2+} for hyperactivation may be provided from a storage organelle in the base of the flagellum. Pharmacological agents known to release Ca^{2+} from stores induce hyperactivation in bull sperm, even in the absence of available extracellular Ca^{2+} (Ho and Suarez, 2001). Receptors for inositol 1,4,5-trisphosphate (IP_3), which gate channels that release Ca^{2+} from intracellular stores, and calreticulin, the Ca^{2+} binding protein of reticular stores, were localized by antibodies to the base of the flagellum within a portion of the redundant nuclear envelope (RNE) (Ho and Suarez, 2001, 2003). The RNE is a cluster of membrane vesicles that originates from the nuclear envelope during condensation of the nucleus during spermiogenesis (Franklin, 1968; Toshimori *et al*., 1985). Unlike other excess organelles, the RNE is not discarded in the residual cytoplasm or cytoplasmic droplets and this is probably because it is not truly redundant but rather serves as an important Ca^{2+} store.

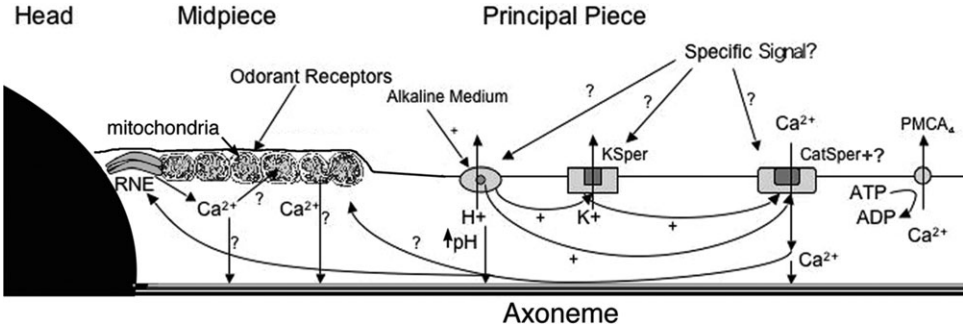

Figure 2: Schematic representation of mechanisms that may regulate hyperactivation.
RNE, redundant nuclear envelope; $PMCA_4$, plasma membrane Ca^{2+}-$ATPase_4$.

There is evidence for a functional RNE Ca^{2+} store in human sperm that responds pharmacologically as a ryanodine receptor-gated store, rather than as an IP_3 receptor-gated store (Harper *et al*., 2004; Harper and Publicover, 2005).

The acrosome has also been identified as a functional Ca^{2+} store in mammalian sperm (Walensky and Snyder, 1995; O'Toole *et al*., 2000; Herrick *et al*., 2005), including human sperm (Bedu-Addo *et al*., 2007; Lawson *et al*., 2007), with a role primarily in the process of acrosomal exocytosis. Because Ca^{2+} has been observed to rise in the flagella of acrosome-reacted sperm as well as in the head, leading to an increase in intensity of hyperactivation (Suarez and Dai, 1995), it is possible that Ca^{2+} released from this store contributes in some way to hyperactivation.

The CatSper channels are confined to the principal piece of the flagellum, whereas the RNE Ca^{2+} store lies in close association with the mitochondria at the base of the midpiece mitochondrial sheath (Figs 2 and 3). It is not known whether the Ca^{2+} for hyperactivation normally comes from both the RNE and plasma membrane channels, or whether the RNE serves instead to modulate hyperactivated motility. Mouse sperm loaded with fluorescent Ca^{2+} indicator and treated with cell-permeant cyclic nucleotides or with alkaline high potassium medium to activate CatSper channels showed an instantaneous increase in Ca^{2+} throughout the principal piece which subsequently took 3–6 s to spread through the midpiece and reach the head. This response was not seen in sperm from CatSper null mutants (Xia *et al*., 2007). Due to the presence of RNE, mitochondria and Ca^{2+} buffers in the cytoplasm, the observed spread of increased Ca^{2+} to the head is highly unlikely to be solely the result of simple diffusion from the flagellar principal piece (reviewed in Clapham, 2007). Nevertheless, the participation of RNE Ca^{2+} stores in spreading the CatSper-induced increase or, for that manner, in normal physiological triggering of hyperactivation *in vivo*, has not been investigated.

In intact sperm, as in many other cell types, cytoplasmic Ca^{2+} is maintained at physiologically appropriate levels by Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers, which pump Ca^{2+} out of the cell or into intracellular stores (reviewed in Jimenez-Gonzalez *et al*., 2006; Clapham, 2007). The primary mechanism of removing excess Ca^{2+} from sperm is a plasma membrane Ca^{2+}-ATPase (PMCA) (Wennemuth *et al*., 2003a, b; Harper *et al*., 2005; Jimenez-Gonzalez *et al*., 2006). PMCA has been localized to the principal piece of the flagellum (Wennemuth *et al*., 2003a, b; Okunade *et al*., 2004; Schuh *et al*., 2004), which is also the location of the CatSper Ca^{2+} channels (Carlson *et al*., 2005; Jin *et al*., 2007). Male mice that are null mutants for the gene encoding $PMCA_4$ are infertile, because their sperm become immotile during the course of capacitation instead of hyperactivating (Okunade *et al*., 2004). The mitochondria of the immotile sperm show abnormal density patterns in transmission electron micrographs, indicative of Ca^{2+} overload (Okunade *et al*., 2004). Intracellular Ca^{2+} levels of motile null sperm were higher than those of wild-type sperm (Schuh *et al*., 2004).

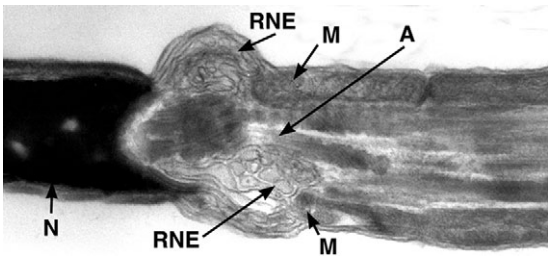

Figure 3: Transmission electron micrograph of bull sperm, showing the redundant nuclear envelope.
N, nucleus; M, mitochondrion; RNE, redundant nuclear envelope; A, axoneme. Micrograph courtesy of Dr Han-Chen Ho and was prepared as described in Ho and Suarez (2001).

In human sperm, a secretory pathway Ca^{2+}-ATPase has been immunolocalized to the midpiece and rear head and suggested to play a role in clearance of Ca^{2+} released from the RNE store (Harper *et al*., 2005). There is also evidence for a Na^+/Ca^{2+} exchanger in human sperm, localized primarily to the postacrosomal region and flagellar midpiece (Krasznai *et al*., 2006; Jimenez-Gonzalez *et al*., 2006). Its role in clearance of excess Ca^{2+} from the cytoplasm, at least in mouse sperm, is known to be minor compared with that of PMCA Ca^{2+} pumps (Wennemuth *et al*., 2003a, b).

cAMP signaling activates motility and enhances beat frequency, but does not induce deep flagellar bending characteristic of hyperactivation

Activation of sperm motility is a prerequisite for hyperactivation, in the sense that sperm that cannot swim cannot become hyperactivated. Activation is dependent on cAMP produced by the form of adenylyl cyclase known as sAC or SACY. Sperm from SACY null mice are unable to activate fully, although they show some sluggish movement (Esposito *et al*., 2004; Xie *et al*., 2006). The null sperm are also unable to undergo protein tyrosine

phosphorylation associated with capacitation and do not hyperactivate (Hess *et al*., 2005; Xie *et al*., 2006). When sperm from SACY null mice are activated using cell-permeant cAMP-AM, they proceed to hyperactivate under capacitating conditions (Marquez and Suarez, 2008). Thus, cAMP produced by SACY is necessary to activate sperm and, in that sense, is a prerequisite for hyperactivation. SACY is stimulated by Ca^{2+} and HCO_3^- (Chen *et al*., 2000; Xie *et al*., 2006; Carlson *et al*., 2007). Stimulating SACY in sperm that are already motile increases flagellar beat frequency (Wennemuth *et al*., 2003a, b; Xie *et al*., 2006; Carlson *et al*., 2007). A detailed study showed that HCO_3^- stimulation of SACY in motile mouse sperm requires the presence of extracellular Ca^{2+}, which acts upstream of HCO_3^-. Furthermore, the normal source of HCO_3^- is CO_2 that diffuses into sperm (Carlson *et al*., 2007). The beat cross frequency of motile human sperm is also increased by HCO_3^- (Luconi *et al*., 2005); however, increased beat frequency does not constitute hyperactivation, which is instead characterized by an increase in flagellar bend amplitude. The increase in bend amplitude usually comes at the expense of beat frequency, because it takes the flagellum longer to generate a larger bend (Suarez *et al*., 1991; Mortimer *et al*., 1997).

The cAMP generated by SACY activates protein kinase A, which phosphorylates serine or threonine resides on proteins, thereby turning on a signaling pathway that leads to phosphorylation of tyrosine residues on other proteins. Increased protein tyrosine phosphorylation in flagella is associated with hyperactivated motility in hamster (Si and Okuno, 1999) and monkey sperm (Mahony and Gwathmey, 1999). Tyrosine phosphorylation has also been associated with heat-induced hyperactivation in human sperm (Chan *et al*., 1998). Tyrosine phosphorylation, dephosphorylation and rephosphorylation of an 80-kDa protein in hamster sperm flagella are associated with the acquisition, loss and reacquisition of temperature-dependent hyperactivation (Si, 1997). However, bull sperm do not hyperactivate when incubated under conditions that increase protein tyrosine phosphorylation and enable sperm to undergo the acrosome reaction; and furthermore, when uncapacitated bull sperm are treated with procaine or caffeine, they immediately hyperactivate without showing an increase in tyrosine phosphorylation (Marquez and Suarez, 2004). Thus, the role of tyrosine phosphorylated proteins is not entirely clear.

Generation of cAMP can also stimulate Ca^{2+} influx, through either cyclic nucleotide-gated Ca^{2+} channels (Wiesner *et al*., 1998) or CatSper channels (Xia *et al*., 2007) to contribute to hyperactivation.

Increased ATP and PH are required by the axoneme to produce hyperactivation

When Triton X-100-demembranated bull sperm were reactivated in the presence of ATP and 400–1000 nM Ca^{2+}, the flagella began to beat asymmetrically. However, to produce the deep flagellar bending characteristic of full-blown hyperactivation, higher levels of ATP were required in the reactivation solution than were required to produce activated motility, indicating an increased ATP requirement for hyperactivation (Ho *et al*., 2002).

Lactate production plays a key role in providing ATP for motility in general and, consequently, for hyperactivation. A germ-cell-specific form of lactate dehydrogenase, LDH-C_4, is responsible for most of the LDH activity in sperm (Odet *et al*., 2008). Male mice that are null mutants for *Ldhc* are infertile. The sperm activate normally, but, when incubated in capacitation medium, the null sperm show a decline in motility over time and hyperactivation never develops (Odet *et al*., 2008).

Hyperactivation of demembranated sperm requires a pH of 7.9–8.5, whereas activation can occur at a pH as low as 7.0, indicating that the cytoplasmic pH increases in the axonemal compartment of intact sperm at the time of hyperactivation (Ho *et al*., 2002). If so, then alkaline pH plays a double role in supporting hyperactivation, because CatSper channels are activated by alkaline intracellular pH (Kirichok *et al*., 2006). A rise in pH that activates CatSper channels would also directly stimulate hyperactivated bending at the axoneme (Fig. 2). Treating bull sperm with NH_4Cl to raise intracellular pH stimulated a rise in intracellular Ca^{2+} and hyperactivation; furthermore, the NH_4Cl treatment stimulated a more intense hyperactivation than the Ca^{2+} ionophore ionomycin, even though ionomycin produced a larger increase in intracellular Ca^{2+} (Marquez and Suarez, 2006). Intracellular pH increases during capacitation of mouse sperm (Zeng *et al*., 1996) and thus would stimulate hyperactivation by activating CatSper channels and triggering asymmetrical bending of the axoneme.

A weakly outwardly rectifying K^+ current, dubbed KSper, has been detected in mouse sperm and proposed to be caused by the product of the testis-specific *mSlo3* gene (Navarro *et al*., 2007). The efflux of K^+ through this channel hyperpolarizes the plasma membrane and, in doing so, increases the driving force for Ca^{2+} influx through CatSper channels. Like CatSper activity, the KSper K^+ current is stimulated by alkaline pH; therefore, efflux of H^+ from flagella could affect Ca^{2+} influx directly through CatSper channels and indirectly through activation of KSper K^+ currents (Navarro *et al*., 2007) (Fig. 2).

Relationship of hyperactivation to capacitation

The physiological changes that confer on sperm the ability to fertilize are collectively called 'capacitation' (Yanagimachi, 1994). These changes include hyperactivation and developing the capacity to undergo the acrosome reaction in response to specific triggers (referred to as 'acrosomal responsiveness'). Although hyperactivation usually occurs *in vitro* at some point during the capacitation process, the pathways leading to hyperactivation and acrosomal responsiveness are not completely coupled. Boatman and Robbins (1991) found that HCO_3^- is essential for both acrosomal responsiveness and hyperactivation of hamster sperm *in vitro*, but hyperactivation requires higher levels of HCO_3^-. Hyperactivation can occur independently of acrosomal responsiveness in mouse and hamster sperm. Sperm from male mice with a *t* complex haplotype ($t^{w32}/+$), a region of inversions in chromosome 17, showed hyperactivated motility prematurely when incubated under capacitating conditions, while acrosomal responsiveness occurred on schedule (Neill and Olds-Clarke, 1987; Olds-Clarke, 1989). In hamster sperm, hyperactivation develops fully in capacitation medium an hour or more before acrosome reactions are seen (Suarez and Dai, 1995). Procaine rapidly initiates hyperactivation in uncapacitated guinea pig and bull sperm (Mujica *et al*., 1994; Ho *et al*., 2002) and bull sperm hyperactivated by procaine do not undergo tyrosine phosphorylation associated with acrosomal responsiveness (Marquez and Suarez, 2004). Bull sperm incubated under conditions that promote

acrosomal responsiveness do not hyperactivate (Marquez and Suarez, 2004). Thus, the processes of hyperactivation and development of acrosomal responsiveness are not completely tied to each other.

When human sperm are incubated in capacitation medium, an average of 10–20% develop motility patterns that look like hyperactivation (Burkman, 1984; Buffone *et al*., 2005).

Sperm from fertile men show higher levels of hyperactivation (~12%) than sperm from asthenozoospermic infertility patients (~4%) (Buffone *et al*., 2005). However, the levels shown by the normal samples are so low that we cannot be certain that incubation in capacitating medium is the optimal means of hyperactivating human sperm or if additional stimulatory factors play a role in hyperactivating sperm *in vivo*.

Factors triggering hyperactivation *in vivo* are poorly understood

Unfortunately, the physiological trigger that switches on hyperactivation *in vivo* remains elusive nearly 40 years after hyperactivation was first reported by Yanagimachi (1970). Hyperactivation must occur at the right place and time to achieve fertilization. There is evidence that hyperactivated sperm cannot traverse the uterotubal junction (Gaddum-Rosse, 1981); therefore the oviduct is the best place to seek triggering molecules. The trigger could be secreted by the oviductal epithelium or introduced into the oviduct by the oocyte–cumulus mass. Hormones, ions and secretions in the oviductal luminal fluid vary during the estrous cycle (Nichol *et al*., 1992).

Human follicular fluid has a dose-dependent effect on flagellar beat frequency and hyperactivation (Yao *et al*., 2000). It was suggested that progesterone is the active factor, because it enhances hyperactivation (Sueldo *et al*., 1993). However, progesterone did not stimulate hyperactivation in at least one study (Kay and Robertson, 1998). Furthermore, the identity of the progesterone receptor is unknown (Modi *et al*., 2007) and localization of putative progesterone receptors is confined to the human sperm head (Blackmore and Lattanzio, 1991).

Cumulus cells that enter the ampulla with the oocyte may secrete signals for hyperactivation. Supernatant from the culture of cumulus oophorus cells increased curvilinear velocity and side-to-side movement of the heads of human sperm (Fetterolf *et al*., 1994).

However, despite evidence from *in vitro* experiments for a role of follicular fluid and cumulus in initiating hyperactivation, no specific signals have been unequivocally identified. Furthermore, there is evidence that sperm hyperactivate before cumulus and follicular fluid enter the oviduct. Hyperactivated sperm have been recovered from the rabbit oviduct before ovulation (Cooper *et al*., 1979; Overstreet and Cooper, 1979) and hyperactivated mouse sperm could be seen through the walls of transilluminated oviducts before ovulation (Suarez and Osman, 1987). On the other hand, steroid hormones in follicular fluid of antral follicles that are still in the ovary could reach sperm in the oviductal isthmus via vascular counter-current transfer from veins leaving the ovary to arteries supplying the wall of the isthmus (Hunter *et al*., 1983).

The oviductal epithelium could secrete hyperactivation-signaling molecules; however, none have been identified. Alternatively, there may be no specific macromolecular trigger, but rather a change in ionic environment, particularly an increase in the pH of oviduct fluid that activates the CatSper channels and raises intracellular pH to initiate hyperactivation. The pH in the lumen of the rhesus monkey oviducts was measured using miniaturized pH electrodes and found to be 7.1–7.3 during the follicular phase. At ovulation there was a sudden increase to 7.5–7.8, which was maintained throughout the luteal phase (Maas *et al*., 1977). This pH increase could possibly be the primary factor inducing hyperactivation in the oviduct.

Hyperactivation may be modulated by chemotactic factors

Chemotaxis has been well documented in sperm of some marine invertebrates. Very low concentrations of specific peptides secreted by sea urchin oocytes attract sperm. In brief, binding of the peptide to sperm increases intracellular cyclic nucleotide levels, then intracellular Ca^{2+}, which increases flagellar bend asymmetry and swimming path curvature (reviewed by Kaupp *et al*., 2008).

Peptide ligands like those of sea urchin oocytes have not been identified in mammals, but there is evidence that odorant-like molecules function as chemoattractants to mammalian sperm. Antibodies to conserved amino acid sequences of odorant receptors specifically labeled the flagellar midpiece of mature dog and rat sperm, as well as the base of the flagellum of mature rat sperm (Vanderhaeghen *et al*., 1993; Walensky *et al*., 1995). Some of these antibodies also detected odorant receptors in western blots of protein extracts of hamster and human sperm (Walensky *et al*., 1995). A human odorant receptor specific to the testis was identified, cloned and functionally expressed in human embryonic kidney cells, where stimulation by specific odorant molecules produced Ca^{2+} signals (Spehr *et al*., 2003). Human sperm respond to the floral odorant bourgeonal by producing Ca^{2+} signals and orienting in a gradient of the odorant (Spehr *et al*., 2003, 2004). Thus, it is thought that a molecule resembling bourgeonal guides human sperm to the oocyte *in vivo*. Such a molecule is currently the object of an intense search.

Human sperm have also been observed responding to gradients of progesterone with oscillations in intracellular Ca^{2+} superimposed on a rise in Ca^{2+}. The flagella of responding sperm showed temporary increases in bend amplitude that corresponded to the oscillatory Ca^{2+} peaks (Harper *et al*., 2004). Because cumulus cells secrete progesterone, a gradient could develop in the vicinity of the cumulus mass in the oviduct. Nevertheless, despite functional evidence for its existence, a progesterone receptor has yet to be identified in human sperm (Correia *et al*., 2007; Modi *et al*., 2007). The cytokine Rantes has also been implicated in human sperm chemotaxis (Isobe *et al*., 2002) and its receptors have been localized to the sperm heads (Muciaccia *et al*., 2005). For reviews on sperm chemotaxis, see Eisenbach (2007) and Kaupp *et al*. (2008).

The relationship of the signaling pathways that switch on hyperactivation and chemotaxis is currently a mystery. There is evidence that both hyperactivation and chemotactic responses involve a rise in Ca^{2+}; however, the Ca^{2+} rise produced by activating CatSper channels to induce hyperactivation in mouse sperm originates in the principal piece of the flagellum (Xia *et al*., 2007), while the Ca^{2+} rise detected in response to the odorant

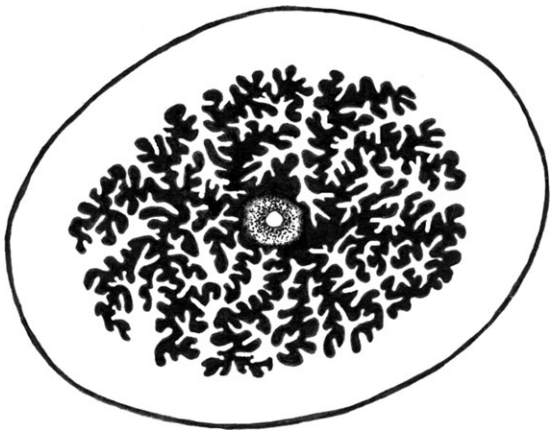

Figure 4: A diagram, drawn to scale, of the human oocyte in cumulus (centre) within the ampulla of the oviduct (Fallopian tube). Adapted from Suarez and Pacey (2006).

bourgeonal originates in the midpiece of human sperm (Spehr *et al*., 2004).

Because hyperactivation occurs in the oviduct far from the oocyte and even before ovulation (Cooper *et al*., 1979; Overstreet and Cooper, 1979; Suarez and Osman, 1987), it is likely that sperm are already hyperactivated when they receive odorant signals. Perhaps chemotactic factors act on hyperactivated sperm to trigger brief releases of Ca^{2+} from the RNE store that modulate the flagellar beating pattern just long enough to re-direct the path of the sperm.

Chemotactic factors could serve to direct sperm toward the ampulla, toward the cumulus mass in the ampulla and/or toward the oocyte within the cumulus mass. In mice, the cumulus mass fills the entire lumen in a substantial region of the oviductal ampulla and makes an easy target for sperm; however, mouse sperm may require guidance to locate oocytes within the mass. In humans, the cumulus mass is a very small target for sperm, because it does not fill the ampullar lumen and the lumen is divided into complex branched channels by mucosal folds (Fig. 4). Without guidance by chemotaxis, human sperm could easily pass by the cumulus mass.

Last word: what is hyperactivation in human sperm and how can we assess it?

Numerous studies in various species have indicated that hyperactivated motility is critical to achieving fertilization *in vivo*, because it assists sperm in reaching the oocyte through the mucus-filled lumen of the oviduct and then enables sperm to penetrate the zona pellucida. Thus, measuring the ability of human sperm to hyperactivate in response to physiological stimuli can be a useful assay to include in the battery of tests for infertility, particularly for those clinicians who believe that intracytoplasmic sperm injection should be used only after exhausting other treatments, including *in vitro* fertilization.

Although human sperm have been reported to swim in patterns characteristic of hyperactivation *in vitro*, the motility of human sperm has not been video recorded within the oviduct or even in flushings of the oviduct. Thus we cannot be certain of what exactly constitutes hyperactivation in human sperm. Given the ethical and technical challenges for getting this information, identifying hyperactivation in clinical samples will have to depend on information learned from studies of model species. Hyperactivation, as defined from studies of model species, has been observed in human sperm incubated under capacitating conditions (Morales *et al*., 1988; Mortimer and Mortimer, 1990; Mortimer and Swan, 1995b); however, there is no standardization of capacitating conditions among published studies nor is there standardization of CASA settings used to detect hyperactivation.

Incubation of human sperm in capacitation medium leads to a gradual increase in incidence of hyperactivation, usually with an average maximum of 20% or less. If specific physiological triggers for hyperactivation were found in model species that could be used on human sperm to produce a rapid and more substantial response, this would enable the development of a fertility assay that would provide higher resolution of the level of fertility of a sample.

In conclusion, hyperactivation is critical to fertilization and reliable tests are needed for assessing the ability of human sperm samples to undergo hyperactivation normally.

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