Ca\textsuperscript{2+} -stores in sperm: their identities and functions

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Abstract

Intracellular Ca\textsuperscript{2+} stores play a central role in the regulation of cellular [Ca\textsuperscript{2+}]\textsubscript{i} and the generation of complex [Ca\textsuperscript{2+}] signals such as oscillations and waves. Ca\textsuperscript{2+} signalling is of particular significance in sperm cells, where it is a central regulator in many key activities (including capacitation, hyperactivation, chemotaxis and acrosome reaction) yet mature sperm lack endoplasmic reticulum and several other organelles that serve as Ca\textsuperscript{2+} stores in somatic cells. Here, we review i) the evidence for the expression in sperm of the molecular components (pumps and channels) which are functionally significant in the activity of Ca\textsuperscript{2+} stores of somatic cells and ii) the evidence for the existence of functional Ca\textsuperscript{2+} stores in sperm. This evidence supports the existence of at least two storage organelles in mammalian sperm, one in the acrosomal region and another in the region of the sperm neck and midpiece. We then go on to discuss the probable identity of these organelles and their discrete functions: regulation by the acrosome of its own secretion and regulation by membranous organelles at the sperm neck (and possibly by the mitochondria) of flagellar activity and hyperactivation. Finally, we consider the ability of the sperm discretely to control mobilisation of these stores and the functional interaction of stored Ca\textsuperscript{2+} at the sperm neck/midpiece with CatSper channels in the principal piece in regulation of the activities of mammalian sperm.


[Ca\textsuperscript{2+}]\textsubscript{i}: a central regulator in sperm function

Regulation of cellular activity, in response to signals from other cells or from the extracellular environment, can occur at a number of levels. Long-term regulation is achieved by control of gene expression. This process can occur through control of translation and/or transcription and also by more subtle regulation of mRNA transcripts, and by regulation of turnover of the protein product. Effects of this type are typically exerted over time periods measured in hours rather than minutes or seconds. Regulation of cellular activity over shorter time periods is achieved by rapid, ‘post-translational’ modification of the function of proteins already present. Various pathways have been characterised, by which the actions of extracellular signals such as hormones, growth factors and transmitters are transduced, leading to appropriate modification of protein function. One such mechanism is through changes in the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}).

In sperm, which lack endoplasmic reticulum (ER) and have a highly condensed nucleus, the regulation of function by translation/transcription (if it occurs at all) will be very limited. Post-translational mechanisms must, therefore, control all activities of the cell. Regulation of protein function through Ca\textsuperscript{2+} signalling is central to a range of activities that are pivotal to sperm function, including hyperactivation, chemotaxis and acrosome reaction (Publicover et al. 2007). Impairment of Ca\textsuperscript{2+} signalling in sperm is associated with male subfertility (Krausz et al. 1995, Baldi et al. 1999, Espino et al. 2009).

Signalling though [Ca\textsuperscript{2+}]\textsubscript{i} is achieved by permitting Ca\textsuperscript{2+} to enter the cytoplasm (where concentration is maintained very low) from the extracellular space and/or from intracellular organelles, where the Ca\textsuperscript{2+} concentration is up to four orders of magnitude higher. Signal initiation requires merely that Ca\textsuperscript{2+} permeable membrane channels are opened, allowing the ions to flow down their electrochemical gradient. The presence of Ca\textsuperscript{2+} channels in the plasma membrane of sperm cells is well established, as is their significance in the key activities of sperm. A number of thorough reviews...
on the various types and distribution of these channels are available (Darszon et al. 1999, Felix 2005, Jimenez-Gonzalez et al. 2006, Navarro et al. 2008). Here, we will review evidence for the existence, identity and role(s) of Ca\(^{2+}\) storage organelles in sperm.

**Ca\(^{2+}\) stores in somatic cells and their associated Ca\(^{2+}\) transporters**

Somatic cells contain a number of membrane-bound organelles that undertake various biochemical reactions vital to the maintenance of cellular homeostasis and viability (Berridge et al. 1998). Many of these organelles also act as Ca\(^{2+}\) reservoirs or Ca\(^{2+}\) stores which contribute to the regulation of Ca\(^{2+}\)-dependent processes (Michelangeli et al. 2005). In order to be classified as a *bona fide* Ca\(^{2+}\) store, an organelle must have at least two types of Ca\(^{2+}\) transporters, enabling both loading of the store and release of stored Ca\(^{2+}\) in a controlled fashion.

**Ca\(^{2+}\) uptake and release mechanisms**

Ca\(^{2+}\) accumulation into stores normally occurs against the electrochemical gradient for the ion and therefore requires expenditure of energy. Typically this is achieved by ATPase ‘pumps’ such as the sarcoplasmic–ER Ca\(^{2+}\) ATPase (SERCA) or secretory pathway Ca\(^{2+}\) ATPases (SPCA), though Ca\(^{2+}\) exchangers (co-transporters) may also be used. By contrast, controlled release of Ca\(^{2+}\) can be achieved by gating of Ca\(^{2+}\)-permeable ion channels in the membrane of the organelle. These are usually regulated by second messengers (or putative second messengers) such as inositol 1,4,5-trisphosphate (IP\(_3\)), cyclic ADP ribose (cADP-ribose), nicotinic acid ADP (NAADP) and even by Ca\(^{2+}\) itself, via a Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism (CICR; Bootman et al. 2001). The difference in mechanisms for uptake and release of stored Ca\(^{2+}\) has significant effects upon the rates at which Ca\(^{2+}\) translocation occurs. SERCAs must undergo multiple binding and conformational states during translocation of Ca\(^{2+}\) and transport only a few ions per ATPase molecule per second, whereas a single release channel can transport 100 000’s of Ca\(^{2+}\) in the same period (Taylor 1995).

**Ca\(^{2+}\) storage organelles**

**Endoplasmic reticulum**

In the early 1980s it was first demonstrated that the ER acted as a Ca\(^{2+}\) store that could release its Ca\(^{2+}\) in the presence of the agonist-generated second messenger IP\(_3\) (Berridge 2002). This release was later shown to occur via activation of IP\(_3\) receptors (IP\(_3\)Rs), IP\(_3\)-activated Ca\(^{2+}\) channels located on the ER membranes (Michelangeli et al. 1995). From analogous studies on striated muscle sarcoplasmic reticulum (SR), it was shown that ER membranes also contain SERCA pumps for Ca\(^{2+}\) accumulation and ryanodine receptor (RyR) type Ca\(^{2+}\) channels, named for their sensitivity to the drug ryanodine, but activated in vivo by Ca\(^{2+}\) itself (CICR) and possibly by cADP-ribose (Michelangeli et al. 2005). Though there seems little doubt that the ER is the primary store of Ca\(^{2+}\) that is used in intracellular Ca\(^{2+}\) signalling, other organelles may also play a role (Michelangeli et al. 2005).

**Nuclear, golgi and lysosomal Ca\(^{2+}\) storage**

Immunohistochemical and biochemical studies have shown that the nuclear envelope, the outer membrane of which is continuous with the ER, also contains both SERCA Ca\(^{2+}\) pumps and IP\(_3\)R Ca\(^{2+}\) channels (Lanini et al. 1992, Humbert et al. 1996). RyR type Ca\(^{2+}\) channels have also been identified on the nuclear membrane (Gerasimenko et al. 2003). In some cells the nuclear membrane forms a complex tubular network which penetrates deep into the nucleus and which is particularly enriched in IP\(_3\)Rs (Echevarria et al. 2003). This has lead to the suggestion that Ca\(^{2+}\) mobilisation, leading to localised increases in [Ca\(^{2+}\)] within distinct regions of the nucleus, may affect gene transcription.

The Golgi apparatus, involved in both post-translational protein modification and protein trafficking, has also been shown to contain IP\(_3\)Rs and SERCA Ca\(^{2+}\) pumps (Surroca & Wolf 2000). These transporters are localised to the cis Golgi region, while membranes of the trans Golgi region contain the SPCA pump (Missiaen et al. 2004, Wootton et al. 2004), which has different transport properties compared to SERCA.

A role for lysosomes in Ca\(^{2+}\) signalling is suggested by the observation that they release Ca\(^{2+}\) when treated with the NAD metabolite and putative second messenger NAADP, which activates the NAADP-sensitive Ca\(^{2+}\) channel (Churchill et al. 2002, Kinnear et al. 2004) of the two-pore channel family (Calcraft et al. 2009). These organelles are believed to be filled by a H\(^{+}\)/Ca\(^{2+}\) exchanger utilising the proton gradient across the membrane maintained by the vacuolar H\(^{+}\) ATPase (Churchill et al. 2002).

**Mitochondria**

It has been known for some time that mitochondria can accumulate Ca\(^{2+}\) into the matrix space, primarily through the mitochondrial Ca\(^{2+}\) uniporter (MCU) located on the inner mitochondrial membrane. Ca\(^{2+}\) uptake is driven by the negative membrane potential of the mitochondrial matrix. Recent studies have shown the MCU to be a Ca\(^{2+}\) channel of relatively low conductance, with a complex gating mechanism (Kirichok et al. 2004). Controlled release of mitochondrial Ca\(^{2+}\) can occur through a Na\(^{+}\)/Ca\(^{2+}\) exchanger (Bernardi 1999). Under conditions where ‘resting’ [Ca\(^{2+}\)]\(_{i}\) is...
elevated, Ca\(^{2+}\) uptake by mitochondria both activates a number of key tricarboxylic acid cycle dehydrogenases and also acts as a Ca\(^{2+}\) sink in order to buffer cytosolic Ca\(^{2+}\) levels (Gunter et al. 2004). If excessive mitochondrial Ca\(^{2+}\) accumulation occurs this can lead to activation of the permeability transition pore, which permits release from the mitochondrial matrix of factors that initiate cell death (Orrenius et al. 2003, Dong et al. 2006, Jeong & Seol 2008). However, under physiological conditions mitochondria also play an important role in Ca\(^{2+}\) buffering and signalling, shaping (and often extending) the kinetics of Ca\(^{2+}\) signals (Bianchi et al. 2004, Rimessi et al. 2008).

Over the last few years researchers have begun to investigate potential interactions between different Ca\(^{2+}\) stores, some recent evidence indicating that such interactions may contribute to the complexity of spatio-temporal intracellular [Ca\(^{2+}\)] profiles (Michelangeli et al. 2005). Current research is now focussing on identifying these interactions and assessing their roles in controlling complex physiological processes.

Do sperm have Ca\(^{2+}\) stores?

In somatic cells the ER is the primary Ca\(^{2+}\) storage organelle. A mature sperm has no recognisable ER but does have a nuclear membrane, an acrosome (a single cap-shaped vesicle that surrounds the anterior nucleus), mitochondria (which are concentrated in the midpiece) and some poorly-defined, irregular membranous structures in the region of the sperm neck from where the cytoplasmic droplet has been shed (Fig. 1a). Since organelles other than the ER can participate in storage and release of Ca\(^{2+}\) in somatic cells (see section Ca\(^{2+}\) storage organelles above), any or several of the membranous structures of sperm may act as releasable Ca\(^{2+}\) stores.

Components of Ca\(^{2+}\) storage organelles expressed in sperm

IP\(_3\) receptors

The first clear evidence that intracellular organelles in mature mammalian sperm might act as Ca\(^{2+}\) stores was the finding of Walensky & Snyder (1995) that components of the phosphoinositide signalling system are present in mammalian sperm. Both the G protein Ga\(_{q/11}\) and the β1 isoform of phospholipase C (PLC), which generates the Ca\(^{2+}\)-mobilising intracellular ligand IP\(_3\), were identified in the acrosomal (anterior head) region. IP\(_3\)Rs were also present, primarily in the anterior head, though a second, smaller concentration of receptors was detected in the anterior midpiece. IP\(_3\)Rs were enriched in acrosomal fractions and were lost from the sperm into the medium upon acrosome reaction, consistent with localisation to the outer acrosomal membrane.

Figure 1 Localisation of Ca\(^{2+}\) store components in sperm. (a) Electron microscope image of a human sperm showing the localisation of the acrosome, nucleus and mitochondria. (b) Immunolocalisation of IP\(_3\)Rs (green) and nuclear pore complex proteins (red) in bovine sperm. IP\(_3\) receptors are localised over the acrosome (asterisks) and also at the sperm neck (arrows). Nuclear pore complex proteins indicate site of redundant nuclear envelope. (c) Phase image of the cells in panel b. (d) Immunolocalisation of secretory pathway Ca\(^{2+}\) ATPase (SPCA) (green) in human sperm. SPCA is restricted to the neck and midpiece. In many cells there is a particular concentration at the sperm neck (arrows). (e) Phase image of the cells in panel d. (f) Immunolocalisation of secretory pathway Ca\(^{2+}\) ATPase (SPCA) (green) in sperm of the sea urchin Strongylocentrotus purpuratus. SPCA localises to the giant mitochondrion in each cell (arrows). (g) Phase image of the cells in panel f. Panels b and c from Ho & Suarez (2003) with permission. Panels f and g from Gunaratne & Vacquier (2006) with permission.

Tomes et al. (1996) identified PLCγ in the head of mouse sperm. The enzyme was transferred to the particulate fraction during capacitation. Stimulation of the sperm with solubilised zona pellucida increased tyrophostin-sensitive PLC activity, suggesting that the γ isoform of PLC was activated during induction of acrosome reaction. Since these initial reports, the presence of IP\(_3\)Rs in the sperm of a number of mammals has been confirmed (Dragileva et al. 1999, Kuroda et al. 1999,
SERCAs are present in sperm their role is far from clear
sea urchin (Gunaratne & Vacquier 2006; Fig. 1f and g). If and e). Similar results were obtained with sperm of the varied somewhat between species and between studies, two concentrations of IP$_3$Rs were generally reported, one over the acrosome and the other at the sperm neck or anterior midpiece (Fig. 1b). In sea urchin sperm an IP$_3$-binding protein has also been identified. An antibody against the type 1 IP$_3$R recognised a protein present in the sperm plasma membrane (Zapata et al. 1997).

Ryanodine receptors
The evidence regarding expression of RyRs in mamma-
lian sperm is less clear. We have observed staining of mature human sperm in the region of the sperm neck both with BODIPY FL-X ryanodine (a fluorescently-tagged ryanodine derivative) and with antibodies against RyRs 1 and 2 (Harper et al. 2004, Lefievre et al. 2007). By contrast, others have reported no staining with BODIPY FL-X ryanodine in bovine sperm (Ho & Suarez 2001) and staining only for RyR3 in mature rodent sperm (Treveno et al. 1998). The conductance of RyRs (> 100 pS; Zalk et al. 2007) is particularly high for Ca$^{2+}$-permeable channels so it is possible that RyRs, if present in sperm, are expressed at extremely low levels. Only one or two channels may be present in each cell, Ca$^{2+}$ flux being regulated by the proportion of time for which the channel is open as it flickers between open and closed states.

Ca$^{2+}$ store pumps and Ca$^{2+}$ chelating proteins
Rossato et al. (2001) used the BODIPY derivative of thapsigargin, a highly potent and specific blocker of SERCAs (Treiman et al. 1998), to probe for the presence of SERCAs in human sperm. Similarly to staining patterns for IP$_3$Rs, localisation of BODIPY thapsigargin was observed over the acrosome and also the midpiece. More recently, Lawson et al. (2007), using antibodies to SERCA type 2, obtained a similar pattern of staining in human, bovine and mouse sperm. SERCA2 was also detected by western blot. Subcellular fractionation showed that binding occurred primarily in the membrane fraction of the cells and also suggested that different splice variants of SERCA2 were present in different subcellular locations. By contrast, using immuno-localisation and western blotting, we were unable to detect SERCAs in human sperm using a pan-SERCA antibody, but did detect SPCA1, another intracellular Ca$^{2+}$ pump. SPCA1 immunostaining was observed only at the sperm neck/midpiece (Harper et al. 2005; Fig. 1d and e). Similar results were obtained with sperm of the sea urchin (Gunaratne & Vacquier 2006; Fig. 1f and g). If SERCAs are present in sperm their role is far from clear since mobilisation of stored Ca$^{2+}$ in sperm by exposure to thapsigargin requires high (non-specific) doses of the drug (Harper et al. 2005; see section Evidence for functional calcium storage in sperm below).

A characteristic of Ca$^{2+}$ stores in somatic cells is the protein calreticulin, which acts as a chelator of Ca$^{2+}$ within the storage organelle. This protein is present in the acrosome of developing rat sperm (Nakamura et al. 1992, 1993) and is present in both the acrosomal and neck regions of human and bovine sperm (Naaby-Hansen et al. 2001, Ho & Suarez 2003).

Evidence for functional calcium storage in sperm
Direct assessment of uptake and release of Ca$^{2+}$ by sperm organelles or organelle membranes has been attempted in only a few studies. Walensky & Snyder (1995) measured accumulation and release of $^{45}$Ca$^{2+}$ in digitonin-permeabilised rat sperm and demonstrated an ATP-dependent accumulation of Ca$^{2+}$ into an intracellular site that was sensitive to thapsigargin (10 µM). Accumulated Ca$^{2+}$ was released (partially) by 10 µM IP$_3$. Spungen & Breitbart (1996) reported that purified acrosomal membranes from bovine sperm possessed a thapsigargin-sensitive Ca$^{2+}$ uptake pump and a cAMP-activated Ca$^{2+}$-release channel. These authors suggested that generation of cAMP (and consequent mobilisation of stored Ca$^{2+}$) could occur upon interaction with the zona pellucida (Breitbart & Spungen 1997, Breitbart 2002).

An alternative approach has been indirectly to assess Ca$^{2+}$ movements attributable to store uptake and release in intact sperm by using fluorescent Ca$^{2+}$ indicators to monitor cytoplasmic [Ca$^{2+}$]. Blackmore (1993) showed that treatment of human sperm with the SERCA inhibitor thapsigargin, to release Ca$^{2+}$ from intracellular stores, caused a sustained increase in [Ca$^{2+}$]i, due to opening of channels at the plasma membrane. No elevation of [Ca$^{2+}$]i was seen when extracellular [Ca$^{2+}$] was buffered with EGTA but upon subsequent addition of Ca$^{2+}$ to the extracellular medium there was a sustained rise in [Ca$^{2+}$]i. Rossato et al. (2001) and Williams & Ford (2003) reported similar observations but in these studies a transient (and much smaller) increase in [Ca$^{2+}$]i was also observed when the drug was applied to cells bathed in Ca$^{2+}$-free saline, confirming that mobilisation of stored Ca$^{2+}$ was indeed occurring. Similar types of response have been observed in sperm of rams (Dragileva et al. 1999), mice (O’Toole et al. 2000) and sea urchins (Gonzalez-Martinez et al. 2004). The simplest interpretation of these observations would be that sperm possess an intracellular store (or stores) of Ca$^{2+}$ that can be mobilised by treatment with thapsigargin. Mobilised Ca$^{2+}$ may sometimes be insufficient to cause a detectable elevation of [Ca$^{2+}$]i, but nevertheless can induce Ca$^{2+}$ influx through store-operated (capaci-
tive) Ca$^{2+}$ channels (see section Store-operated Ca$^{2+}$ channels in sperm below).

The mechanism by which thapsigargin mobilises stored Ca$^{2+}$ in sperm is not clear. Rossato et al. (2001) reported effects of the drug on Ca$^{2+}$ handling by human

sperm at 10–100 nM and Meizel & Turner (1993) observed dose-dependent induction of acrosome reaction at similar doses. These observations are consistent with studies on somatic cells where 50% block of SERCA activity (or 50% maximal Ca\(^{2+}\) store mobilisation) occurs at <100 nM and often <10 nM thapsigargin (Treiman et al. 1998, Wootton & Michelangeli 2006). However, most studies on the effects of thapsigargin on sperm Ca\(^{2+}\) signalling have used micromolar doses (1–20 μM), with negligible effects being observed at doses ≤5 μM (e.g. Dragileva et al. 1999, Williams & Ford 2003, Harper et al. 2005). Cyclopiazonic acid (CPA), another widely used SERCA inhibitor, mobilised Ca\(^{2+}\) in human sperm at high doses (maximal effect at 100 μM; Rossato et al. 2001) but completely fails to mobilise Ca\(^{2+}\) at lower doses (Williams & Ford 2003, Harper et al. 2005) that could be considered both saturating and specific (Wootton & Michelangeli 2006). Thus, though it appears that SERCA (at least SERCA2) is expressed in mammalian sperm (see section Ca\(^{2+}\) stores in sperm), such that mechanisms for Ca\(^{2+}\) mobilisation and accumulation at the two sites within the cell may be discrete (section Separation of store-regulated activities below; Publicover et al. 2007).

Ca\(^{2+}\) stores in sperm. De Blas et al. (2002) used human sperm permeabilised with streptolysin-O and labelled with fluo3 directly to visualise Ca\(^{2+}\) stores. Fluorescence (indicating the presence of Ca\(^{2+}\)-containing organelles) was again observed in the acrosomal region and at the midpiece. Acrosomal fluorescence was significantly reduced when the cells were exposed either to BAPTA-am (a membrane-permeant Ca\(^{2+}\) chelator) or to a combination of Br-A23187 (Ca\(^{2+}\) ionophore) and EGTA, but labelling in the sperm midpiece showed less sensitivity to these treatments. Herrick et al. (2005) were able to observe Ca\(^{2+}\) stores in intact, live mouse sperm by exploiting the ability of manganese to quench fluorescence of the Ca\(^{2+}\) reporter fura-2. Cells were loaded with fura-2 then exposed to manganese, which entered the cytoplasm, thus quenching fluorescence of the dye, but was excluded from intracellular organelles. As in permeabilised cells, fluorescence was localised to the acrosomal and neck/midpiece regions. This pattern of labelling can also be observed when intact (non-permeabilised) mouse or human sperm are loaded with a low-affinity Ca\(^{2+}\) dye. In this case the dye highlights the high Ca\(^{2+}\) concentrations inside the Ca\(^{2+}\) storage organelles but does not fluoresce significantly at the much lower Ca\(^{2+}\) concentration in the cytoplasm (Herrick et al. 2005, Morris J unpublished data; Fig. 2a). Intriguingly, immunolocalisation studies indicate that the ‘toolkits’ of these two stores may differ (section Components of Ca\(^{2+}\) storage organelles expressed in sperm), such that mechanisms for Ca\(^{2+}\) mobilisation and accumulation at the two sites within the cell may be discrete (section Separation of store-regulated activities below; Publicover et al. 2007).

The acrosomal store

There is no dispute that the storage organelle in the acrosomal region of the sperm head is the acrosome (Fig. 1a) itself. Ca\(^{2+}\) release channels (IP\(_3\)Rs) in this region occur primarily (possibly exclusively) in the outer acrosomal membrane (see section Components of Ca\(^{2+}\) storage organelles expressed in sperm). Thus, the acrosomal store regulates Ca\(^{2+}\) concentration in the peri-acrosomal cytoplasm, its pumps and channels being lost during acrosome reaction when compound fusion occurs between the outer acrosomal membrane and the underlying plasmalemma.

Ca\(^{2+}\) storage at the sperm neck/midpiece

The identity of the Ca\(^{2+}\) storage organelle in the neck/midpiece region of the sperm is less clear. Suarez et al. have demonstrated the presence of IP\(_3\)Rs and calreticulin (a Ca\(^{2+}\) binding and storage protein) at the neck region of bovine and hamster sperm, in the region occupied by the redundant nuclear envelope

Location and identity of the Ca\(^{2+}\) storage organelle(s) in sperm

Localisation of the components of intracellular Ca\(^{2+}\) storage organelles (pumps and channels) shows two concentrations of staining in sperm, one over the anterior head and the other over the sperm neck and midpiece (section Components of Ca\(^{2+}\) storage organelles expressed in sperm). Antimonate staining to identify calcium deposits within human sperm showed a similar distribution (Chandler & Battersby 1976). Thus, at least two organelles, in different parts of the cell, serve as
nuclear pore complex proteins (markers for the RNE) only partial overlapped that for IP$_3$Rs (Ho & Suarez 2003). Immunogold labelling of electron microscope sections showed that calreticulin and IP$_3$Rs were associated with membrane cisternae that did not contain nuclear pores and were apparently a separate compartment of the RNE (Ho & Suarez 2001, 2003). No staining was associated with mitochondria. Pharmacological manipulations designed to activated these receptors (e.g. thimerosal) or to inhibit intracellular Ca$^{2+}$ pumps (5–20 μM thapsigargin) mobilised Ca$^{2+}$ in the region of the sperm neck and had functional effects (see below) on motility (Ho & Suarez 2001, 2003). Naaby-Hansen et al. (2001) observed co-localisation of IP$_3$Rs and calreticulin in both the acrosome and neck region of human sperm. Immunogold staining for calreticulin showed that this protein was present in the acrosome (particularly at the equatorial segment) and also in vesicles in the sperm neck (adjacent to the nucleus) and in the cytoplasmic droplet (Fig. 2c). These vesicles were closely apposed to the plasma membrane (Naaby-Hansen et al. 2001).

A further candidate for intracellular storage of Ca$^{2+}$ in the neck/midpiece region of sperm is accumulation and release by mitochondria (see section Mitochondria). Mitochondria of mammalian sperm have been shown to take up Ca$^{2+}$ in situ (Storey & Keyhani 1973, 1974, Babcock et al. 1976, Vijayaraghavan & Hoskins 1990). In mouse sperm the contribution of mitochondrial Ca$^{2+}$ buffering was marginal under resting conditions but became more significant when plasma membrane Ca$^{2+}$ pumps were inhibited, conditions under which resting Ca$^{2+}$ may be elevated (Wennemuth et al. 2003). Occasionally we have observed strong fluorescence, apparently localized to the mitochondria, in human sperm labelled with Ca$^{2+}$-reporting dyes (Fig. 2d and e), suggesting that these organelles were accumulating large amounts of Ca$^{2+}$. This was particularly characteristic of one donor who was known to be fertile and did not appear to be associated with reduced cell viability or function. ‘Conventional’ mitochondrial Ca$^{2+}$ uptake and release does not contribute significantly to the store-mediated Ca$^{2+}$-oscillations that occur in the posterior head and midpiece of human sperm stimulated with low doses of progesterone or with NO. Uncoupling of mitochondrial respiration (with 2,4-dinitrophenol or CCCP) does not inhibit these Ca$^{2+}$ oscillations and can even activate them when the store in this region has been sensitized by NO$\cdot$ (Machado-Oliveira et al. 2008; Fig. 2f). An intriguing possibility is that the sperm mitochondrial inner membrane bears Ca$^{2+}$-ATPases which permit Ca$^{2+}$ accumulation supported by glycolytically generated ATP. SPCA clearly localizes to the giant mitochondrion of sea urchin sperm (Gunnaratne & Vacquier 2006) and staining of human sperm for SPCA often shows both a concentration at the sperm neck and a more extended area of staining throughout the midpiece (Fig. 1d and e). Furthermore, localization of

**Figure 2** Intracellular Ca$^{2+}$ storage sites in mammalian sperm. (a) Pseudo colour image of MagFluo-4 loaded sperm showing warm colours show high [Ca$^{2+}$]. This low-affinity Ca$^{2+}$ indicator highlights the high concentrations of Ca$^{2+}$ in the acrosomal store and at the sperm neck/midpiece (arrows). (b) Electron micrograph showing posterior of the nucleus and the cytoplasmic droplet of a bovine sperm. Large numbers of membranous cisternae (parts of RNE and/or a discrete population of calreticulin containing vesicles – RNE/Crv) can be seen in this region. (c) Electron micrograph showing posterior of the nucleus and the cytoplasmic droplet of a human sperm. This section has been labelled with gold-conjugated anti-calreticulin antibodies. Green arrow shows small calreticulin-containing vesicle in anterior of cytoplasmic droplet. Yellow arrow shows large calreticulin-containing vesicle in the cytoplasmic droplet adjoining the midpiece. (d) Electromicrograph of human sperm labelled with Oregon Green BAPTA 1. The NO$\cdot$ donor spermine NONOate causes protein S-nitrosylation, which sensitises the Ca$^{2+}$ store in the neck/midpiece causing a slow elevation of [Ca$^{2+}$]. (Machado-Oliveira et al. 2008). Upon application of the mitochondrial uncoupler CCCP, to collapse the mitochondrial inner membrane potential, there is a pronounced rise in [Ca$^{2+}$]$_i$ (probably due to release of mitochondrial Ca$^{2+}$) which is then followed in some cells by Ca$^{2+}$ transients and oscillations (green, pink, brown and turquoise traces) in the sperm neck/midpiece. These transients and oscillations reflect cyclical release and re-uptake of Ca$^{2+}$ stored at the sperm neck/midpiece by a mechanism that does not require an intact mitochondrial membrane potential. When dithiothreitol (DTT) is applied, reversing protein S-nitrosylation and removing the sensitising effect of NO on the Ca$^{2+}$ store (Machado-Oliveira et al. 2008), the cells recover despite the continued presence of the mitochondrial uncoupler. Panel b from Ho & Suarez (2003) with permission, panel c from Naaby-Hansen et al. (2001) with permission.
STIM1, a marker of Ca\(^{2+}\) stores (see section Store-operated Ca\(^{2+}\) channels in sperm below), also stains the length of the midpiece. Two distinct ‘stripes’ of staining are often discernible in STIM1 stained cells, consistent with localization to the mitochondria and similar to the pattern of staining seen when mitochondria are Ca\(^{2+}\)-loaded (Fig. 3). However, it should be noted that, in sperm of the sea urchin *Stronglocentrotus purpuratus*, mitochondrial inhibitors and uncouplers cause mobilization of stored Ca\(^{2+}\) followed by sustained Ca\(^{2+}\) influx, apparently due to mobilization of mitochondrial Ca\(^{2+}\) and consequent activation of store-operated Ca\(^{2+}\) channels (Ardón et al. 2009).

**Store-operated Ca\(^{2+}\) channels in sperm**

A common observation in somatic cells is that mobilisation of stored Ca\(^{2+}\) (leading to a fall in [Ca\(^{2+}\)]\(_i\) inside the storage organelle) causes activation of Ca\(^{2+}\) influx though store-operated Ca\(^{2+}\) channels at the plasma membrane. This process is called ‘store-operated’ or ‘capacitative’ Ca\(^{2+}\) entry. It appears that store-operated influx encompasses ‘a family of Ca\(^{2+}\)-permeable channels, with different properties in different cells’ (Parekh & Putney 2005). Evidence in support of the occurrence of store operated Ca\(^{2+}\) influx in mammalian and sea urchin sperm has been reported by a number of laboratories, all of whom observed increased influx of Ca\(^{2+}\) into sperm in response to manoeuvres designed to mobilise stored Ca\(^{2+}\) (Blackmore 1993, Dragileva et al. 1999, O’Toole et al. 2000, Rossato et al. 2001, Hirohashi & Vaccquer 2003, Williams & Ford 2003, Ardón et al. 2009, Espino et al. 2009).

Some members of the canonical transient receptor potential (TRPC) channel superfamily have been suggested as candidate store-operated channels in somatic cells (Birnbaumer et al. 1996, Abramowitz & Birnbaumer 2009). In mouse sperm, several TRPC channels are expressed and have been shown to be localised over the anterior sperm head (Jungnickel et al. 2001, Castellano et al. 2003, Sutton et al. 2004, Stamboulian et al. 2005). Channels incorporating TRPC2 play a role in the zona pellucida-induced Ca\(^{2+}\) influx that leads to acrosome reaction (Jungnickel et al. 2001), though whether activation of these channels is a response to Ca\(^{2+}\) store depletion is still not clear (Florman et al. 2008; see section The acrosomal store). More recently, the proteins of the STIM and ORAI families have been proposed to play key roles in capacitative Ca\(^{2+}\) influx. STIM1 is the putative sensor for detection of Ca\(^{2+}\) store status and ORAI1 is thought to form the Ca\(^{2+}\)-permeable membrane channel (Strange et al. 2007, Wang et al. 2008). These proteins may also combine/interact with TRPCs to form and regulate store-operated channels (Abramowitz & Birnbaumer 2009, Kim et al. 2009). The role(s) of these channels in sperm are far from clear but there is evidence to suggest that they may be important in acrosome reaction in mammalian and echinoderm sperm (O’Toole et al. 2000, Gonzalez-Martinez et al. 2004; see section The acrosomal store). We have examined the expression in human sperm of STIM and ORAI (K Nash & L Lefievre, unpublished data). Both western blotting and immunolocalisation confirm the presence of these proteins and suggest that they are present primarily at the sperm neck and midpiece, though lower levels of expression over the acrosomal region may also occur (Fig. 3).

**Roles of Ca\(^{2+}\) stores in sperm**

Since at least two intracellular Ca\(^{2+}\) stores are present in sperm, in different locations and potentially with different mechanisms of filling and mobilisation (Publicover et al. 2007), it is likely that these organelles have different roles in the regulation of sperm function.

**The acrosomal store**

The acrosomal store is strongly implicated in regulation of exocytosis of the acrosomal vesicle itself (acrosome reaction). Mouse sperm stimulated with zona pellucida glycoprotein ZP3 show a large, transient influx of Ca\(^{2+}\) (Arnoult et al. 1999) that may reflect activation of a T-type voltage-operated Ca\(^{2+}\) channel, though the identity of this channel is not yet established (Florman et al. 2008). In parallel to this Ca\(^{2+}\) influx there is believed to be a G-protein dependent elevation of pH\(_i\) and also activation of PLC leading to generation of IP\(_3\) (Florman et al. 2008). Male mice that are null for PLC\(_{A4}\) show reduced fertility associated with failure of the sperm to undergo acrosome reaction upon binding to the zona pellucida (Fukami et al. 2001). Further investigation of sperm from these animals showed that they were unable to generate a [Ca\(^{2+}\)]\(_i\) signal in response to solubilised zona pellucida, whereas the cells could respond normally to 5 \(\mu\)M thapsigargin.

Figure 3 STIM1 is expressed at the sperm neck and midpiece. (a) Immunolocalisation of STIM1 in human sperm. (b) Shows immunofluorescence of STIM1 overlaid on a phase image of the same cells. The fluorescence appears as two ‘stripes’ (as is seen in Ca\(^{2+}\)-loaded mitochondria; Fig. 2d), with a brighter spot in the region of the RNE and calreticulin-containing vesicles (arrows) anterior to the mitochondria (arrow in c).
(Fukami et al. 2003). De Blas et al. (2002) used streptolysin-O treated (permeabilised) human sperm directly to observe the status of the acrosomal Ca\textsuperscript{2+} store (see section Location and identity of the Ca\textsuperscript{2+} storage organelle(s) in sperm above). Using this approach, they were able to show that mobilisation of acrosomal Ca\textsuperscript{2+} through IP\textsubscript{3}-sensitive channels was required for induction of acrosome reaction by the small GTPase Rab3A. Herrick et al. (2005) used labelling of Ca\textsuperscript{2+} stores in intact mouse sperm (see section Location and identity of the Ca\textsuperscript{2+} storage organelle(s) in sperm above) to show a clear association between mobilisation of acrosomal Ca\textsuperscript{2+} (by 20 \textmu M thapsigargin) and acrosome reaction. In cells bathed in medium containing no added Ca\textsuperscript{2+} and supplemented with 5 mM EGTA thapsigargin was still effective in inducing acrosome reaction. They concluded that mobilisation of the acrosomal store can be sufficient to induce acrosome reaction, such that the acrosome can be viewed as a Ca\textsuperscript{2+}-storage organelle that is capable of regulating its own secretion (Herrick et al. 2005).

After the initial activation of signalling that occurs upon contact with the egg vestments, there is a sustained influx of Ca\textsuperscript{2+} that is apparently required for acrosome reaction, both in mouse and sea urchin sperm (O’Toole et al. 2000, Gonzalez-Martinez et al. 2004). In mouse sperm, the plasma membrane channels incorporating TRPC2 subunits are implicated in this process (Jungnickel et al. 2001), potentially being activated by a store operated mechanism (O’Toole et al. 2000), though other mechanisms of activation are also possible (Florman et al. 2008). A role for store operated Ca\textsuperscript{2+}-influx in induction of acrosome reaction is consistent with observations that treatment of mammalian sperm with thapsigargin (to mobilise stored Ca\textsuperscript{2+}) causes both elevation of [Ca\textsuperscript{2+}]\textsubscript{i} and acrosome reaction (Blackmore 1993, Meizel & Turner 1993, Dragileva et al. 1999, Rossato et al. 2001, Williams & Ford 2003), both these effects being dependent upon influx of extracellular Ca\textsuperscript{2+}. Since store operated Ca\textsuperscript{2+} influx may involve a combination of TRPC and ORAI subunits (section Store-operated Ca\textsuperscript{2+} channels in sperm), ORAI may also participate in this process. Store-operated Ca\textsuperscript{2+} influx is also implicated in acrosome reaction in sea urchin spermatozoa stimulated with egg jelly (Gonzalez-Martinez et al. 2004).

Models for activation of SNARE (membrane fusion) proteins during acrosome reaction typically incorporate a two-stage Ca\textsuperscript{2+} signal, but mobilisation of stored Ca\textsuperscript{2+} is the final ‘trigger’ of acrosome reaction (e.g. Mayorga et al. 2007, Zarelli et al. 2009). Requirement for store-operated Ca\textsuperscript{2+} influx downstream of mobilisation of the acrosomal store is thus not absolutely established. However, it should be noted that in experimental situations mobilisation of stored Ca\textsuperscript{2+} might be exaggerated, generating a [Ca\textsuperscript{2+}]\textsubscript{i} signal that is larger and more effective than that occurring upon zona pellucida binding.

Recently, the model (described above) for activation of sperm Ca\textsuperscript{2+} signalling by zona pellucida has been challenged. Xia & Ren (2009) reported that, in epididymal mouse sperm, the only functional plasma membrane Ca\textsuperscript{2+} channels were formed by CatSpers, a family of sperm-specific, plasma membrane ion channel subunits. CatSpers are localised to the principal piece of the flagellum, where they form weak voltage-sensitive, Ca\textsuperscript{2+}-permeable channels that are activated by elevated pH\textsubscript{i} and mediate hyperactivation (Navarro et al. 2008). Solubilised zona pellucida induced a [Ca\textsuperscript{2+}]\textsubscript{i} elevation in 66% of sperm that initiated (within 20 s of stimulation) at the principal piece and then spread forward, taking almost 3 s to reach the sperm head (Xia & Ren 2009). In 37% of cells, a second (delayed) response occurred a few minutes after stimulation. Zona pellucida could not induce the first elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in any CatSper null sperm, but the delayed response occurred in 18% of these cells. Since zona pellucida receptors are likely to be in the sperm head activation of CatSpers in the principal piece of the flagellum is probably indirect, possibly via zona pellucida-induced elevation of pH\textsubscript{i}. CatsSper null sperm were able to undergo acrosome reaction in response to stimulation with zona pellucida, leading the authors to speculate that it was the delayed phase of the Ca\textsuperscript{2+} signal (possibly Ca\textsuperscript{2+} store generated) that induced acrosome reaction. The spread of elevated [Ca\textsuperscript{2+}]\textsubscript{i} from the principal piece into the head of wild-type cells is probably an active process (Xia & Ren 2009) and may well reflect mobilisation of Ca\textsuperscript{2+} stores by CICR. However, this model cannot easily be reconciled with the key role of voltage operated Ca\textsuperscript{2+} channels in the established model described above, for which there is a considerable body of evidence. Clearly, there is a need for further work in this area.

**Effects of mobilisation of Ca\textsuperscript{2+} stored at the neck/midpiece**

The store located in the region of the sperm neck functions as a regulator of sperm motility. Ho & Suarez showed that manoeuvres designed to mobilise this store (application of thapsigargin or the IP\textsubscript{3}R agonist thimerosal) caused elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in the neck region and hyperactivation in bovine sperm, these effects being independent of [Ca\textsuperscript{2+}]\textsubscript{i} (Ho & Suarez 2001, 2003). Assessment of mitochondrial function and pharmacological manipulation of the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger indicated that the observed effects did not reflect activity of conventional mitochondrial Ca\textsuperscript{2+} uptake and release mechanisms (Ho & Suarez 2003). They went on to show a similar effect of store mobilisation in mouse sperm from both wild type mice and also in a proportion of sperm from mice null for CatSpers (Marquez et al. 2007).
It appears that stored Ca\textsuperscript{2+} in the neck/midpiece region of human sperm acts similarly. In these cells, treatments that induce Ca\textsuperscript{2+} influx can ‘switch on’ cyclical mobilisation of this store (causing cytoplasmic [Ca\textsuperscript{2+}]\textsubscript{i} oscillations) apparently due to a form of CICR (Kirkman-Brown et al. 2004, Bedu-Addo et al. 2005, Harper et al. 2005; section Evidence for functional calcium storage in sperm). In many of the cells that show oscillations an increased excursion of the flagellum, often associated with asymmetrical bending of the midpiece, occurs during the [Ca\textsuperscript{2+}]\textsubscript{i} peaks. Flagellar activity ‘relaxes’ during the intervening troughs (Harper et al. 2004, Bedu-Addo et al. 2005, Machado-Oliveira et al. 2008; Fig. 4). 4-Aminopyridine, an extremely potent inducer of hyperactivation in human sperm (Gunter et al. 2004) causes reversible, repeatable mobilisation of Ca\textsuperscript{2+} stored in the neck/midpiece. In many cells, Ca\textsuperscript{2+} mobilisation is accompanied by (and apparently induces) sustained, asymmetric bending of the proximal flagellum, while, the distal flagellum continues to beat. Upon removal of 4-aminopyridine, [Ca\textsuperscript{2+}]\textsubscript{i} falls and the flagellar bend ‘relaxes’ (Costello S unpublished data). Investigations of the Ca\textsuperscript{2+} dependence of 4-AP-induced hyperactivation clearly show that, as in mouse and bovine sperm, mobilisation of stored Ca\textsuperscript{2+} is sufficient to initiate hyperactivation, but also suggest that store-operated Ca\textsuperscript{2+} influx contributes to maintenance of this mode of motility. Castellano et al. (2003) observed that blockers of store operated channels caused inhibition of motility in human sperm.

**Separation of store-regulated activities**

Ca\textsuperscript{2+} mobilisation from the acrosome and from the store(s) in the sperm neck/midpiece regulate different activities. It is, therefore, important that they can be controlled separately. In mammals acrosome reaction is believed to occur at the surface of the zona pellucida. Sperm-zona pellucida interaction activates signalling cascades leading to acrosomal exocytosis (Florman et al. 2008; Fig. 5). The acrosomal content then disperses slowly (Harper et al. 2008), its content probably aiding penetration of the zona pellucida matrix. Though sperm in the early stages of acrosome reaction may bind to and go on to penetrate the zona pellucida (Buffone et al. 2008), it is likely that those that undergo acrosome reaction prematurely will be severely compromised in their ability to fertilise. It is therefore vital that stimuli that mobilise Ca\textsuperscript{2+} stored in the midpiece/neck region, for regulation of motility, should not ‘accidentally’ activate the acrosomal store. In human sperm stimulated with progesterone, large [Ca\textsuperscript{2+}]\textsubscript{i} oscillations at the sperm neck and consequent regulation of flagellar activity cause no detectable increase in the occurrence of acrosome reaction (Harper et al. 2004). Also, in an elegant study on hamster sperm, Suarez & Dai (1995) observed that [Ca\textsuperscript{2+}]\textsubscript{i} had increased to a greater extent in the midpiece than in the head in hyper-activated sperm, while the reverse was true for acrosome-reacted sperm.

How might this be achieved? The store(s) in the sperm neck/midpiece appears to be mobilised by CICR. In human sperm a minimal level of Ca\textsuperscript{2+} influx at the plasma membrane is required to support cyclical Ca\textsuperscript{2+} mobilisation (Ca\textsuperscript{2+} oscillations) but pharmacological blockade of IP\textsubscript{3}Rs (with 2-APB) or of PLC (with U73122 or neomycin) has no effect. Thus, this activity does not seem to require agonist-stimulated generation of IP\textsubscript{3}. In fact, after stimulation with progesterone to induce [Ca\textsuperscript{2+}]\textsubscript{i} oscillations, many cells continue to oscillate after removal of the agonist, presumably because, in these cells, Ca\textsuperscript{2+} ‘leak’ at the plasma membrane can support CICR once it has been initiated (Harper et al. 2004). The putative expression of RyRs in the sperm neck/midpiece (section Ryanodine receptors) is consistent with Ca\textsuperscript{2+} mobilisation by CICR, but why are IP\textsubscript{3}Rs (section Inositol trisphosphate receptors) also expressed here? First, IP\textsubscript{3}Rs may play a role in CICR. It is known that these receptors can support this process provided that an adequate ‘background’ level of IP\textsubscript{3} is present (Berridge 1993). Such a background level of IP\textsubscript{3} may be present in capacitated sperm, particularly since hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate IP\textsubscript{3} may be activated by elevation of [Ca\textsuperscript{2+}]\textsubscript{i} (Thomas & Meizel 1989). Second, during the burst of IP\textsubscript{3} generation that follows zona pellucida binding and...
mediates emptying of the acrosomal store, the store in the midpiece/sperm neck may be strongly activated through its IP$_3$Rs. In addition, zona binding mat activate CatSpers (Xia & Ren 2009; section The acrosomal store). If either or both these processes occur, arrival of the sperm at the zona pellucida will initiate a combination of acrosome reaction and intense hyperactivation to facilitate penetration of the zona pellucida (Fig. 5). In this context, it is noteworthy that hyperactivation is intensified in acrosome reacted hamster sperm (induced by zona pellucida) and that in these cells [Ca$^{2+}$]$_i$, is increased in the flagellum (Suarez & Dai 1995).

**Outlook**

Only 10 years ago the presence of Ca$^{2+}$ stores in sperm was a matter for debate (Publicover & Barratt 1999). The presence of these stores is now well established and there is little doubt that they enable the cell to generate Ca$^{2+}$-signals that vary in size, ‘shape’ and location within the cell, permitting discrete control of different Ca$^{2+}$-regulated functions. However, there are many aspects of the activation and control of store mobilisation of which we are still ignorant and on which future work should be focussed.

The identity and characteristics of the store located at the sperm neck/midpiece is far from clear. It is likely that Ca$^{2+}$ storage here comprises more than one structure. Furthermore, the nature of the pumps and channels that are functional in this region is disputed. There is evidence for expression and or function of SERCAs, SPCAs, IP$_3$Rs and RyRs in the Ca$^{2+}$ stores of the neck/midpiece (see sections Evidence for functional calcium storage in sperm and Ca$^{2+}$ storage at the sperm neck/midpiece) and it may be that these Ca$^{2+}$ handling ‘tools’ are all expressed in this region of the cell but used in discrete ways to regulate functionally separate Ca$^{2+}$ storage compartments.

Another area of great interest is the question of whether Ca$^{2+}$ stores in sperm are functional in freshly ejaculated cells. Is delay of filling of the store(s) or delay of store ‘priming’ (development of sensitivity to stimulation) a mechanism by which premature activation of Ca$^{2+}$-regulated processes is controlled? It has been suggested recently that Ca$^{2+}$ mobilisation from the RNE might be regulated during capacitation by activity of Src kinase which is localised to this region of human sperm and is activated during capacitation (Varano et al. 2008). Furthermore, residence in the female tract may affect sensitivity of Ca$^{2+}$ mobilisation. For instance, NO$^-$, which is generated by endothelial cells of the oviduct, sensitises Ca$^{2+}$ mobilisation from the store in the neck/midpiece of human sperm (Machado-Oliveira et al. 2008).

Finally, the relationship between mobilisation of stored Ca$^{2+}$, influx of Ca$^{2+}$ at the sperm plasma membrane, hyperactivation and acrosome reaction must be elucidated. CatSper channels are required for normal hyperactivation of mouse sperm. Cells null for these channels cannot hyperactivate and the mice are sterile (Navarro et al. 2008). More recently they have been implicated in acrosome reaction (see section The acrosomal store). Since stored Ca$^{2+}$, at least at the neck/midpiece of human sperm, can be mobilised by CICR, Ca$^{2+}$-influx through CatSpers may recruit stored Ca$^{2+}$, in addition to Ca$^{2+}$ entering through the plasma membrane (Fig. 5). The observations of Xia et al. (Xia et al. 2007, Xia & Ren 2009) that the elevation of [Ca$^{2+}$]$_i$, that occurs upon opening of CatSper channels can propagate to the sperm head is consistent with this.
suggestion. Direct pharmacological mobilisation of stored Ca\textsuperscript{2+} can itself induce hyperactivation in wild type mouse sperm bathed in Ca\textsuperscript{2+}-free medium and also in a proportion of sperm from mice null for CatSpers (Marquez et al. 2007). Thus, store mobilisation alone is apparently sufficient to induce hyperactivation (Fig. 5). An important part of the function of CatSper channels in supporting hyperactivation may be to induce CICR at the sperm neck/midpiece.

Recent findings have revealed unexpected sophistication in the Ca\textsuperscript{2+} signalling capability of sperm (Publicover et al. 2007). It may be that there is considerably more to functioning of the Ca\textsuperscript{2+} store in sperm than we currently know.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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