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Mobilisation of stored calcium in the neck region of human sperm – a mechanism for regulation of flagellar activity

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ABSTRACT Calcium signalling plays a pivotal role in sperm physiology, being intimately involved in the regulation of acrosome reaction, chemotaxis and hyperactivation. Here we describe briefly the mechanisms of calcium regulation in somatic cells and the ways in which these mechanisms have been adapted to function in mature spermatozoa. We then consider recent data from this and other laboratories on the responses of sperm to three compounds: progesterone and nitric oxide (both products of the cumulus oophorus) and 4-aminopyridine. All of these compounds induce calcium signals in the posterior sperm head and neck region and, when applied at appropriate concentrations, modify flagellar activity, causing asymmetric bending of the proximal flagellum. We argue that these effects reflect a common mode of action, mobilisation of calcium stored in the sperm neck region. Finally we consider the nature of calcium signalling pathways in sperm. We suggest that this highly specialised and extremely polarised cell, though working with the same calcium signalling 'tools' as those of somatic cells, employs them to generate unusually 'hard-wired' calcium signals that do not act to integrate stimuli. 'Leakage' between these calcium signalling pathways will generate inappropriate responses, compromising functioning of the cell.

KEY WORDS: *sperm, calcium, nitric oxide, calcium store, hyperactivation*

Introduction

Fluctuations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) mediate rapid transmission of information from extracellular signals to internal response systems in all cells (Berridge, 1997). $[Ca^{2+}]_i$ is, in many ways, ideally suited for encoding of information in cells. Cytosolic concentration is maintained at extremely low levels compared to that in the surrounding extracellular medium, so Ca^{2+} flux into the cytoplasm, from an effectively limitless reservoir, can occur 'instantly' upon gating of Ca^{2+}-permeable channels. Moreover, movement of 'small' numbers of ions can cause a large change in P_{Ca} (Kretsinger, 1979). The use of $[Ca^{2+}]_i$ to encode information in the cytoplasm is, however, dependent upon the ability of the cell to generate signals of sufficient precision and complexity. In cells where $[Ca^{2+}]_i$ is used simultaneously to regulate multiple processes or where failure to localise a Ca^{2+} signal to its site of action may induce inappropriate or even toxic effects (Johnson and Chang, 2000; Berridge, 2006; Petersen *et al.,* 2006), resolution of signals into specific domains of elevated $[Ca^{2+}]$ (microdomains) will be particularly important. Conversely, in many cells $[Ca^{2+}]_i$ itself, and/or components of the Ca^{2+} signalling apparatus (such as membrane Ca^{2+} channels; Evans and Zamponi, 2006), are sites at which signal pathways converge. In this situation $[Ca^{2+}]_i$ signalling acts as an integrative mechanism for diverse inputs (Fig. 1A).

Though understanding of the physiology of male germ cells is (arguably) some way behind that for somatic cells, it is well established that changes in Ca^{2+}-signalling regulate or contribute to the regulation of many aspects of mammalian sperm function (Darszon *et al.,* 2005; Felix, 2005; Jimenez-Gonzalez *et al.,* 2006; Zhang *et al.,* 2006; Publicover *et al.,* 2007). $[Ca^{2+}]_i$ has been shown to increase during capacitation in several mammalian species (Yanagimachi, 1994) including human (Baldi *et al.*, 1991;

Abbreviations used in this paper: Ca^{2+} - calcium ion; EGTA, ethylene glycol tetraacetic acid; IP_3R, inositol tris phosphate receptor; NO, nitric oxide; PMCA, plasma membrane Ca^{2+}-ATPase; RNE, redundant nuclear envelope; RyR, ryanodine receptor; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase; sGC, soluble guanylate cyclase; SOC , store-operated channel; SPCA, secretory pathway Ca^{2+}-ATPase.

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Garcia and Meizel, 1999) and has both negative and positive actions on capacitation and related signalling events (Visconti *et al.*, 2002). Ca^{2+} plays a central role in both acrosome reaction and sperm chemotaxis, with spermatozoa failing to perform either of these crucial functions in the absence of extracellular Ca^{2+} (Yanagimachi, 1994; Eisenbach and Giojalas, 2006). Furthermore, Ca^{2+} apparently switches on hyperactivation (Ho *et al.*, 2002), a swimming pattern characterized by asymmetric flagellar beating and the development of high-amplitude flagellar waves, that is essential for fertilization. Here we briefly review current knowledge of $[Ca^{2+}]_i$ regulation in somatic cells and in sperm, then go on to examine the actions on human sperm of three diverse compounds, all of which modulate flagellar activity, apparently by mobilisation of stored Ca^{2+}. Finally we consider the nature of sperm Ca^{2+}-signalling as compared to that in more complex cells.

Cellular $[Ca^{2+}]$ regulation

$[Ca^{2+}]_i$ in resting cells is strongly buffered (typically $<10^{-7}$ M) whereas $[Ca^{2+}]_o$ and $[Ca^{2+}]$ in intracellular organelles is in the range 10^{-4}-10^{-3} M (Berridge, 2004). Regulation of $[Ca^{2+}]_i$ is achieved by a combination of pumps and channels on both the plasma and intracellular membranes (Fig. 1B). At the plasma membrane Ca^{2+} ATPases and/or Na^+-Ca^{2+} exchangers extrude Ca^{2+} from the cell. Most cells express both Ca^{2+} ATPases and Na^+-Ca^{2+} exchangers, but the relative contribution of the two components of membrane Ca^{2+} extrusion varies widely (Guerini *et al.,* 2005). These pumps can be regulated in various ways including a simple feedback of $[Ca^{2+}]_i$ itself, which stimulates activity of plasma membrane Ca^{2+}-ATPases through the ubiquitous Ca^{2+} binding protein calmodulin (Guerini *et al.,* 2005). Within the cell, the vast majority of Ca^{2+} is either bound to cytosolic proteins or sequestered into membranous organelles. The endoplasmic reticulum is the primary Ca^{2+} storage organelle (the sarcoplasmic reticulum in muscle), Ca^{2+} being pumped into the lumen of the compartment by sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPases. Other organelles also act as Ca^{2+} stores, including the nuclear envelope, lysosomes and the golgi apparatus. Recently it has become apparent that a second type of ATPase, the secretory pathway Ca^{2+}-ATPase contributes significantly to Ca^{2+} accumulation into storage organelles, particularly those derived from the golgi (Wuytack *et al.,* 2003).

A diversity of Ca^{2+} channels are used to generate $[Ca^{2+}]_i$ signals (Fig. 1). At the plasma membrane there are voltage-operated Ca^{2+} channels (VOCCs; Ca^{2+}-permeable channels sensitive to membrane potential), those activated by cyclic nucleotides (CNG channels), channels activated by ligand binding (both direct binding of the ligand to the channel and indirect activation via G-proteins) and channels which open upon mobilization of Ca^{2+} from intracellular stores (capacitative or store-operated Ca^{2+} entry). At intracellular membranes there are at least two types of channels for mobilizing stored Ca^{2+}. Inositol trisphosphate receptors (IP_3Rs) are activated by generation of inositol trisphosphate (IP_3) which occurs at the plasma membrane. Ligand-activation of phospholipase C results in breakdown of inositol 4,5 bisphosphate to IP_3 and diacylglycerol. IP_3 is a small hydrophilic molecule that can diffuse through the cytoplasm to intracellular membranes, where it binds directly to the IP_3R, causing it to open and releasing stored Ca^{2+} (Fig. 1B). Ryanodine receptors (RyRs; so-called because of their sensitivity to the plant alkaloid ryanodine) are sensitive to elevation of Ca^{2+} concentration (both cytoplasmic and in the lumen of the membranous organelle). These receptors appear primarily to 'amplify' or propagate $[Ca^{2+}]_i$ signals generated by Ca^{2+} influx or store mobilization, a process called Ca^{2+}-induced release of Ca^{2+} (Fig. 1B). RyRs in mammalian cells are also regulated by two other soluble molecules, cyclic ADP ribose (cADPR) and nicotinic acid-adenine dinucleotide phosphate (NAADP), both of which are generated in response to ligand binding at the plasma membrane and cause elevation of $[Ca^{2+}]_i$ (Fliegert *et al.,* 2007).

In male germ cells the structural complexity that is characteristic of somatic cells (particularly the network of intra-membranous organelles) is greatly reduced during spermiogenesis. However, studies of Ca^{2+}-regulation in sperm suggest that the 'standard' components and Ca^{2+}-signaling capabilities are retained, though possibly in modified form (Wennemuth *et al.,* 2003; Publicover *et al.,* 2007). Immunohistochemistry and Western blotting experiments show that several types of VOCCs are present (Felix, 2005; Jagannathan *et al.,* 2006) and there is also good evidence for expression of ligand-activated channels (Meizel, 2004) and cyclic nucleotide-regulated channels (Weyand *et al.,* 1994; Gauss *et al.,* 1998; Galindo *et al.,* 2000; Kobori *et al.,* 2000) in mammalian and invertebrate sperm, In addition, a number of studies have shown that capacitative Ca^{2+} influx occurs in sperm (Blackmore, 1993; 1999; Dragileva *et al.,* 1999; O'Toole *et al.,* 2000; Rossato *et al.,* 2001), indicating that store-operated channels are also present. Ca^{2+}-ATPase extrusion pumps in the plasma membrane are probably the greatest contributors to Ca^{2+} buffering in mammalian sperm (Wennemuth *et al.,* 2003) and are potentially involved in regulating $[Ca^{2+}]$ changes during capacitation (Fraser and McDermott, 1992; DasGupta *et al.*, 1994). Plasma membrane Ca^{2+} ATPase4 is expressed at high levels in the flagellum (Fig. 1B) and is very important in sperm motility (Okunade *et al.,* 2004; Schuh *et al.,* 2004). A plasma membrane Ca^{2+} ATPase is also expressed in sea urchin sperm, localized to the sperm head. Pharmacological inhibition of this pump disrupts control of $[Ca^{2+}]_i$ in the sperm and causes loss of flagellar activity (Gunaratne *et al.,* 2006). Na^+-Ca^{2+} exchangers are also present in sperm and contribute significantly to $[Ca^{2+}]_i$ regulation (Wennemuth *et al.,* 2003). It has been hypothesized that the Na^+-Ca^{2+} exchanger in sperm is regulated by caltrin, a low molecular weight protein originating from seminal plasma that binds to spermatozoa and is released during capacitation (Rufo *et al.*, 1984). A Ca^{2+}/H^+ exchanger system has also been implicated in the regulation of intracellular Ca^{2+} (Fraser, 1995).

Intracellular Ca^{2+} storage and stimulus-activated Ca^{2+} mobilization in somatic cells is dependent primarily upon the endoplasmic reticulum. It might therefore be expected that this aspect of Ca^{2+}-signalling would be largely lost in mature sperm. However, studies from many laboratories have demonstrated mobilization of stored Ca^{2+} in sperm, the most convincing evidence being that stimulus-induced Ca^{2+} signals can be observed in sperm incubated in Ca^{2+}-free or very low Ca^{2+} medium (Dragileva *et al.*, 1999; O'Toole *et al.,* Ho and Suarez, 2001; Rossato *et al.*, 2001; Harper *et al.,* 2004; Gunaratne and Vacquier, 2006; Bedu-Ado *et al.,* 2007). Treatment of sperm with pharmacological blockers of sarcoplasmic endoplasmic reticulum Ca^{2+} ATPases (primarily thapsigargin; Thastrup *et al.*, 1990) to mobilize stored Ca^{2+} has

also provided data consistent with the existence of Ca^{2+} stores (Blackmore, 1993; Meizel and Turner, 1993; Dragileva *et al.*, 1999; O'Toole *et al.*, 2000; Rossato *et al.*, 2001; Dorval *et al.*, 2003; Williams and Ford, 2003). However, interpretation of these data is complicated by the fact that most studies have used very high doses of thapsigargin (discussed in Harper *et al.*, 2005) and the existence of sarcoplasmic endoplasmic reticulum Ca^{2+} ATPases in sperm is controversial (Harper *et al.*, 2005; Gunaratne and Vacquier 2006b; Lawson *et al.*, 2007; see above). Though a specific (diagnostic) pharmacological tool is not available, it appears that both human and sea urchin sperm express the non-sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase Ca^{2+} store pump secretory pathway Ca^{2+} ATPase and that this pump plays a significant role in sperm Ca^{2+} storage (Harper *et al.*, 2005;

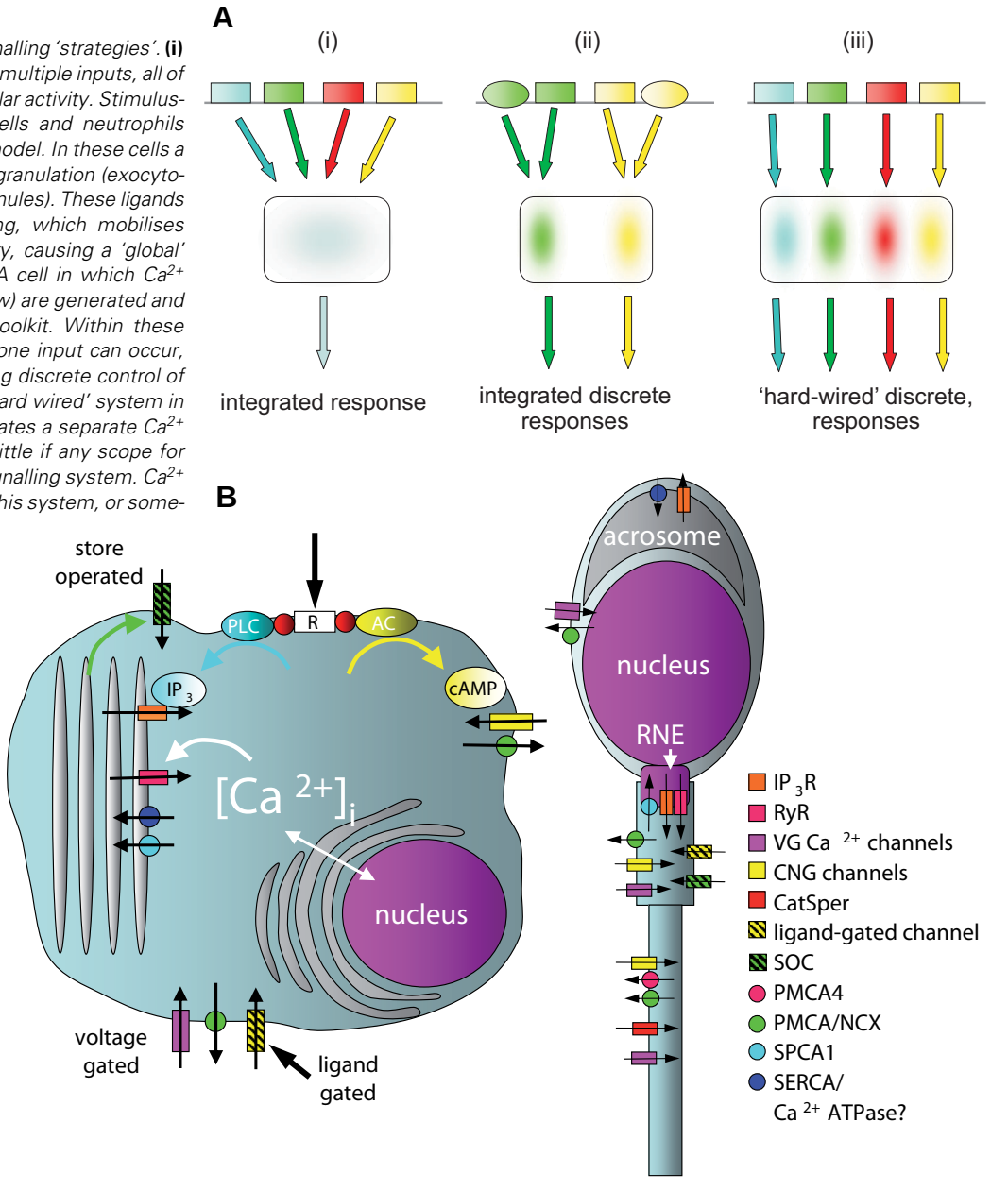

Fig. 1. Ca^{2+} signalling. (A) *Ca^{2+} signalling 'strategies'.* **(i)** *Situation in which $[Ca^{2+}]_i$ integrates multiple inputs, all of which are regulating the same cellular activity. Stimulus-induced Ca^{2+}-signalling in mast cells and neutrophils shows some resemblance to this model. In these cells a range of ligands may cause cell degranulation (exocytosis of a population of exocytotic granules). These ligands act by inducing receptor clustering, which mobilises stored Ca^{2+} and permits Ca^{2+} entry, causing a 'global' $[Ca^{2+}]_i$ signal (Furuno, 2005).* **(ii)** *A cell in which Ca^{2+} domains (shown in green and yellow) are generated and contained by the Ca^{2+}-signalling toolkit. Within these domains integration of more than one input can occur, but domains are separated, allowing discrete control of Ca^{2+} regulated processes.* **(iii)** *A 'hard wired' system in which each type of stimulus generates a separate Ca^{2+} signal in a separate domain, with little if any scope for integration of inputs by the Ca^{2+}-signalling system. Ca^{2+} signalling in sperm appears to use this system, or something close to it (see text).* **(B)** *Simplified summary of $[Ca^{2+}]_i$ signalling 'toolkits' in a somatic cells (left) and a sperm (right). Somatic cell diagram does not include all known or putative components. Sperm signalling components shown are 'best guess' on the basis of available evidence. In the somatic cell $[Ca^{2+}]_i$ is controlled by a range of channels (shown by rectangles) and pumps (shown by circles) in the plasma and intracellular membranes. Pumps and channels for uptake and mobilisation of stored Ca^{2+} are shown on the stack of intracellular membranes on the left. These are situated primarily on the endoplasmic reticulum but are also present on nuclear and Golgi membranes. Agonist binding and trans-membrane voltage regulate Ca^{2+}-permeable channels in the plasma membrane. Agonist-induced generation of IP_3 and cAMP regulate Ca^{2+}-permeable channels in intracellular (blue arrow) and plasma (yellow arrow) membranes respectively. Mobilisation of stored Ca^{2+} activates store-operated channels in the plasma membrane (green arrow). Ca^{2+}-ATPase pumps and Na^+/Ca^{2+} exchangers extrude Ca^{2+} at the plasma membrane (green circles), SERCAs and SPCAs pump Ca^{2+} into intracellular organelles (blue circles). In the sperm the same components are present but many show a clearly restricted distribution. Complexity of intracellular membranes is greatly reduced and the endoplasmic reticulum is lost. Intracellular pumps and channels are present on the acrosome and in the sperm neck region on the redundant nuclear envelope and calreticulin containing vesicles. CatSpers (specific to sperm; red rectangle) are restricted to the principal piece of the sperm. PMCA4 is the primary plasma membrane Ca^{2+} ATPase in mammalian sperm and is restricted to the principal piece of the flagellum (Okunade* et al., *2004; Schuh* et al., *2004). AC, adenyl cyclase; CNG channel, cyclic nucleotide regulated channel; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+}-ATPase; R, ligand receptor; RNE, redundant nuclear envelope; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase; SOC, store-operated channel; SPCA, secretory pathway Ca^{2+}-ATPase.*

Gunaratne and Vacquier 2006).

There is evidence for the presence of at least three discrete intracellular Ca^{2+} stores in mammalian sperm. The acrosome is able to store Ca^{2+} (Fig. 1B), and has IP_3Rs on its outer membrane (Walensky and Snyder, 1995; Kuroda *et al.*, 1999; Rossato *et al.*, 2001; De Blas *et al.,* 2002; Fig. 1B). Mobilisation of Ca^{2+} from this store is necessary for acrosome reaction, the acrosomal store controlling it's own exocytosis (Herrick *et al.,* 2005). A second Ca^{2+} store (or stores) is present in the sperm neck region (Fig. 1B). The identity of the membranous compartment in which Ca^{2+} is stored is not yet clear, but it appears to involve the redundant nuclear envelope (RNE) and/or calreticulin-containing vesicles in the cytoplasmic droplet (Ho and Suarez, 2002; Naaby Hansen *et al.,* 2001; Ho and Suarez, 2003; Harper *et al.*, 2004, 2005). Both IP_3R and RyRs have been localized to this region (Fig. 1B) and the store may therefore respond both directly to IP_3 and also by Ca^{2+}-induced Ca^{2+} release. In addition, as in somatic cells, sperm mitochondria can accumulate Ca^{2+}. Wennemuth *et al.,* (2003) estimated that the contribution of the mitochondrial Ca^{2+} uniporter to the rate of Ca^{2+} clearance was similar to that of Na^{+}-Ca^{2+} exchangers, whereas the plasma membrane Ca^{2+} ATPase removed Ca^{2+} approximately 3 times more rapidly.

Ca^{2+} signals in sperm

The very small size and rapid motility of sperm are such that application of standard cell physiological techniques is technically demanding. Furthermore, studies on the physiological activity and agonist-induced responses of cell populations can mask important events that occur in only a proportion of 'normal' and responsive cells, a particular problem with human sperm (Lefievre *et al.,* 2003). However, technical and experimental innovations have contributed greatly to overcoming these problems such that the last 10 years have seen great progress in our understanding of sperm physiology in general and Ca^{2+} signaling in particular (Publicover *et al.,* 2007). It is now apparent that vertebrate and invertebrate sperm can generate a number of highly sophisticated, spatiotemporal Ca^{2+} signals that selectively can regulate specific activities (acrosome reaction, chemotaxis, hyperactivation). Here we concentrate primarily on the $[Ca^{2+}]_i$ signals that occur in human sperm exposed to three diverse compounds; progesterone, nitric oxide and 4-aminopyridine (a highly effective inducer of hyperactivation in human sperm; Gu *et al.,* 2004) and we consider the ability of the sperm, a cell with an extremely small cytoplasmic volume, to use Ca^{2+} to regulate a number of discrete activities.

Progesterone, Ca^{2+} signalling and regulation of sperm function

Progesterone is present in high (micromolar) concentrations in the follicular fluid (Osman *et al.,* 1989; Thomas and Meizel, 1989) and is synthesized, both before and after ovulation, by the cells of the cumulus oophorus that surround the egg. For human spermatozoa, progesterone is the only biological agonist for which detailed study has been undertaken (Kirkman-Brown *et al.,* 2002). Exposure to progesterone, at nanomolar to micromolar doses, causes an immediate (within seconds) increase in $[Ca^{2+}]_i$ in both capacitated and non-capacitated human sperm (Thomas and

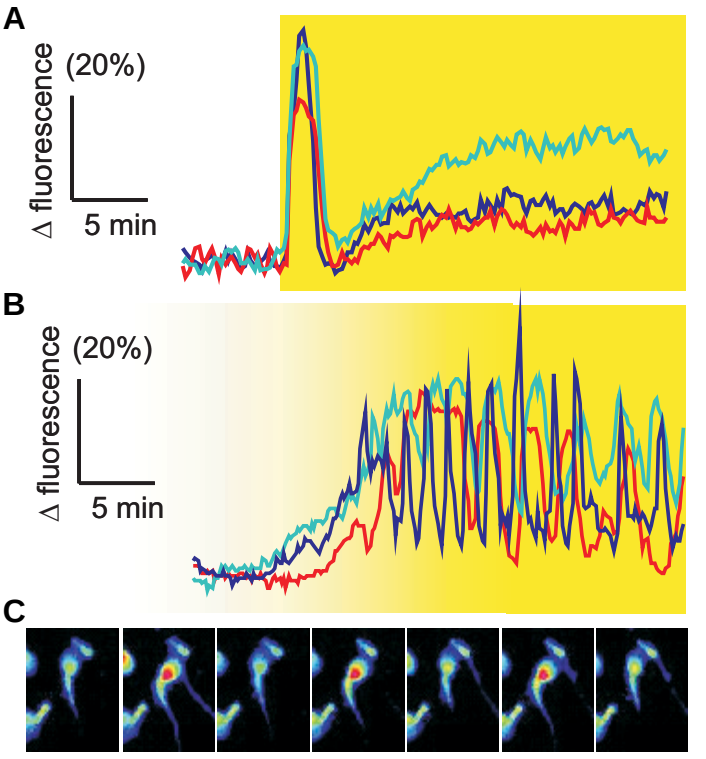

Fig. 2. Ca^{2+} signals evoked by progesterone in immobilised human sperm. (A) *Biphasic elevation of $[Ca^{2+}]_i$ (change in fluorescence of Oregon Green BAPTA 1) in response to application of 3.1 μM progesterone (shown by yellow shading) as a concentration 'step'. Responses of three separate cells are shown.* **(B)** *Response to a progesterone applied as a logarithmic concentration gradient (0-3.1 μM; shown by yellow shading). Cells show a slow rise in $[Ca^{2+}]_i$ (change in fluorescence of Oregon Green BAPTA 1) upon which, in up to 50% of cells, oscillations are superimposed. Responses of three separate cells are shown each of which generated $[Ca^{2+}]_i$ oscillations.* **(C)** *Series of pseudo-coloured images (warm colours show high $[Ca^{2+}]_i$) of a sperm that is generating Ca^{2+} oscillations. The seven images show peaks and troughs for three complete cycles. During each of the $[Ca^{2+}]_i$ peaks there is a marked flexure (a clear change in angle between the midline of the head and proximal flagellum), which is always in the same direction and 'relaxes' during $[Ca^{2+}]_i$ troughs. Images are at approximately 50 s intervals.*

Meizel, 1989; Blackmore *et al.,* 1990, 1991; Baldi *et al.,* 1991; Bedu-Addo *et al.,* 2005). In capacitated cells exposure to progesterone causes a dose-dependent induction of acrosome reaction, apparently dependent upon this Ca^{2+} influx (Yanagimachi, 1994b; Blackmore *et al.,* 1990; Baldi *et al.,* 1991; Harper *et al.,* 2003). The kinetics of acrosome reaction within a population of cells closely follow the kinetics of $[Ca^{2+}]_i$ elevation (Harper *et al.,* 2006). The very short latency of the response to progesterone, and the ability of progesterone to exert a similar effect even when presented as a membrane-impermeant conjugate, indicate a mode of action involving a cell surface receptor for progesterone and not the classical intracellular receptor that regulates gene transcription (Blackmore *et al.,* 1991; Meizel and Turner, 1991). The nature of this receptor for progesterone is still unresolved, but it is noteworthy that exposure to the steroid induces a prolonged, relative or complete refractory period to further stimulation (Aitken *et al.,* 1996; Harper *et al.,* 2003). This suggests that the receptor

activation–signal transduction pathway is subject to a rapid down regulation process that has a profound and prolonged effect on the fertilizing physiology of the sperm (Aitken *et al.,* 1996).

Stimulation of human sperm with micromolar doses of progesterone increases $[Ca^{2+}]_i$ in a biphasic manner (Meizel *et al.,* 1997: Kirkman-Brown *et al.,* 2000: Bedu-Addo *et al.,* 2005: Bedu-Addo *et al.,* 2007; Fig. 2A). An initial, rapid elevation of $[Ca^{2+}]_i$ is observed which initiates in the mid-head region, peaks within 15 s (at 37°C) and then decays over the following 40-60 s (Blackmore *et al.,* 1990: Foresta *et al.,* 1993: Plant *et al.,* 1995: Aitken *et al.,* 1996: Tesarik *et al.,* 1996: Meizel *et al.,* 1997: Harper *et al.,* 2003). The amplitude (but not the kinetics) of the transient $[Ca^{2+}]_i$ response shows strong dose sensitivity over the range of 0.3 nM-3 μM, saturating at ≈ 300 nM (ED_{50} ≈ 30-50 nM) in both sperm populations (Baldi *et al.,* 1991) and at the single cell level (Harper *et al.,* 2003). The frequency of occurrence (% cells responding to progesterone) is largely dose-independent (Harper *et al.,* 2003). This $[Ca^{2+}]_i$ transient is apparently mediated by Ca^{2+} influx since it is greatly inhibited in the presence of La^{3+} (a non selective Ca^{2+} channel blocker), and is abolished when the extracellular medium is supplemented with the Ca^{2+} chelator EGTA (Blackmore *et al.,* 1990: Plant *et al.,* 1995: Aitken *et al.,* 1996: Bedu-Addo *et al.,* 2007). However, recent data suggest that the Ca^{2+} signaling cascade activated by progesterone may be more complex. Though the population response to progesterone is not detectable in EGTA-buffered medium, approximately 5% of cells bathed in this medium for 4-5 min do respond to progesterone with a small $[Ca^{2+}]_i$ transient (Bedu-Addo *et al.,* 2007). More prolonged incubation abolishes this response, suggesting that stored Ca^{2+} is mobilized by progesterone but that incubation in EGTA-containing medium rapidly depletes the progesterone-mobilised store (Bedu-Addo *et al.,* 2007). If EGTA is not used but Ca^{2+} is simply omitted from the saline (nominally Ca^{2+}-free, conditions) $[Ca^{2+}]_o$ is <5 μM (Harper *et al.,* 2004). Under these conditions a response to progesterone is clearly detectable. The $[Ca^{2+}]_i$ transient is smaller, has a longer latency of activation (at least 4 s) and peaks later. We have concluded that the transient response of human spermatozoa to progesterone is a combination of Ca^{2+}-influx and mobilization of a labile, EGTA-sensitive store. (Bedu-Addo *et al.,* 2007).

Perhaps more significantly, it appears that progesterone not only mobilises stored Ca^{2+}, but elicits Ca^{2+} signals in sperm of a complexity comparable with those observed in somatic cells. When sperm are stimulated with a progesterone gradient (to represent more closely the stimulus that a sperm will encounter upon approaching the oocyte) there is no initial transient (as described above) but all cells show a slow, tonic rise in $[Ca^{2+}]_i$ upon which (in up to 50% of cells) Ca^{2+} oscillations in the rear of the sperm head and the midpiece are superimposed (Harper *et al.,* 2004; Fig. 2B). Oscillations can also be induced by application of 3 μM progesterone as a concentration 'step', following the initial $[Ca^{2+}]_i$ transient, but the number of cells in which this signalling pattern occurs is low (10-20%; Harper *et al.,* 2004; Kirkman-Brown *et al.,* 2004; Aitken and McLaughlin, 2007). These $[Ca^{2+}]_i$ oscillations persist when saline in the recording chamber (sEBSS) is replaced with saline with no added Ca^{2+} (nominally Ca^{2+}-free, see above) and their amplitude and definition often increases, indicating that they are primarily dependent on store mobilisation. Intriguingly, when sEBSS is replaced by nominally Ca^{2+}-free saline before 3 μM progesterone is applied, the proportion of cells in which progesterone induces oscillations is markedly increased (≈50%; Bedu-Addo *et al.,* 2007; CV Harper, unpublished data). However, when cells were transiently (4 min) exposed to EGTA-buffered saline, then superfused with nominally Ca^{2+}-free saline prior to stimulation, oscillations occurred in only 1% of cells (Bedu-Addo *et al.,* 2007). Thus it appears that, when presented 'physiologically', progesterone causes a modest Ca^{2+} influx (with no initial transient component) and activates mobilisation of an EGTA-sensitive Ca^{2+} store that, under appropriate circumstances, will cyclically empty and refill, producing a series of $[Ca^{2+}]_i$ oscillations. But what is this store and how is it mobilised?

Characterization of progesterone-induced $[Ca^{2+}]_i$ oscillations in human sperm suggests that they are generated by a form of Ca^{2+}-induced Ca^{2+} release. Oscillations were not affected by inhibition of IP_3 generation or by 2-aminoethoxydiphenyl borate, a membrane-permeant inhibitor of IP_3Rs. In contrast, pharmacological modulators of RyRs, which mediate Ca^{2+}-induced Ca^{2+} release, had marked effects on oscillations. Ryanodine rapidly altered the frequency of oscillations (in a dose-dependent manner) and both ryanodine and caffeine converted irregular Ca^{2+} 'ripples' into an organised series of transients (Harper *et al.,* 2004). Ryanodine and 4-chloro-m-cresol (a potent agonist of RyR 1 and 2; Zorzato *et al.,* 1993; Choisy *et al.,* 1999; Matyash *et al.,* 2002) both elevate $[Ca^{2+}]_i$ in human sperm, inducing oscillations in ≈12% and ≈9% of cells respectively (Machado-Oliveira, unpublished data). This is consistent with our conclusion that tonic, low-level Ca^{2+} influx is required to maintain cyclic filling and emptying of the store (Harper *et al.,* 2004). Using both BODIPY ryanodine (live cells; Harper *et al.,* 2004) and antibodies against RyR 1 and 2 (a gift from Professor FA Lai, University of Cardiff) we have observed staining primarily in the neck region of human sperm. Calreticulin-containing vesicles occur in this region of human sperm (Naaby-Hansen *et al.,* 2001). These findings are in agreement with our observation that $[Ca^{2+}]$ oscillations are primarily localised to this region (Harper *et al.,* 2004) and suggest that it is the sperm neck Ca^{2+} store(s) (see above) that generates these complex Ca^{2+} signals. Studies by Suarez and colleagues have shown the presence of a Ca^{2+} store in this region in bovine sperm, which they identified as the redundant nuclear envelope (RNE; Ho and Suarez, 2001; 2003). Their work showed that this structure bore IP_3Rs (as do the calreticulin-containing vesicles of human sperm) but they did not detect RyRs. Mobilization of this store appears to play a role in regulation of the flagellum in bovine and murine sperm (Ho and Suarez, 2001; 2003; Marquez *et al.,* 2007), being sufficient at least to initiate hyperactivation.

As noted above (in the section on cellular calcium regulation), the presence (and more particularly the function) of sarcoplasmic endoplasmic reticulum Ca^{2+} ATPases in sperm is disputed (Harper *et al.,* 2005), though recent studies do appear to confirm that this ATPase is present (Lawson *et al.,* 2007). However, progesterone-induced $[Ca^{2+}]_i$ oscillations show very little sensitivity to the SERCA-inhibitor thapsigargin (Harper *et al.,* 2004, 2005), indicating that SERCAs do not contribute significantly to filling of the progesterone-mobilised Ca^{2+} store. Recently another intracellular Ca^{2+} store pump, secretory pathway Ca^{2+} ATPase, has been detected in both human and sea urchin sperm. Immunohistochemistry suggests that this ATPase is localised to the posterior head and/or midpiece (Harper *et al.,* 2005; Gunaratne and

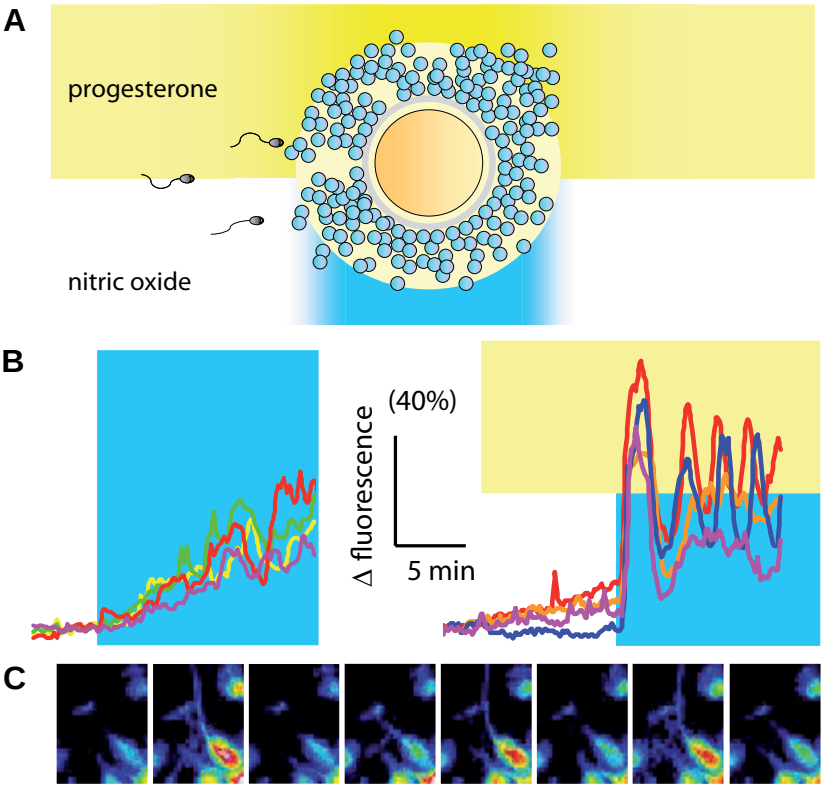

Fig. 3. Ca^{2+} signals evoked by nitric oxide in immobilised human sperm. (A) *Model for probable progesterone (yellow) and nitric oxide (blue) stimulus profiles encountered by the sperm during approach the oocyte. Progesterone shows a clear and probably chemotactic concentration gradient within and around the oocyte-cumulus complex. Nitric oxide is present at significant concentration within the cumulus due to tonic activity of nitric oxide synthase in cumulus cells, but does not form a concentration gradient by diffusion due to its reactivity and consequent short half-life.* **(B)** *Left: slow rise in $[Ca^{2+}]_i$ (change in fluorescence of Oregon Green BAPTA 1) upon exposure to 100 μM spermine NONOate. Responses of four separate cells are shown. Right: Treatment with 100 pM progesterone (yellow shading) causes a small increase in $[Ca^{2+}]_i$ in some cells. In all of these 4 cells (including one [blue trace] which showed no detectable response to 100 pM progesterone) subsequent application of spermine NONOate (blue shading) caused a large transient increase in $[Ca^{2+}]_i$ followed by oscillations. In some cells the response to nitric oxide under these conditions was very different (see text).* **(C)** *Series of pseudo-coloured images (warm colours show high $[Ca^{2+}]_i$) of a sperm stimulated with nitric oxide after pre-treatment with 100 pM progesterone. Image series shows peaks and troughs of the initial $[Ca^{2+}]_i$ transient and the first two Ca^{2+} oscillations. During each of the $[Ca^{2+}]_i$ peaks there is a marked flexure (a clear change in angle between the midline of the head and proximal flagellum), which is always in the same direction and 'relaxes' during $[Ca^{2+}]_i$ troughs. Images are at approximately 50 s intervals.*

Vacquier, 2006) and it appears to participate in refilling of the store responsible for progesterone-induced Ca^{2+} oscillations (Harper *et al.,* 2005).

The ability of progesterone to induce acrosome reaction in mammalian sperm is, in the context of sperm function, surprising. If progesterone induces acrosome reaction *in vivo*, in sperm approaching the cumulus-oocyte complex, or even in cells entering the cumulus, this will reduce, or even negate, their ability to fertilise (Lui *et al.,* 2006). We have argued that induction of acrosome reaction by progesterone may reflect failure of the sperm's $[Ca^{2+}]_i$ homeostatic mechanisms, potentially allowing removal of poor quality cells from the 'race' to fertilise (Harper and Publicover, 2005). It appears that regulation of flagellar activity, modulating motility and/or chemotaxis, may be the primary function of progesterone-induced $[Ca^{2+}]_i$ signalling. Micromolar doses of progesterone directly stimulate hyperactivated motility in human and other mammalian sperm (Uhler *et al.,* 1992; Yang *et al.,* 1994; Jaiswal *et al.,* 1999; Gwathmey *et al.,* 2000). We consider this to be a reflection of Ca^{2+}-induced Ca^{2+} release, from the membranous store(s) in the neck region of the sperm that occurs upon progesterone stimulation. Increased flagellar excursion and flagellar asymmetry occur during the initial progesterone-induced $[Ca^{2+}]_i$-transient both in cells incubated under standard conditions and in cells bathed in medium with no added Ca^{2+} (Bedu-Addo *et al.,* 2007). In contrast, when cells are briefly pre-treated with EGTA before returning them to medium with no added Ca^{2+}, which apparently abolishes progesterone-induced store mobilisation (see above), the effect of progesterone-treatment on flagellar motility is lost (Bedu-Addo *et al.,* 2007). Furthermore, during $[Ca^{2+}]_i$ oscillations, which do not induce acrosome reaction, some cells show bursts of flagellar activity (Harper *et al.,* 2004) and many show flagellar asymmetry (unilateral bending of the proximal flagellum; Fig. 2C) synchronised to the peaks of the Ca^{2+} oscillations. Thus we propose that treatment with progesterone induces phasic mobilisation of the sperm neck store, causing intermittent modulation of flagellar activity. In bovine sperm, tonic mobilisation of Ca^{2+} from what is apparently the equivalent structure, causes hyperactivation (Ho and Suarez, 2001; 2003).

Nitric oxide and sperm Ca^{2+} signalling and interaction with progesterone

Studies in our laboratory indicate that the cells of the cumulus oophorus express nitric oxide synthase. Furthermore, cumulus cells *in vitro* synthesise NO; production greatly exceeding any that may in occurring sperm (WCL Ford and L Lefievre, unpublished data; Machado-Oliveira *et al.,* in prep). Thus as sperm approach or enter the cumulus they probably experience increased concentrations of NO (Fig. 3A). Application of NO to mammalian sperm *in vitro* has functional effects. A number of laboratories have reported that treatment of human sperm with NO donors induces acrosome reaction (Zamir *et al.,* 1995; Herrero *et al.,* 1997; Revelli *et al.,* 2001) and there is also good evidence that exposure to NO has effects on sperm motility. High concentrations of NO, particularly after prolonged incubation, can be toxic to sperm, reducing motility severely (Joo *et al.,* 1999; Wu *et al.,* 2004; 2004b), but exposure to low concentrations of NO donors over short periods is reported to stimulate motility (Herrero *et al.,* 1994; Zhang and Zheng, 1996; Yeoman *et al.,* 1998). Since stimulation of acrosome reaction and hyperactivation may both involve mobilisation of Ca^{2+}, we have investigated whether incu-

bation of human sperm with NO donors causes modulation of $[Ca^{2+}]_i$.

Slow release donors of NO (such as spermine NONOate) cause a sustained rise in $[Ca^{2+}]_i$ in human sperm (Fig. 3B). This effect of NO is modest (far smaller than the response to progesterone) but persistent, and is not modulated by omission of Ca^{2+} from the saline (nominally Ca^{2+}-free). In contrast, the response to treatment with 8-bromo-cGMP, which will activate cyclic-nucleotide regulated membrane Ca^{2+} channels, is reduced by >80% under these conditions. Thus the action of NO on $[Ca^{2+}]_i$ of human sperm reflects primarily the mobilisation of a Ca^{2+} store. Furthermore, use of pharmacological agents and manipulation of Ca^{2+} fluxes (see above) strongly suggests that this action of NO is not due to activation of soluble guanylate cyclase (sGC) but is a direct effect of the S-nitrosylation of sperm proteins (Machado-Oliveira *et al.,* in prep). RyR are modulated both positively and negatively by NO, primarily by S-nitrosylation; Stoyanovsky *et al.,* 1997; Zahradnikova *et al.,* 1997; Xu *et al.,* 1998; Hart and Dulhunty, 2000; Li *et al.,* 2000; Heunks *et al.,* 2001; Zima and Blatter, 2006) and we have shown that RyR 2 is a target for S-nitrosylation in human sperm (Lefievre *et al.,* 2007). Since the action of progesterone to mobilise stored Ca^{2+} in human sperm involves RyR or RyR-like proteins, we have investigated possible interaction between these agonists. In initial experiments where progesterone (3.2 µM) was applied to sperm pre-treated with spermine NONOate, the $[Ca^{2+}]_i$ transient response (see Fig. 2A) was particularly large in many cells and was often prolonged. Even more intriguing is our observation that pre-treatment with progesterone at very low concentration (100 pm – 1 nM) before application of a nitric oxide donor (a sequence of events that is more likely to reflect the stimuli encountered *in vivo*; Fig. 3 A,B) can cause dramatic modification of the response to NO (Machado-Oliveira *et al.,* unpublished data). 100 pM progesterone induces a small rise in $[Ca^{2+}]_i$ in some cells, but in many there is no discernible effect. However, the response to subsequent application of the NO donor spermine NONOate is, in many cells, transformed. NO, instead of causing a tonic elevation of $[Ca^{2+}]_i$, induces an immediate, large, Ca^{2+} transient which is often followed by Ca^{2+} oscillations (Fig. 3B). Since progesterone-induced, phasic mobilisation of Ca^{2+} stored in the sperm neck region causes phasic modulation of flagellar beat (see above), we investigated flagellar activity in sperm that show a $[Ca^{2+}]_i$ transient upon exposure to NO. In poorly immobilised cells bending of the proximal flagellum, leading to flagellar asymmetry, is clearly associated with the NO-induced $[Ca^{2+}]_i$ transient and subsequent oscillations (Fig. 3C). We observed no effect on acrosomal status. Intriguingly, we have found that the effect of this stimulus protocol is bi-modal. Exactly the same treatment can induce a transient reduction in $[Ca^{2+}]_i$ upon application of NO. Both types of response can occur in cells from the same semen sample, but we do not observe extreme versions of both response patterns in the same experiment. The basis for the remarkable variability of response in cells exposed to this protocol is far from clear, but it may well be relevant that, though S-nitrosylation can potentiate opening of RyR (Stoyanovsky *et al.,* 1997; Xu *et al.,* 1998), inhibition of these Ca^{2+} channels can occur under strongly nitrosylating conditions or at high doses of NO (Zahradnikova *et al.,* 1997; Hart and Dulhunty, 2000; Zima and Blatter, 2006). Mobilisation of stored Ca^{2+} by NO may thus have a bell-shaped dose response curve similar to that for the action of IP_3 on IP_3Rs.

We propose that in their effects both on mobilisation of stored Ca^{2+} and on regulation of flagellar activity, progesterone and NO converge at a key site, resulting in complex interaction. Since progesterone and NO are both products of the cumulus oophorous (Machado-Oliveira *et al.,* in prep), the strong synergistic effect seen in some cells (Fig. 3B) is potentially of considerable significance *in vivo*. The site of this agonist interaction is yet to be established, but as described above, the RyR must be a prime candidate. At least two mechanisms might contribute to the synergistic interaction of progesterone and NO (Fig. 4): i) Low dose progesterone may provide a tonic, 'background' influx of Ca^{2+} that allows the store to fill up, such that a an immediate, significant release of Ca^{2+} occurs upon exposure to NO. NO itself might enhance this effect since generation of cGMP may lead to the activation of Ca^{2+} permeable channels in

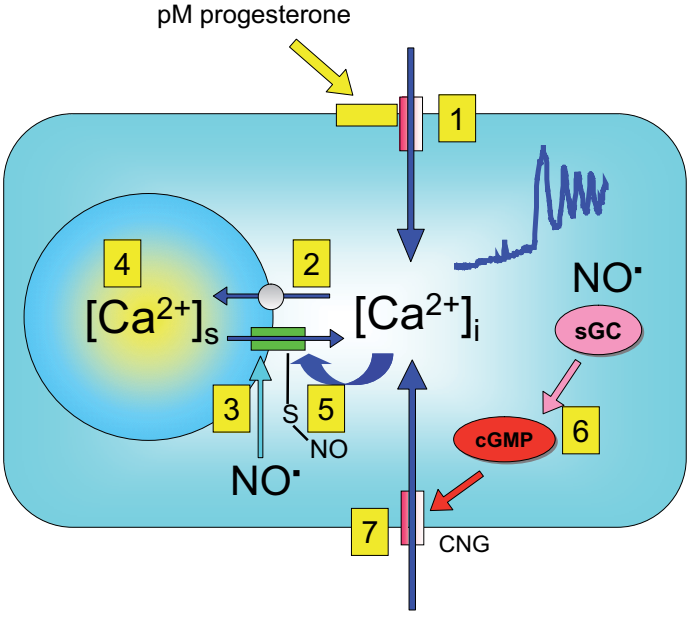

Fig. 4. Tentative model for synergistic interaction of pM concentrations of progesterone and nitric oxide in mobilising Ca^{2+} from the intracellular store in the neck region of human sperm. *Gating of Ca^{2+} permeable channels by progesterone causes a modest increase in Ca^{2+} 'leak' into the sperm* **(1)**, *leading to elevation of resting $[Ca^{2+}]_i$ and accumulation of Ca^{2+} into the store ($[Ca^{2+}]_s$)* **(2)**. *Application of nitric oxide ($NO^{\bullet}$) results in nitrosylation of ryanodine receptors (green rectangle) in the membrane of the sperm neck store* **(3)**. *Depending on conditions in the cell prior to nitric oxide exposure, this may increase or reduce open probability of ryanodine receptors (see text). Where nitrosylation increases open probability of ryanodine receptors, Ca^{2+}-mobilisation is enhanced by progesterone pre-treatment because the store has now been filled with Ca^{2+}* **(4)** *and/or because the effect of nitrosylation on ryanodine receptor open probability is enhanced due to elevated cytoplasmic ($[Ca^{2+}]_i$)* **(5)**. *In the absence of progesterone pre-treatment, stimulation of soluble guanylate cyclase (sGC) by nitric oxide, and consequent activation (directly or indirectly) of Ca^{2+} permeable channels in the plasmalemma (CNG; 7) may be necessary to support mobilisation of stored Ca^{2+}.*

the sperm plasmalemma; ii) Exposure to pM concentrations of progesterone clearly increases resting $[Ca^{2+}]_i$ in some cells and it is likely that in others the rise is too small or too localised to be detected. Such an increase may be insufficient to induce Ca^{2+}-induced Ca^{2+} release prior to NO exposure, but might become so after the release mechanism is sensitised by S-nitrosylation (Aracena *et al.,* 2003).

4-aminopyridine, Ca^{2+} mobilisation and hyperactivation

When 4-aminopyridine (2 mM) is applied to a population of human sperm the percentage of hyperactivated cells, as assessed by computer-assisted semen analysis (CASA) increases 'immediately'. The proportion of cells assessed as Sort 7 (hyperactivated) increases from 3-4% to 40-50% (Fig. 5A) and shows little decay over a period of 40 min (Gu *et al.,* 2004; S Costello unpublished data). This effect is similar in amplitude whether cells are prepared by incubation in capacitating medium (supplemented EBSS containing 25 mM bicarbonate, 0.3% BSA) for 5-6 h or for just 1-1.5 h, a point at which changes associated with capacitation are not complete (Bedu-Addo *et al.,* 2005). Thus 4-aminopyridine is one of the most potent inducers of human sperm hyperactivation yet described. The signalling mechanisms that govern hyperactivation are not yet clear, but an increase in intracellular Ca^{2+} in the principal piece and/or mid-piece of the flagellum is apparently key to initiation and maintenance of hyperactivation (Suarez and Dai, 1995; Carlsen *et al.,* 2003; Quill *et al.,* 2003; Marquez *et al.,* 2007). We therefore investigated whether exposure of human sperm to 4-aminopyridine, at a concentration sufficient to induce hyperactivation, caused an elevation of $[Ca^{2+}]_i$.

Application of 2 mM 4-aminopyridine caused a dose dependent, tonic increase in $[Ca^{2+}]_i$ in human sperm. This was clearly visible in fluorimetric records from populations and in imaging experiments a similar sustained, reversible rise in $[Ca^{2+}]_i$ occurred in the neck-midpiece region associated with asymmetric bending of the proximal flagellum (Fig. 5B). 4-aminopyridine is widely used as a K^+ channel blocker (Alexander *et al.,* 2006), but this effect of the drug was apparently independent of any actions on K^+ fluxes (and consequently on voltage-regulated Ca^{2+}-permeable channels) since similar responses occurred when Em was 'clamped' by pre-incubating cells with the K^+-ionophore valinomycin. A possible alternative mechanism of action of 4-aminopyridine on $[Ca^{2+}]_i$ is by mobilisation of stored Ca^{2+}, an effect that has been observed in a number of mammalian cell types (Ishida and Honda, 1993; Cabado *et al.,* 2000; Grimaldi *et al.,* 2001). Furthermore, the amplitude of the response to 4-aminopyridine was not altered when cells were super-fused with 4-aminopyridine in 'nominally Ca^{2+}-free' medium. However, when cells in 'nominally Ca^{2+}-free' medium were pre-treated with bis-phenol, an inhibitor of both sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase and the secretory pathway Ca^{2+} ATPase intracellular Ca^{2+} pumps (Brown *et al.,* 1994; Harper *et al.,* 2005), the response to 4-aminopyridine was greatly reduced. Exposure of human sperm to EGTA-buffered, 'Ca^{2+} free' medium rapidly depletes intracellular Ca^{2+} stores (Harper *et al.,* 2004; Bedu-Addo *et al.,* 2007). Cells incubated in EGTA-buffered saline for 10 -15 min showed no response to 4-aminopyridine, but application of the drug 4-5 min after exposure to EGTA induced a clear response in almost 25% of cells. Under these circumstances the rise in $[Ca^{2+}]_i$ was transient and was of diminished amplitude. Thus it appears that at least part of the elevation in $[Ca^{2+}]_i$ that occurs in human sperm exposed to 4-aminopyridine reflects the mobilisation of stored Ca^{2+}, though the prolonged effect is probably dependent upon Ca^{2+}-influx (see below).

We used CASA to examine whether mobilisation of stored Ca^{2+} might contribute to the strong hyperactivating action of 4-aminopyridine on human sperm. The ability of 4-aminopyridine dramatically to increase the proportion of hyperactivated cells was not inhibited by simple omission of Ca^{2+} from the medium, the ability of the drug to induce hyperactivation being, if anything, enhanced. Furthermore, following brief re-suspension in EGTA buffered saline, application of 2 mM 4-aminopyridine induced hyperactivation in a proportion of cells only slightly lower than that in controls (standard medium; Fig. 5A). However, after incubation in EGTA-buffered medium for 20 min the proportion of motile cells was decreased (by one third) and the hyperactivating effect of 4-aminopyridine was significantly reduced (though not abolished; Fig. 5A). It appears that mobilisation of Ca^{2+} from a labile, EGTA-sensitive store, contributes to the ability of 4-aminopyridine to induce hyperactivation. These findings are consistent with previous reports that extra-cellular calcium is not a requirement for initiation of hyperactivation in bovine and murine sperm. Suarez and colleagues concluded that mobilisation of stored Ca^{2+} (possibly from the redundant nuclear envelope) is able to initiate but cannot sustain hyper-activated motility (Ho and Suarez 2001; Marquez *et al.,* 2007). Significantly, treatment of human sperm with 4-aminopyridine did not increase the occurrence of acrosome reaction in cells incubated under capacitating conditions.

Though 4-aminopyridine may initiate hyperactivation by mobilising stored Ca^{2+}, the ability to cause prolonged elevation of $[Ca^{2+}]_i$ (and maintained hyperactivation) must require activation of Ca^{2+} permeable channels in the plasma membrane. In many cells store depletion triggers Ca^{2+} influx via capacitative Ca^{2+} influx (store operated calcium channels), which act to maintain a prolonged Ca^{2+} signal and to refill the mobilised store (Fig. 1B). Thus, depletion of stored calcium in sperm, probably from stores in the neck region of the cell (Ho and Suarez, 2001; Naaby-Hansen *et al.,* 2001; Harper *et al.,* 2004) could activate local store-operated Ca^{2+}-channels in the flagellum to promote and prolong hyperactivation. 4-aminopyridine may potentiate activity of such channels (Grimaldi *et al.,* 2001). However, it has been demonstrated recently that 4-aminopyridine greatly enhances activity of the pH-sensitive sperm K^+ current I_{KSper} in mouse sperm principal piece by cytoplasmic alkalinisation (Navarro *et al.,* 2007). Since the sperm-specific, pH-sensitive Ca^{2+}-permeable CatSper is similarly localised (Ren *et al.,* 2001; Kirichok *et al.,* 2006) and is also pH sensitive (Kirichok *et al.,* 2006), an alternative explanation is that 4-aminopyridine induces Ca^{2+}-influx in the sperm tail (and sustained hyperactivation) by activation of $I_{CatSper}$. To investigate this possibility, we super-fused sperm with saline adjusted to pH 8.5. In simple medium human sperm are 'pH-compliant', pH_i being approximately 0.4 units less than the external pH over a range from 7.2 to 8.2 (Hamamah *et al.,* 1996). Elevation of pH_o from 7.5 to 8.5 might thus be expected to raise pH_i by as much as one unit. As expected, upon super-fusion with medium at pH 8.5 (replacing standard medium at pH 7.4) we observed a sustained increase in $[Ca^{2+}]_i$, probably reflecting activation of CatSper channels. Surprisingly, this pre-treatment did not occlude the response to

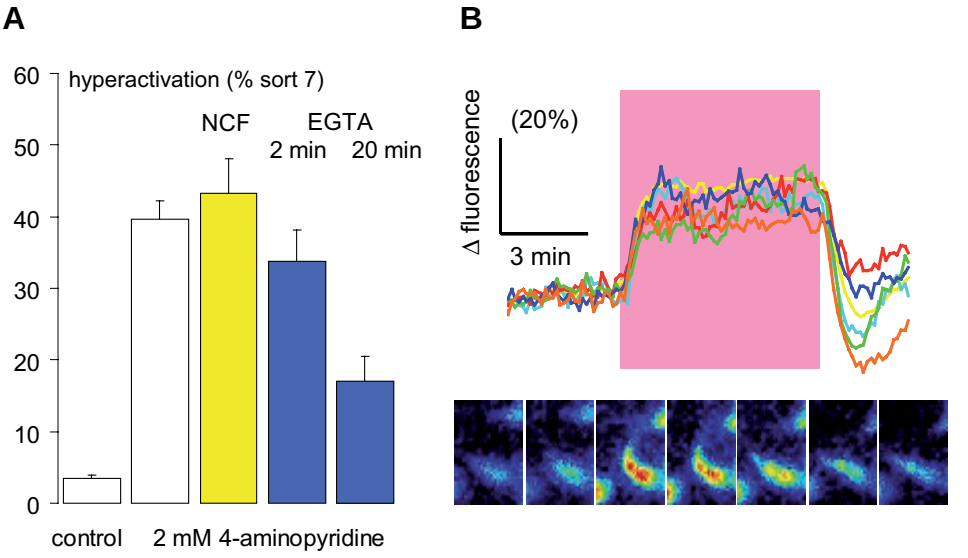

Fig. 5. Hyperactivation and Ca^{2+} signals in 4-aminopyridine-treated in human sperm. **(A)** *Stimulation of hyperactivation by 2 mM 4-aminopyridine. The percentage of hyperactivated cells (sort 7) approximately 1-2 min after application of 4-aminopyridine is increased more than 10-fold compared to that in untreated cells (white bars). Omission of Ca^{2+} from the medium does not inhibit this effect (yellow bar). When cells are suspended in EGTA-buffered medium prior to treatment (blue bars) the ability of 4-aminopyridine to stimulate hyperactivation is gradually lost, the response being <50% of that in controls after 15-20 min.* **(B)** *Upper panel shows 'typical' effect of 4-aminopyridine on $[Ca^{2+}]_i$ (change in fluorescence of Oregon Green BAPTA 1) in human sperm. Upon superfusion with 2 mM 4-aminopyridine (pink shading) fluorescence rises by 20-30% within 1-2 min and stabilises. Upon washout of 4-aminopyridine $[Ca^{2+}]_i$ immediately falls to levels at or near those observed before treatment, sometimes with an 'undershoot' of 2-3 min duration. Responses of 6 separate cells are shown. Lower panel shows a series of pseudo-coloured images (warm colours show high $[Ca^{2+}]_i$) of a cell treated with 2 mM 4-aminopyridine. First two images are prior to treatment, images 3 and 4 are during 4-aminopyrine super-fusion and images 5-7 are during and after washout. Note that there is a marked flexure (a clear change in angle between the midline of the head and proximal flagellum) during 4-aminopyridine exposure that 'relaxes' immediately upon washout of the drug.*

subsequent application of 4-aminopyridine. These results suggest that the 4-aminopyridine induced Ca^{2+}-influx may not be solely pH/CatSper dependent and that modulation of capacitative Ca^{2+} influx may play a role in this effect. We tentatively conclude that hyperactivation of human sperm that occurs upon exposure to 4-aminopyridine may, in a number of ways, parallel the induction of hyperactivation in bovine sperm induced by manoeuvres designed to mobilise stored Ca^{2+} (Ho and Suarez, 2001; 2003)

The Ca^{2+} signal repertoire of sperm – an unusually hard-wired cell?

In this review we have concentrated on our current focus of interest, the mobilisation of stored Ca^{2+} in the neck region of the sperm and its putative contribution to the regulation of motility and hyperactivation. But this is only a small part of a much more complex pattern of $[Ca^{2+}]_i$ signalling in sperm (Publicover *et al.,* 2007). Studies of the zona pellucida-induced acrosome reaction show a central and complex role for Ca^{2+} signalling, but also clearly show that the pathway and Ca^{2+}-signalling components involved are different to those described above (Florman *et al.,* 1998; O'Toole *et al.,* 2000; Tomes, 2007). More recently, work on responses of sperm to chemotactic cues has shed new light on the Ca^{2+}-mediated signalling events that underlie chemotaxis (Eisenbach and Giojalas, 2006; Kaupp *et al.,* 2006; Spehr *et al.,* 2006). Perhaps not surprisingly, hyperactivation, acrosome reaction and chemotactic responses each involve a $[Ca^{2+}]_i$ signal with discrete spatio-temporal characteristics. Should we be surprised that sperm can do this?

The mature sperm cell is clearly 'simplified', lacking much of the intracellular structure and organisation that is present in undifferentiated germ cells. However, not only do sperm retain the molecular components of the somatic cell Ca^{2+} signalling toolkit, but they even introduce new ones of their own (CatSpers; see contribution by D Clapham). Furthermore, the extreme level of structural and functional polarisation that is seen in a mature sperm 'pre-adapts' the cell for flexible use of a diffusible messenger, despite its small size. It appears that (at least for rapidly acting stimulus-response pathways) sperm use a small number of parallel but separate Ca^{2+} signalling pathways. On the basis of the available data we would suggest that agonists of acrosome reaction, hyperactivation and chemotaxis each mobilise a different source of Ca^{2+}, in different places and with different $[Ca^{2+}]_i$ kinetics. In the case of stored Ca^{2+} the mechanism of store mobilisation may even differ between the acrosomal and neck stores. Progesterone and NO apparently converge in their effects on the Ca^{2+} store at the sperm neck, but whether each of the Ca^{2+} signalling pathways of sperm integrate multiple inputs is not yet clear. Chemotaxis is particularly interesting in this respect. Chemotaxis in human sperm can apparently be induced by several agonists (Eisenbach and Giojalas, 2006) and therefore via several receptors, yet convergence of input from more than one chemical gradient would potentially 'confuse' the orientation of the cell. One possible answer is that transduction pathways of chemo-attractants do converge, but activation of the receptors occurs sequentially, as the sperm ascends the female tract, rather than simultaneously (Eisenbach and Giojalas, 2006). Thus Ca^{2+}-signalling in sperm, though similar in most ways to that in somatic cells, may be more 'hard-wired' than in most cells, having direct and 'simple' connections between inputs and outputs of Ca^{2+}-signalling pathways in order to minimise potentially dangerous cross-talk in a very small cell (Fig. 1A (iii)). Inevitably some spatial overlap of signals occurs, but each signal has its own temporal and spatial characteristics which may be sufficiently selective to activate a specific target (Johnson and Chang, 2000). In human sperm populations, where many cells are (according to standard semen analysis) not normal, inappropri-

ate 'leakage' between Ca^{2+} signal pathways may not be an uncommon event and, in extreme cases, will cause idiopathic male subfertility. Premature induction of acrosome reaction by high doses of progesterone, which would render the cell unable to fertilise (Harper and Publicover, 2005) is an example of such signal 'leakage'.

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