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Cell penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced [Ca$^{2+}$]$_i$ signal in human sperm

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Abstract.

Previous work has provided evidence for involvement of store operated channels (SOCs) in \([\text{Ca}^{2+}]_i\) signalling of human sperm, including a contribution to the transient \([\text{Ca}^{2+}]_i\) elevation that occurs upon activation of CatSper, a sperm-specific cation channel localised to the flagellum, by progesterone. To further investigate the potential involvement of SOCs in the generation of \([\text{Ca}^{2+}]_i\) signals in human sperm we have used cell-penetrating peptides containing the important basic sequence KIKKK, part of the STIM-Orai activating region/CRAC activation domain (SOAR/CAD) of the regulatory protein stromal interaction molecule 1 (STIM1). SOAR/CAD plays a key role in controlling the opening of SOCs, which occurs upon mobilisation of stored \(\text{Ca}^{2+}\). Resting \([\text{Ca}^{2+}]_i\) temporarily decreased upon application of KIKKK peptide (3-4 min) but scrambled KIKKK peptide had a similar effect, indicating that this action was not sequence specific. However, in cells pre-treated with KIKKK the transient \([\text{Ca}^{2+}]_i\) elevation induced by stimulation with progesterone decayed significantly more slowly than in parallel controls and in cells pre-treated with scrambled KIKKK peptide. Examination of single cell responses showed that this effect was due, at least in part, to an increase in the proportion of cells in which the initial transient was maintained for an extended period, lasting up to 10 minutes in a sub-population of cells. We hypothesise that SOCs contribute to the progesterone-induced \([\text{Ca}^{2+}]_i\) transient and that interference with the regulatory mechanisms of SOC delays their closure, causing a prolongation of the \([\text{Ca}^{2+}]_i\) transient.

**Key words:** spermatozoa, cell penetrating peptide, stromal interaction molecule 1, store-operated channels
Introduction

$[\text{Ca}^{2+}]_i$ plays a central role in the control of sperm function. Maturation of the ejaculated sperm to acquire competence to fertilise (capacitation), the regulation of motility pattern (behaviour and chemotaxis) and control of the secretion of the acrosomal vesicle (acrosome reaction), which releases hydrolytic enzymes and exposes proteins required for fertilisation at the sperm surface, are all controlled separately through $[\text{Ca}^{2+}]_i$ signals. $\text{Ca}^{2+}$ channels and $\text{Ca}^{2+}$ stores are present in mammalian sperm (Costello et al., 2009, Darzon et al., 2011, Publicover et al., 2007) and both play important roles in sperm function. CatSper, a sperm-specific cation channel localised to the flagellum, is the primary $\text{Ca}^{2+}$-influx channel in mammalian sperm, is central to the regulation of $[\text{Ca}^{2+}]_i$, and experiments in CatSper-null mice have shown that the channels play a key role in both regulation of motility and the early phase of zona pellucida-induced acrosome $[\text{Ca}^{2+}]_i$ signalling (Carlson et al., 2003, Xia and Ren, 2009). Intriguingly, ‘late’ $[\text{Ca}^{2+}]_i$, responses persisted in the mutant mice and the zona-pellucida-induced acrosome reaction was not inhibited (Xia and Ren, 2009). $\text{Ca}^{2+}$ stores are present both within the acrosome and at the sperm neck region (Costello et al., 2009). Release of $\text{Ca}^{2+}$ stored in the acrosome is required for completion of the acrosome reaction and the $\text{Ca}^{2+}$ stored at the sperm neck plays a key role in regulation of motility (Alasmari et al., 2013, Costello et al., 2009).

The characteristics of $[\text{Ca}^{2+}]_i$ signals generated by store mobilisation in human sperm are consistent with the occurrence of capacitative $\text{Ca}^{2+}$ entry (CCE) mediated by store-operated $\text{Ca}^{2+}$ channels (SOCs) (Blackmore, 1993, Lefievre et al., 2012, Park et al., 2011). SOCs are activated by interaction with STIM (stromal interaction molecule), a sensor molecule present in the membrane of the $\text{Ca}^{2+}$ store that detects depletion of stored $\text{Ca}^{2+}$. Upon store mobilization, STIM redistributes, moving to a position adjacent to the plasma membrane and forming ‘puncta’, where it interacts with the channel proteins (Orai and possibly members of the TRPC [transient receptor potential canonical] family), causing SOCs to open (Cahalan, 2009). STIM1, Orai proteins and TRPC proteins have all been
detected in human sperm (Castellano et al., 2003, Darszon et al., 2012, Lefievre et al., 2012). STIM1 is localised primarily to the neck region/midpiece and the acrosome, the areas where Ca\(^{2+}\) stores are present (Lefievre et al., 2012).

Progesterone, a product of the cumulus cells which surround the oocyte, is the best characterised agonist of Ca\(^{2+}\) signalling in human sperm. Progesterone directly activates CatSper channels, causing an immediate and transient Ca\(^{2+}\) elevation which peaks within 30 s and decays within 1-2 min (Blackmore et al., 1990, Lishko et al., 2011, Strunker et al., 2011). This is followed by a prolonged [Ca\(^{2+}\)]\(_i\) plateau. In a sub-population of cells this plateau phase includes repeated [Ca\(^{2+}\)]\(_i\) elevations (oscillations) (Aitken and McLaughlin, 2007, Harper et al., 2004, Kirkman-Brown et al., 2004, Sanchez-Cardenas et al., 2014) which are typically irregular/chaotic but are ‘organised’ by ryanodine and by caffeine, consistent with Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release (CICR) from the store at the sperm neck (Harper et al., 2004). These oscillations modify flagellar beat (Harper et al., 2004) and may suppress the acrosome reaction (Sanchez-Cardenas et al., 2014). We recently showed that a low concentration of 2-aminoethoxydiphenyl borate (2-APB; 5 \(\mu\)M), which potentiates SOC activity by promoting interaction with STIM, enhanced the progesterone transient at the sperm neck (but not the flagellum) (Lefievre et al., 2012). The [Ca\(^{2+}\)]\(_i\) plateau phase was not enhanced by 2-APB pre-treatment but application of 2-APB during the plateau caused generation of irregular [Ca\(^{2+}\)]\(_i\) noise and spikes. Patch clamp experiments showed that 2-APB inhibited currents through CatSper channels. These observation suggest that store emptying by CICR and consequent activation of SOCs amplify the initial CatSper-mediated [Ca\(^{2+}\)]\(_i\) transient and generate [Ca\(^{2+}\)]\(_i\) oscillations.

Use of cell penetrating peptides (CPPs) to traffic targeted peptides and small proteins across the plasma membrane offers an alternative potentially more specific technique than pharmacological intervention. Rab3A and GST, both ~25kDa, are delivered into sperm when fused to a polyarginine CPP (RRRQRRKRRRQ) at the C terminus (Lopez et al., 2007) and the translocation of exogenous Rab3A is sufficient to trigger acrosomal exocytosis. We recently investigated the ability of a number of fluorescently labelled CPPs to translocate into sperm, identifying effective CPPs (Jones et al., 2013). We have now exploited this technique to further investigate functional expression of SOCs in
human sperm and their potential participation in the [Ca\(^{2+}\)]\(i\) response to progesterone by using CPPs to target functioning of SOCs in sperm. Though there are competing models for regulation of SOC activity, the importance of a region within the STIM molecule termed CAD (CRAC-activating domain) or SOAR (STIM Orai activating region) is clear (Yuan et al., 2009). Whereas the full cytoplasmic segment of STIM1 is a poor activator of Orai1, the CAD/SOAR domain is more effective (Korzeniowski et al., 2010). This suggests that in the full length form intramolecular interactions maintain STIM inactive until store depletion occurs. Unfolding of STIM then allows interaction with Orai. SOAR includes an internal basic patch which may interact with an acidic region on Orai to stabilise such folding (Korzeniowski et al., 2010, Soboloff et al., 2011, Kim and Muallem, 2011). To further investigate the potential involvement of SOCs in the generation of [Ca\(^{2+}\)]\(i\) signals in human sperm, we have used a CPP (KIKKK) that mimics the basic patch on the SOAR region of STIM. KIKKK may act directly to stimulate CCE by binding Orai or may interact with the acidic patch on STIM, thus interfering with the refolding that would normally render STIM quiescent upon store refilling.

**Methods**

*Preparation and capacitation of spermatozoa*

Healthy donors were recruited in accordance with the Human Fertilisation and Embryology Authority Code of Practice (Version 8) and gave informed consent. Protocols were approved by the University Ethical Committee (University of Birmingham Life and Health Sciences ERC 07-009). Semen collected by masturbation after 2-3 days of sexual abstinence was allowed to liquefy for ≈30 min (37°C, 6% CO\(_2\)). Cells were harvested by direct swim-up into supplemented Earle’s balanced salt solution (sEBSS), as described previously (Harper et al., 2003) and adjusted to 6 x 10\(^6\) cells/ml. Aliquots of 200 µl were left to capacitate (37°C, 6% CO\(_2\)) for 4-5 hours.
**Salines:** sEBSS contained (mM) 118.4 NaCl, 5.4 KCl, 0.81 MgSO₄·7H₂O, 25.0 NaHCO₃, 1.02 NaH₂PO₄, 5.5 C₆H₁₂O₆, 2.5 C₂H₂NaO₄, 19.0 CH₃CH(OH)COONa, 1.8 CaCl₂·2H₂O, and 15 HEPES (pH 7.35, 285-295 mOsm), supplemented with 0.3% (w/v) fatty acid free bovine serum albumin (BSA).

Initial experiments with complete sEBSS showed no effect of the peptides used here but when peptides were applied in BSA-free medium clear effects of peptide treatment occurred. We therefore completed preparation of spermatozoa and transfer to the recording chamber in complete medium but the cells were then superfused with BSA-free EBSS for approx. 5 min before recording commenced. No-peptide controls were run under similar conditions.

**Microwave-enhanced Synthesis of Proteomimetic Bioportides**

A polycationic sequence derived from human STIM (STIM³⁷¹-³⁹²; KQLLVAKEGAEKIKKRNTLFG) [KIKKK] and a scrambled homologue (LKNKFKGVKLAEIEKQALKGTR) [scrambled KIKKK] were synthesized using microwave-assisted solid phase peptide synthesis. Syntheses (0.1 mmole scale) were performed using a Discover SPS Microwave Peptide Synthesizer (CEM Microwave Technology Ltd, Buckingham, UK) with fibre optic temperature control employing an N-α-Fmoc protection strategy with O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) activation on Rink amide methylbenzhydryl amine resins pre-loaded with the first amino acid (AnaSpec, Inc. Cambridge Bioscience Ltd, Cambridge, UK). Deprotection with 7 ml of 20% piperidine was performed for 3 min at 50 W/75°C. A majority of amino acid (AA) coupling reactions were accomplished with a 4-fold molar excess of Fmoc-protected AA with HCTU and diisopropylethylamine (DIEPA), molar ratio of 1:1:2 (AA/HCTU/DIEPA), in 4 ml for 10 min at 25 W/75°C. Arg coupling was performed in two stages: 30 min 0 W/~25°C followed by 5 min at 17 W/75°C. N-terminal acylation with 6-carboxy-tetramethylrhodamine (TAMRA; Novabiochem, Beeston, UK) yielded fluorescent peptides to confirm efficient uptake efficacy (Jones et al., 2013).
Crude peptides were purified to apparent homogeneity using reverse phase semi-preparative scale high performance liquid chromatography. The predicted masses of all peptides used (average M + H+) were confirmed to an accuracy of +1 by matrix-assisted laser desorption ionization time of flight mass spectrometry (Jones et al., 2008). The ability of KIKKK and scrambled KIKKK to penetrate sperm was initially tested using TAMRA-labelled versions of the peptides. KIKKK accumulation appeared to exceed that of the scrambled peptide (see Supplementary Figure 1), probably reflecting specific binding of KIKKK after membrane translocation.

**Single cell imaging of [Ca\(^{2+}\)]:** Loading of cells with Oregon Green BAPTA 1 (Invitrogen) and time-lapse fluorescence imaging was as described previously (Nash et al., 2010). All experiments were performed at 25±0.5°C in a continuous flow of medium (sEBSS). Images were captured at 0.1 Hz using a 40x oil-immersion objective and a Q Imaging Rolera-XR cooled CCD camera or Andor Ixon 897 EMCCD camera controlled by iQ software (Andor Technology, Belfast, UK) (Nash et al., 2010).

KIKKK and scrambled KIKKK were applied by addition to the perfusion header at 5 μM, a concentration that provides optimal loading of mammalian spermatozoa with CPP within minutes without compromising membrane integrity / cell viability and distinguishes clearly between peptides with high and low translocation efficiency (Jones et al., 2013).

**Data analysis:**

Analysis of images, background correction and normalisation of data were performed using IQ (Andor; Northern Ireland) and Excel (Microsoft) as described previously. Unless stated otherwise, the region of interest was drawn around the posterior head and neck region (PHN) of each cell. Raw intensity values were imported into Microsoft Excel and normalised using the equation

\[
\Delta F = \left[ \frac{(F - F_{\text{rest}})}{F_{\text{rest}}} \right] \times 100\% ,
\]

where ΔF is % change in intensity at time t, F is fluorescence intensity at time t and F_{rest} is the mean of ≥10 determinations of F during the control period. For comparing responses between experiments we calculated and plotted R_{tot}, the mean ΔF of all cells (n=25-75) in the experiment.
Amplitude of the progesterone-induced \([\text{Ca}^{2+}]_i\) transient was calculated from the three points spanning the peak of the \(\Delta F_{\text{mean}}\) trace. Amplitude at 3 min after progesterone application was calculated using 6 consecutive points spanning 2.5-3.5 min after application of progesterone.

To calculate the proportion of cells giving a significant response to stimulation we used the mean and 95% confidence intervals of the 10 data points immediately preceding stimulation and the three points spanning the peak of change in fluorescence in the \(R_{\text{tot}}\) trace. The response is considered significant if the difference between control and response peak is greater than the sum of the two confidence intervals. Where one sample is much less variable than the other (which is the case when the control \([\text{Ca}^{2+}]_i\) signal is both stable and ‘quiet’) then alpha approaches 0.05. If both samples show variability (as when the control \([\text{Ca}^{2+}]_i\) signal is ‘noisy’ or if the fluorescence intensity is drifting) the test becomes more stringent, the alpha level falling to \(\approx 0.008\) when the SEM of the two samples is equal.

Values given in the results and in figures are shown ±SEM. For testing significance a value of \(P <0.05\) was considered significant. Calculation of confidence intervals, SEM and testing of statistical significance was carried out using Excel (Microsoft).

Progesterone and all materials for salines were from Sigma, Poole, UK except BSA which was from Stratech Scientific, Newmarket, UK.

Results

We have shown previously that CPPs enter mammalian spermatozoa rapidly, with a half-time between 1 and 3 minutes, dependent on the CPP employed (Jones et al., 2013). Therefore, rather than comparing separate groups of treated and non-treated cells, the effects of CPPs on resting \([\text{Ca}^{2+}]_i\) was monitored during application. Exposure of human sperm to 5 µM KIKKK induced a transient decrease in \([\text{Ca}^{2+}]_i\) that was detectable within <30 s, was maximal at \(\approx 90\) s (mean decrease in \(R_{\text{tot}}\) of 6.6 ±3.2 %; \(n=8\) experiments; \(P<0.05\) compared to pre-stimulation) and recovered to control level.
within approximately 3-4 min (figure 1). Application of scrambled KIKKK induced a similar effect (P<0.025 versus pre-stimulation; fig 1). The amplitude of this effect varied between cells (fig 1c), but both KIKKK and scrambled KIKKK induced a significant decrease in fluorescence (see methods) in ≈65% of cells. Twenty percent of cells (19.2 ±2.7%; n=18 experiments) were spontaneously active, generating [Ca\textsuperscript{2+}] oscillations under control conditions. In approximately 40% of these cells, application of CPPs briefly arrested and reset this activity (KIKKK=42.8 ±7.8%, n=9 experiments; scrambled KIKKK=39.8 ±9.0%, n=9 experiments; t>0.8), but oscillation amplitude and kinetics were maintained, indicating that basal Ca\textsuperscript{2+} influx and Ca\textsuperscript{2+} clearance mechanisms were not affected by KIKKK or scrambled KIKKK peptide treatment (fig 1d).

We then investigated whether KKKK could affect Ca\textsuperscript{2+} signals induced by the CatSper agonist progesterone. Experiments were carried out as ‘parallel’ sets, including a control to assess the effect of progesterone with no pre-treatment and experiments using pre-treatment with KIKKK and with scrambled KIKKK peptide. Stimulation of human sperm with 3μM progesterone induces a biphasic [Ca\textsuperscript{2+}]i response, comprising an early [Ca\textsuperscript{2+}]i transient which decays within 2 min, followed by a slowly developing sustained component (Blackmore et al., 1990, Kirkman-Brown et al., 2000). This biphasic response was clearly visible in R\textsubscript{tot} plots (mean normalised responses of all cells in an experiment) of control experiments (fig 2a). Exposure to 5μM KIKKK CPP prior to stimulation with 3μM progesterone did not affect the mean amplitude or rise time of the [Ca\textsuperscript{2+}]i transient in R\textsubscript{tot} traces (P>0.75 compared to parallel controls and cells pre-treated with scrambled peptide; fig 2a). However, in KIKKK-pre-treated cells the decay of the transient was clearly slower than in control experiments (untreated and pre-treated with scrambled peptide). During the steepest part of the falling phase of the [Ca\textsuperscript{2+}]i transient (from 1.5 to 2 min after progesterone application) the rate of decay was significantly reduced in KIKKK-pre-treatment experiments compared to parallel controls (no pre-treatment and scrambled peptide pre-treatment; fig 2c) such that in 8/9 experiments the amplitude of the early sustained response (R\textsubscript{tot} assessed 2.5-3.3 min after application of progesterone) was significantly greater in cells pre-treated with KIKKK (fig 2b; P<0.03; paired t-test). This difference between
control and pre-treated cells persisted into the plateau phase of the \([Ca^{2+}]_i\) signal in some sets of experiments but this was inconsistent and at 7.5 min after progesterone addition the three conditions did not differ statistically (P>0.2).

Though \([Ca^{2+}]_i\) responses to progesterone occur in ≈98% of human sperm (Harper et al., 2003, Kirkman-Brown et al., 2000), the response of the cells, as assessed by the amplitude and ‘shape’ of the \([Ca^{2+}]_i\) signal, can vary greatly within a sample (Harper et al., 2003, Kirkman-Brown et al., 2003). Pre-treatment with 5 µM KIKKK slightly reduced the proportion of cells that generated a significant \([Ca^{2+}]_i\) response to progesterone compared to controls (mean control =98.5±1.0%; KIKK=95.9±1.3%; n=9 P<0.05, paired-T test on arcsine transformed data), a similar effect occurring with scrambled KIKK (96.1 ±1.3%; n=9; P>0.7 compared to KIKKK). However, inspection of single cell traces showed that there was a marked increase in a sub-population of ‘atypical’ responses where the decay of \([Ca^{2+}]_i\) transient was greatly delayed such that the \([Ca^{2+}]_i\) response appeared monophasic rather than the normal biphasic pattern (fig 3a,b). Assessment of the frequency of prolonged responses by visual inspection (defined as no discernible decay of the \([Ca^{2+}]_i\) signal for ≥3 min after application of progesterone) gave values of 8.8% in control experiments with no pre-treatment (424 cells; 9 experiments), 6.3% after pre-treatment with scrambled peptide (474 cells; 9 experiments; P=0.20 compared to control; chi-square) and 15.2% after pre-treatment with KIKKK peptide (408 cells; 9 experiments; P=1.9*10^-7 compared to scrambled; chi square). To further quantify the occurrence of prolonged \([Ca^{2+}]_i\) transients in KIKKK pre-treated cells we calculated for each cell the mean amplitude (normalised fluorescence) of the early sustained \([Ca^{2+}]_i\) signal for the period from 3-5 min after progesterone application (immediately after termination of a normal \([Ca^{2+}]_i\) transient) and normalised this value to the amplitude of the preceding transient in that cell (S/T ratio). For control cells the S/T ratio was 0.36 ±0.03 (424 cells, 9 experiments) and this was not significantly altered in cells pre-treated with scrambled peptide (0.39 ±0.06; 474 cells, 9 experiments) (P=0.63). However, in cells pre-treated with KIKKK this ratio was significantly increased (P<0.003) to 0.55 ±0.05 (408 cells, 9 experiments). The frequency distribution of S/T ratios was clearly bi-modal. In control and scrambled peptide pre-treated cells most S/T ratios formed a peak with a mode of ≈0.4 but there was
also a clear shoulder at 1.0, reflecting cells in which the initial [Ca\textsuperscript{2+}], ‘transient’ had not decayed within 3 min (fig 3d). In KIKKK pre-treated cells the ratios were higher (mode increased from ≈0.4 to 0.55) and the occurrence of ratios >0.9 was significantly increased (P<0.05; chi square). We conclude that pre-treatment with KIKKK increases the proportion of cells in which the progesterone-induced [Ca\textsuperscript{2+}], transient persists for several minutes before decaying.

In our previous work on the effects of 2-APB on the progesterone-induced [Ca\textsuperscript{2+}], signal we observed strong enhancement of the sustained Ca\textsuperscript{2+} response that was localised specifically to the midpiece, possibly reflecting enhanced accumulation of Ca\textsuperscript{2+} in the mitochondria due to the effects of the drug on mitochondrial Ca\textsuperscript{2+} export (Lefievre et al., 2012). To assess whether the effect described here might be similar, we investigated the location of the [Ca\textsuperscript{2+}], signal. Prolonged responses occurred simultaneously in the midpiece and PHN of the sperm showing that this effect was primarily on cytoplasmic [Ca\textsuperscript{2+}], and not due to Ca\textsuperscript{2+} accumulation within the mitochondrial matrix (fig. 4).

Discussion

We showed previously, using fluorescently-tagged peptides, that CPPs readily enter mammalian sperm, reaching 50% of their final intracellular concentration within 1-3 min (Jones et al., 2013). In the present study, when KIKKK peptide was applied to human sperm we observed a fall in [Ca\textsuperscript{2+}], which recovered to control levels within 3-4 min, often causing a ‘resetting’ of activity in spontaneously oscillating cells. The kinetics of this effect are consistent with rapid entry of the peptide into human sperm but, since this effect was also seen upon application of scrambled KIKKK, it clearly does not reflect a specific effect of the KIKKK sequence on regulation of SOCs. One intriguing possibility is that, since both KIKKK and scrambled peptides bear a net positive charge (7 basic residues out of a total of 22), it binds electrostatically to the net negative charge on the membrane surface. Until equilibration of the peptide across the membrane is reached, this binding is asymmetric, making the extracellular surface more positive and increasing the voltage gradient within the membrane. This effect, termed charge screening, functionally hyperpolarises the membrane and
can reduce the open probability of voltage-sensitive channels (such as CatSper) sufficiently to inhibit passive Ca\(^{2+}\) influx and lower resting [Ca\(^{2+}\)] (Wilson et al., 1983). As the peptide accumulates within the sperm the asymmetry of this effect will decay, allowing passive Ca\(^{2+}\) influx and [Ca\(^{2+}\)] to return to resting levels.

Stimulation of human sperm with nM-µM concentrations of progesterone elicits an immediate [Ca\(^{2+}\)]\(_i\) transient that is dependent upon Ca\(^{2+}\) influx through CatSper channels (Strunker et al., 2011). Under the conditions of our imaging experiments [Ca\(^{2+}\)]\(_i\) peaks in 15-30 s and decays over 1-2 min (Harper et al., 2003, Kirkman-Brown et al., 2000). In populations of sperm pre-treated with the KIKKK peptide there was no change in the rise-time or amplitude of this [Ca\(^{2+}\)]\(_i\) transient but decay was slower, such that 3 min after progesterone stimulation the [Ca\(^{2+}\)]\(_i\) in pre-treated cells was ≈1.5-fold higher than in control cells or cells pre-treated with scrambled peptide. Analysis of single cell records showed that this effect was not due to a reduced decay rate of Ca\(^{2+}\) clearance but reflected an increase in the proportion of cells in which the transient peak was abnormally prolonged into a plateau. These monophasic responses were not restricted to the midpiece, occurring simultaneously at the PHN, and thus reflect increased cytoplasmic [Ca\(^{2+}\)\(_i\)], rather than Ca\(^{2+}\) accumulation in the mitochondria (fig 4). The slow decay of the sperm population [Ca\(^{2+}\)\(_i\)] signal results from cell-cell variation in the duration of these abnormal [Ca\(^{2+}\)\(_i\)] transients. Interestingly, such prolonged transients also occur in untreated cells, but their incidence was greatly enhanced by pre-treatment with KIKKK.

These observations suggest that SOCs at the sperm neck (Lefievre et al., 2012) become activated and contribute a second Ca\(^{2+}\)-influx pathway. A number of strands of evidence point to rapid recruitment of such a secondary Ca\(^{2+}\) signal downstream of CatSper-mediated Ca\(^{2+}\) influx in human sperm:

1. Low dose (5 µM) 2-APB, which sensitises SOC activation but does not affect CatSper currents, enhanced [Ca\(^{2+}\)\(_i\)] elevation during the initial progesterone-induced transient. This
effect was localised to the sperm neck but did not occur in the flagellum (Lefievre et al., 2012);

(ii) Treatment with 1-10 μM nifedipine significantly reduces the duration (but not amplitude) of the progesterone-induced [Ca\(^{2+}\)] transient, apparently inhibiting a ‘late’ component of the transient which activates 10-20 s after initiation of the response (Kirkman-Brown et al., 2003). Since 10 μM nifedipine did not inhibit CatSper (Strunker et al., 2011) and there is no evidence for activation of nifedipine-sensitive voltage-activated Ca\(^{2+}\) channels by progesterone, we consider it likely that this action is exerted on Ca\(^{2+}\) store mobilisation or on SOCs;

(iii) In progesterone-treated sperm, [Ca\(^{2+}\)], oscillations, consistent with cyclical mobilisation of stored Ca\(^{2+}\), occur after the initial [Ca\(^{2+}\)], transient. These are dependent on a low level of Ca\(^{2+}\)-influx and are sensitive to manipulation of ryanodine receptors (RyRs) (Aitken and McLaughlin, 2007, Harper et al., 2004, Kirkman-Brown et al., 2004, Sanchez-Cardenas et al., 2014).

Since Ca\(^{2+}\) stores, STIM and Orai proteins are present at the sperm neck, the simplest interpretation consistent with these observations is that CatSper-mediated Ca\(^{2+}\)-influx into the flagellum induces secondary mobilisation of stored Ca\(^{2+}\) and activation of CCE. Upon decay of the CatSper-mediated Ca\(^{2+}\) influx, Ca\(^{2+}\) stores will refill and CCE will also decay, dependent upon successful termination of STIM-Orai interaction, such that interference with the regulatory mechanism on STIM delays (though does not prevent) decay of the [Ca\(^{2+}\)]. If this model is correct, pharmacological blockade of SOCs should have effects on the progesterone-induced [Ca\(^{2+}\)], signal. Indeed at high doses of 2-APB (100 μM, which inhibits SOC function (DeHaven et al., 2008, Lis et al., 2007)) the enhancement of the progesterone-induced [Ca\(^{2+}\)], transient seen at low doses is reversed, the sustained component of the signal is suppressed and in some preparations the transient duration is reduced compared to parallel controls (Lefievre et al., 2012). In experiments with the non-specific SOC blocker SKF 96365 (10-30 μM) we observed a rise in [Ca\(^{2+}\)], in most cells, an effect which has been described previously (Jan et al., 1999). When progesterone was subsequently applied, the sustained component was strongly
inhibited and the duration (but not amplitude) of the progesterone-induced $[\text{Ca}^{2+}]_i$ transient was reduced compared to a parallel control experiments (Lefievre, Morris, Publicover; unpublished data). Though the effects of these drugs on the response to progesterone are consistent with activation of SOCs, the effects of SKF 96365 alone are such that they cannot be interpreted.

A peculiarity of SOCs in sperm is that, whereas in most cells STIM is distant from the plasmalemma and must be redistributed to interact with Orai, intracellular membranes of sperm are located close to the plasma membrane such that no redistribution of STIM is discernible in cells that have not been stimulated to mobilise stored $\text{Ca}^{2+}$ (Lefievre et al., 2012). Thus, if the auto-inhibitory interactions in STIM are disrupted, activated SOCs may switch off relatively slowly even if store refilling occurs, leading to prolonged $\text{Ca}^{2+}$ influx.

$[\text{Ca}^{2+}]_i$ signalling in human sperm is crucial in the regulation of a range of functions (Darszon et al., 2011, Publicover et al., 2007). Whereas previous work using pharmacological and immunological techniques suggested that a range of channels contributed to sperm $\text{Ca}^{2+}$-signalling, data obtained from the application of patch clamp has revealed only currents carried by CatSper channels. The reason for this discrepancy is not clear, though whole-cell access and consequent cytoplasmic dialysis of the minute cytoplasmic volume might affect the functioning of some sperm ion channels (Publicover and Barratt, 2012). Evidence for participation of SOCs must therefore be interpreted cautiously, but the data are consistent with a flexible $\text{Ca}^{2+}$-signalling system in which activation of SOCs can contribute a spatially and temporally separate route for influx of $\text{Ca}^{2+}$ into the sperm, which may contribute to the ability of the cell to regulate separately several $\text{Ca}^{2+}$-controlled processes (Alasmari et al., 2013).
Authors' roles

JM, SJ, ML and LL carried out the experimental work and analysis of data; SJP and JH designed the study and wrote the manuscript.

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Conflict of interests

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Figure legends

**Fig. 1. Effect of cell-penetrating peptides on resting [Ca\(^{2+}\)]\(_i\) in human sperm.**

In this, and subsequent figures, data are expressed as % change in fluorescence. (a) shows effect of 5 μM scrambled KIKK peptide, (b); shows effect of 5 μM KIKK peptide. Arrows indicate time of addition of peptide. Traces show mean (±SEM) R\(_{tot}\) for 8 experiments. (c) Single cell traces showing transient fall in [Ca\(^{2+}\)]\(_i\) upon application of KIKKK. (d) shows effect of KIKKK in cells showing spontaneous [Ca\(^{2+}\)]\(_i\) oscillations. In a few cells oscillations ‘paused’ briefly (blue trace) but in the majority they persisted and kinetics appeared unchanged.

**Fig. 2. Pre-treatment with KIKK prolongs the progesterone-induced [Ca\(^{2+}\)]\(_i\) transient in human sperm.**

(a): effects of 5 uM KIKKK peptide (red) and 5 μM scrambled KIKKK peptide (grey) on progesterone-induced biphasic [Ca\(^{2+}\)]\(_i\) response in human sperm. Black shows response to progesterone in cells with no pre-treatment. Each trace is the mean of R\(_{tot}\) plots (average of all cells in an experiment) from 9 experiments (±SEM). Arrows indicate time of addition of progesterone. b: shows enlarged plot of boxed section from panel (a) with error bars (±SEM). c: Mean rate of decay of R\(_{tot}\) (from 1.5 to 2 min after application of progesterone; mean ±SEM from 9 sets of parallel experiments). ** indicates P<0.01 compared to KIKKK pre-treatment (paired t test).

**Fig.3. KIKKK pre-treatment enhances the occurrence of prolonged progesterone-activated [Ca\(^{2+}\)]\(_i\) transients in human sperm.**

(a) and (b) show single cell [Ca\(^{2+}\)]\(_i\) responses from the same (5 μM KIKKK pre-treated) experiment which have been sorted into two populations according to whether the [Ca\(^{2+}\)]\(_i\) transient starts to decay with 3 min of progesterone application (‘conventional’ transient; (a)) or persists at maximum for at
least 3 min after progesterone application (prolonged response (b)). c: shows distribution of the ratio
of S/T ratios ([Ca$^{2+}$], response amplitude at 3 min after progesterone application: amplitude of [Ca$^{2+}$],
transient peak – see inset). Black trace shows control, red trace shows cells pre-treated with 5 μM
KIKKK and grey trace shows cells pre-treated with 5 μM scrambled KIKK. KIKKK pre-treatment
enhances the size of the modal S/T ratio and also increases the size of the shoulder at 0.9-1.1 which
shows cells where the transient is maintained as a plateau for ≥3 min.

**Fig. 4. Prolonged [Ca$^{2+}$], responses in human sperm are not due to mitochondrial Ca$^{2+}$
accumulation.**

‘a’: montage shows a pseudocolour image series of a 5 μM KIKKK-pre-treated cell which shows a
prolonged [Ca$^{2+}$], response. Images are at 1 min intervals (indicated by adjacent numbers).

Progesterone was added just before image 9. Note that increased [Ca$^{2+}$], at the posterior head/neck
(PHN) is maintained for >4 min. ‘b’: four examples showing separate analysis of [Ca$^{2+}$], responses in
the midpiece (green) and PHN region (blue) in the same cell. Responses occur simultaneously in the
PHN and midpiece.
Fig. 2
Fig. 3
Fig. 4