Cell-penetrating peptides, targeting the regulation of store-operated channels, slow decay of the progesterone-induced \([\text{Ca}^{2+}]_i\) 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 localized 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 activating domain (SOAR/CAD) of the regulatory protein stromal interaction molecule 1. SOAR/CAD plays a key role in controlling the opening of SOCs, which occurs upon mobilization 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 pretreated 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 pretreated 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 min in a subpopulation of cells. We hypothesize 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 fertilize (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 fertilization 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 (Publicover et al., 2007; Costello et al., 2009; Darszon et al., 2011) and both play important roles in sperm function. CatSper, a sperm-specific cation channel localized 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 (Costello et al., 2009; Alasmari et al., 2013).

The characteristics of \([\text{Ca}^{2+}]_i\) signals generated by store mobilization in human sperm are consistent with the occurrence of capacitative \(\text{Ca}^{2+}\) entry (CCE) mediated by store-operated channels (SOCs) (Blackmore, 1993; Park et al., 2011; Lefievre et al., 2012). SOCs are activated by interaction with stromal interaction molecule (STIM), a sensor molecule present in the membrane of the \(\text{Ca}^{2+}\) store that detects depletion

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of stored 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 transient receptor potential canonical [TRPC] family), causing SOCs to open (Cahalan, 2009). STIM1, Orai proteins and TRPC proteins have all been detected in human sperm (Castellano et al., 2003; Darzson et al., 2012; Lefevre et al., 2012). STIM1 is localized primarily to the neck region/midpiece and the acrosome, the areas where Ca^{2+} stores are present (Lefevre et al., 2012).

Progesterone, a product of the cumulus cells which surround the oocyte, is the best characterized 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 subpopulation of cells, this plateau phase includes repeated [Ca^{2+}]_i elevations (oscillations; Harper et al., 2004; Kirkman-Brown et al., 2004; Aitken and McLaughlin, 2007; Sanchez-Cardenas et al., 2014), which are typically irregular/chaotic but are ‘organized’ by ryanoxin 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 mM), which potentiates SOC activity by promoting interaction with STIM, enhanced the progesterone transient at the sperm neck (but not the flagellum; Lefevre et al., 2012). The [Ca^{2+}]_i plateau phase was not enhanced by 2-APB pretreatment, 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 observations 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 potentially more specific technique than pharmacological intervention. Rab3A and GST, both small proteins across the plasma membrane offers an alternative possibility for investigating 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 stabilize such folding (Korzeniowski et al., 2010; Kim and Mulleim, 2011; Soboloff et al., 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% CO2). 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 × 10^6 cells/ml. Aliquots of 200 μl were left to capacitate (37°C, 6% CO2) for 4–5 h.

Saline sEBSS contained (mM) 118.4 NaCl, 5.4 KCl, 0.81 MgSO4.7H2O, 25.0 NaHCO3, 1.02 NaH2PO4, 5.5 C6H12O6, 2.5 C3H3NaO3, 19.0 CH3COONa, 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 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; KQLLVA KEGAEIKKKRNTLFG) [KIKKK] and a scrambled homologue (LKNKFKGV KLAIEIQKLGTR) [scrambled KIKKK] were synthesized using microwave-assisted solid-phase peptide synthesis. Syntheses (0.1 mmol 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-tetramethylyuronium hexafluoro phosphate (HCTU) activation on Rink amide methlybenzhydrylamine resins pre-loaded with the first amino acid (AA; 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 AA coupling reactions were accomplished with a 4-fold molar excess of Fmoc-protected AA with HCTU and disopropylthylamine (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 efficiency (Jones et al., 2013).

Crude peptides were purified to apparent homogeneity using semi preparative, reversed-phase 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 (Supplementary Fig. S1), probably reflecting specific binding of KIKKK after membrane translocation.

**Single-cell imaging of \([Ca^{2+}]_i\)**

Loading of cells with Oregon Green BAPTA 1 (Invitrogen) and time-lapse fluorescence imaging was done 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 × 40 oil immersion objective and a Q Imaging Retiga-ER cooled CCD camera or Andor ikon 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 normalization 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 (PHN) region of each cell. Raw intensity values were imported into Microsoft Excel and normalized using the following equation:

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

where \(\Delta F\) is % change in intensity at time t, F is fluorescence intensity at time t and \(F_{\text{rest}}\) is the mean of 10 determinations of F during the control period. For comparing responses between experiments, we calculated and plotted \(R_{\text{rest}}\), the mean \(\Delta F\) of all cells (n = 25–75) in the experiment.

Amplitude of the progesterone-induced \([Ca^{2+}]_i\) transient was calculated from the three points(103,476),(543,549) spanning the peak of the \(\Delta F_{\text{mean}}\) trace. Amplitude at 3 min after progesterone application was calculated using six 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 \(R_{\text{rest}}\) traces. 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 \([Ca^{2+}]_i\) signal is both stable and ‘quiet’), then alpha approaches 0.05. If both samples show variability (as when the control \([Ca^{2+}]_i\) signal is ‘noisy’ or if the fluorescence intensity is drifting), the test becomes more stringent, the alpha level falling to ≈0.008 when the SEM of the two samples is equal.

Values given in the results and in figures are shown as ± 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 saline 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 min, 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 \([Ca^{2+}]_i\) was monitored during application. Exposure of human sperm to 5 μM KIKKK induced a transient decrease in \([Ca^{2+}]_i\), that was detectable within <30 s, was maximal at ≈90 s (mean decrease in \(R_{\text{rest}}\) of 6.6 ± 3.2%; n = 8 experiments; \(P < 0.05\) compared with pre-stimulation) and recovered to control level within ~3–4 min (Fig. 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 \([Ca^{2+}]_i\) oscillations under control conditions. In ~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 \([Ca^{2+}]_i\) influx and \([Ca^{2+}]_i\) clearance mechanisms were not affected by KIKKK or scrambled KIKKK peptide treatment (Fig. 1d).

We then investigated whether KIKKK could affect \([Ca^{2+}]_i\) 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 pretreatment and experiments using pretreatment with KIKKK and with scrambled KIKKK peptide. Stimulation of human sperm with 3 μM progesterone induces a biphasic \([Ca^{2+}]_i\) response, comprising an early \([Ca^{2+}]_i\) 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 \(R_{\text{rest}}\) plots (mean normalized responses of all cells in an experiment) of control experiments (Fig. 2a). Exposure to 5 μM KIKKK CPP prior to stimulation with 3 μM progesterone did not affect the mean amplitude or rise time of the \([Ca^{2+}]_i\) transient in \(R_{\text{rest}}\) traces (\(P > 0.75\) compared with parallel controls and cells pretreated with scrambled peptide; Fig. 2a). However, in KIKKK-pretreated 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 \([Ca^{2+}]_i\) transient (from 1.5 to 2 min after progesterone application), the rate of decay was significantly reduced in KIKKK-pretreatment experiments compared with parallel controls (no pretreatment and scrambled peptide pretreatment; Fig. 2c), such that in 8 of 9 experiments the amplitude of the early sustained response (\(R_{\text{rest}}\) assessed 2.5–3.3 min after application of progesterone) was significantly greater in cells pretreated with KIKKK (Fig. 2b; \(P < 0.03\); paired t-test). This difference between control and pretreated 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 ≈96% of human sperm (Kirkman-Brown et al., 2000; Harper et al., 2003), 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). Pretreatment with 5 μM KIKKK slightly reduced the proportion of cells that generated a significant \([Ca^{2+}]_i\) response to progesterone compared with controls (mean control = 98.5 ± 1.0%; KIKKK = 95.9 ± 1.3%; n = 9, \(P < 0.05\), paired t-test on arcsine transformed data), a similar effect occurring with scrambled KIKKK (96.1 ± 1.3%; n = 9; \(P > 0.7\) compared with KIKKK). However, inspection of single-cell traces showed that there was a marked increase in a subpopulation of
‘atypical’ responses where the decay of \([\mathrm{Ca}^{2+}]\), transient was greatly delayed, such that the \([\mathrm{Ca}^{2+}]\) response appeared monophasic rather than the normal biphasic pattern (Fig. 3a and b). Assessment of the frequency of prolonged responses by visual inspection (defined as no discernible decay of the \([\mathrm{Ca}^{2+}]\) signal for \(\geq 3\) min after application of progesterone) gave values of 8.8% in control experiments with no pretreatment (424 cells; 9 experiments), 6.3% after pretreatment with scrambled peptide (474 cells; 9 experiments; \(P = 0.20\) compared with control; \(x^2\)) and 15.2% after pretreatment with KIKKK peptide (408 cells; 9 experiments; \(P = 1.9 \times 10^{-7}\) compared with scrambled; \(x^2\)). To further quantify the occurrence of prolonged \([\mathrm{Ca}^{2+}]\) transients in KIKKK-pretreated cells, we calculated for each cell the mean amplitude (normalized fluorescence) of the early sustained \([\mathrm{Ca}^{2+}]\) signal for the period from 3 to 5 min after progesterone application (immediately after termination of a normal \([\mathrm{Ca}^{2+}]\) transient) and normalized this value to the amplitude of the preceding transient in that cell (S/T ratio). For control cells, the S/T ratio was \(0.36 \pm 0.03\) (424 cells, 9 experiments) and this was not significantly altered in cells pretreated with scrambled peptide (0.39 \(\pm 0.06\); 474 cells, 9 experiments; \(P = 0.63\)). However, in cells pretreated with KIKKK, this ratio was significantly increased (\(P < 0.003\)) to 0.55 \(\pm 0.05\) (408 cells, 9 experiments). The frequency distribution of S/T ratios was clearly bi-modal. In control and scrambled peptide pretreated cells, most S/T ratios formed a peak with a mode of \(\approx 0.4\), but there was also a clear shoulder at 1.0, reflecting cells in which the initial \([\mathrm{Ca}^{2+}]\) ‘transient’ had not decayed within 3 min (Fig. 3d). In KIKKK-pretreated cells, the ratios were higher (mode increased from \(\approx 0.4\) to 0.55) and the occurrence of ratios >0.9 was significantly increased (\(P < 0.05\); \(x^2\)). We conclude that pretreatment with KIKKK increases the proportion of cells in which the progesterone-induced \([\mathrm{Ca}^{2+}]\) transient persists for several minutes before decaying.

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

Stimulation of human sperm with nM–μM concentrations of progesterone elicits an immediate [Ca\(^{2+}\)] transient that is dependent on Ca\(^{2+}\) influx through CatSper channels (Strunker et al., 2011). Under the conditions of our imaging experiments, [Ca\(^{2+}\)] peaks in 15–30 s and decays over 1–2 min (Kirkman-Brown et al., 2000; Harper et al., 2003). In populations of sperm pretreated with the KIKKK peptide, there was no change in the rise time or amplitude of this [Ca\(^{2+}\)] transient but decay was slower, such that 3 min after progesterone stimulation the [Ca\(^{2+}\)] in pretreated cells was ≈ 1.5-fold higher than in control cells or cells pretreated with the 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\(^{2+}\)], rather than Ca\(^{2+}\) accumulation in the mitochondria (Fig. 4). The slow decay of the sperm population [Ca\(^{2+}\)] signal results from cell–cell variation in the duration of these abnormal [Ca\(^{2+}\)] transients. Interestingly, such prolonged transients also occur in untreated cells, but their incidence was greatly enhanced by pretreatment 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:

(i) Low-dose (5 μM) 2-APB, which sensitizes SOC activation but does not affect CatSper currents, enhanced [Ca\(^{2+}\)] elevation during the initial progesterone-induced transient. This effect was localized 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) channels by progesterone, we consider it likely that this action is exerted on \([\text{Ca}^{2+}]_i\) store mobilization or on SOCs.

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

Since \([\text{Ca}^{2+}]_i\) stores, STIM and Orai proteins are present at the sperm neck, the simplest interpretation consistent with these observations is that CatSper-mediated \([\text{Ca}^{2+}]_i\)-influx into the flagellum induces secondary mobilization of stored \([\text{Ca}^{2+}]_i\) and activation of CCE. Upon decay of the CatSper-mediated \([\text{Ca}^{2+}]_i\)-influx, \([\text{Ca}^{2+}]_i\) stores will refill and CCE will also decay, dependent on successful termination of STIM–Orai interaction, such that interference with the regulatory mechanism on STIM delays (though does not prevent) decay of the \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at high doses of 2-APB [100 μM, which inhibits SOC function (Lis et al., 2007; DeHaven et al., 2008)], the enhancement of the progesterone-induced \([\text{Ca}^{2+}]_i\) signal. Indeed at...
transient was reduced compared with a parallel control experiment (L. Lefievre, J. Morris, S. Publicover; unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data). Though the effects of these drugs on the response to progesterone are consistent (L. Lefievre, J. Morris, S. Publicover, unpublished data).

data obtained from the application of patch clamp have 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 Ca^{2+}-signalling system in which activation of SOCs can contribute a spatially and temporally separate route for influx of Ca^{2+} into the sperm, which may contribute to the ability of the cell to regulate separately several Ca^{2+}-controlled processes (Alasmari et al., 2013).

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Authors’ roles**

J.M., S.J., M.L. and L.L. carried out the experimental work and analysis of data; S.J.P. and J.H. designed the study and wrote the manuscript.

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**Conflict of interest**

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

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