Stimulation of Human Spermatozoa with Progesterone Gradients to Simulate Approach to the Oocyte

INDUCTION OF \([\text{Ca}^{2+}]\), OSCILLATIONS AND CYCLICAL TRANSITIONS IN FLAGELLAR BEATING

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Progesterone is present at micromolar concentrations in the cumulus matrix, which surrounds mammalian oocytes. Exposure of human spermatozoa to a concentration gradient of progesterone (0–3 μM) to simulate approach to the oocyte induced a slowly developing increase in \([\text{Ca}^{2+}]\), upon which, in many cells, slow oscillations were superimposed. \([\text{Ca}^{2+}]\), oscillations often started at very low progesterone (<10 nm), and their frequency did not change during the subsequent rise in concentration. Oscillations also occurred, but in a much smaller proportion of cells, in response to stepped application of progesterone (3 μM). When progesterone was removed, \([\text{Ca}^{2+}]\), oscillations often persisted or quickly resumed. Superfusion with low-\([\text{Ca}^{2+}]\) bathing medium (no added \([\text{Ca}^{2+}]\)) did not prevent \([\text{Ca}^{2+}]\), oscillations, but they could be abolished by addition of EGTA or La³⁺. Inhibitors of sarcoplasmic/endoplasmic reticulum \([\text{Ca}^{2+}]\)-ATPases or inositol trisphosphate signaling had no effect on \([\text{Ca}^{2+}]\), oscillations, but pharmacological manipulation of ryanodine receptors affected both their frequency and amplitude. Staining of live spermatozoa with BODIPY FL-X ryanodine showed localization of ryanodine binding primarily to the caudal part of the head and mid-piece. \([\text{Ca}^{2+}]\), oscillations did not induce acrosome reaction, but in cells generating oscillations, the flagellar beat mode alternated in synchrony with the oscillation cycle. Flagellar bending and lateral movement of the sperm head during \([\text{Ca}^{2+}]\), peaks were markedly increased compared with during \([\text{Ca}^{2+}]\), troughs. This alternating pattern of activity is likely to facilitate zona penetration. These observations show that progesterone initiates unusual and complex store-mediated \([\text{Ca}^{2+}]\), signaling in human spermatozoa and identify a previously unrecognized effect of progesterone in regulating sperm “behavior” during fertilization.

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[Ca²⁺], signaling plays a key role in the functioning of spermatozoa, controlling motility, acrosome reaction (AR),1 and (probably) chemotactic responses (1–4). Sperm express a range of plasma membrane Ca²⁺-permeable channels (5–8) and are believed to possess Ca²⁺ stores. Inositol trisphosphate receptors and sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPases have been localized to the acrosome and mid-piece, and permeabilized human spermatozoa have been shown to contain a releasable store of Ca²⁺ (9–13). The best characterized agonist of [Ca²⁺], signaling in mammalian spermatozoa is the zona pellucida, which surrounds the oocyte. The zona pellucida induces elevations in [Ca²⁺], upon sperm contact, resulting in AR at the zona surface (14, 15). The zona pellucida-activated [Ca²⁺], signal in mouse spermatozoa is initially generated by Ca²⁺ influx through a voltage-operated Ca²⁺ channel. There is good evidence for subsequent participation of a low capacity (or fractionally filled) Ca²⁺ store in this process, but the function of store mobilization is to permit activation of store-operated Ca²⁺ channels rather than to provide a significant source of Ca²⁺ (16). Progesterone, which is synthesized by and present in mammalian cumulus (17–19), also causes elevations in [Ca²⁺], and is the best characterized agonist of human spermatozoa. As with the response to the zona pellucida, stimulation with progesterone induces a “simple” signal by Ca²⁺ influx (20, 21). This involves at least two influx pathways, but their identity is far from clear (22). Intriguingly, a small proportion of progesterone-stimulated human spermatozoa generate large slow [Ca²⁺], oscillations, possibly reflecting store mobilization (23).

That responsiveness of human spermatozoa to progesterone is correlated with fertilization success in vitro (24) indicates its biological significance, but the role of progesterone in fertilization is poorly understood. Micromolar doses of progesterone induce AR, but the relevance of this response for fertilization in vivo is disputed (22, 25). Progesterone is also reported to induce hyperactivation, a vigorous swimming pattern caused by marked changes in flagellar beating. Hyperactivated motility is adopted by sperm as they gain the ability to fertilize and is characteristic of cells retrieved at the site and time of fertilization (4).

Progesterone is present in the cumulus at micromolar concentrations (17), but human spermatozoa respond to the hormone at nanomolar doses (26, 27). Spermatozoa will therefore...
detect cumulus-derived progesterone, distributed by diffusion and/or ciliary currents, prior to encountering the oocyte zona cumulus. Furthermore, the progesterone stimulus encountered by the spermatozoa is likely to occur as a concentration gradient, which the cell ascends as it approaches the oocyte. Previous studies on the action of progesterone on spermatozoa have used stepped application. To understand better the response of human spermatozoa to the progesterone stimulus that they encounter in vivo, we have exposed human spermatozoa to a rising logarithmic progesterone concentration, generated by an unusual mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were cell culture-tested grade where available. Oregon Green BAPTA-1/AM and BODIPY FL-X ryanodine were from Molecular Probes, Inc. Aristolochic acid, caffeine, cyclopiazonic acid, 2,4-dinitrophenol, Me$_2$SO, fluorescein isothiocyanate (FITC), FITC-conjugated *Pium sativum* agglutinin, Pluronic F-127, progesterone, poly-l-lysine, neomycin, ryanodine, tetracaine, thapsigargin, and 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) were from Sigma. (2-Aminoethoxy)diphenylborate, SKF-96365, and U-73122 were products of Calbiochem. Fatty acid-free bovine serum albumin was from JRH Biosciences (Andover, United Kingdom). Drugs were dissolved as a concentrated stock solution in Me$_2$SO (when necessary) and diluted in supplemented Earle’s balanced salt solution (sEBSS; 0.3% bovine serum albumin) before application. Me$_2$SO never exceeded 0.5% and was normal 0.1%. These doses of Me$_2$SO had no effect on [Ca$^{2+}$], in human spermatozoa. The progesterone enzyme-linked immunosorbent assay (ELISA) kit was obtained from DRG Diagnostics.

**Preparation and Capacitation of Spermatozoa—**Donors were recruited at Birmingham Women’s Hospital. Highly motile spermatozoa were harvested into sEBSS as described previously (27) and were left to capacitate (to acquire the ability to fertilize, a process that normally occurs during residence in the female tract) for 6-24 h at 37 °C in 5% CO$_2$. 

**Imaging—**Aliquots were loaded with Oregon Green BAPTA-1/AM and imaged in a continuously perfused chamber as described previously (27). Stepped (3 mM) progesterone stimuli and drugs were applied by addition to the perfusion header. 1 mM La$^{3+}$ was applied in HEPES-buffered saline containing 150 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 5 mM MgCl$_2$, 10 mM glucose, and 0.5% bovine serum albumin (pH 7.4). All experiments were carried out at 25 °C unless stated otherwise. Data acquisition and storage were controlled by a PC running AQM Orca 2001 (Kinetic Imaging Ltd., Nottingham, United Kingdom).

**Generation and Assessment of Gradient Stimuli—**Progesterone gradients were generated using a five-chamber gradient maker (0, 3 nM, 30 nM, 300 nM, and 3 μM progesterone) connected to the inflow of the imaging chamber. Gradient characteristics were assessed in two ways. To determine the time of initiation of the gradient and to assess smoothness/stability, an FITC gradient was generated using 0, 2, 10, 20, and 30 mM FITC. Fluorescence intensity in the imaging chamber was measured at 10-s intervals. The outflow from the imaging chamber was collected at various time points during a number of the gradient experiments, and progesterone was measured using the ELISA kit following the manufacturer’s instructions.

**Single Cell Data Processing—**Data were processed off-line using AQM Orca 2001 as described previously (27). Raw intensity values from the caudal part of the head of each sperm were imported into Microsoft Excel and normalized to pre-stimulus values. At each time point, the normalized fluorescence intensity values ($R$) for each cell were compiled to generate an overall average normalized head fluorescence ($R_{avg}$). The latency of response to gradient stimuli was estimated by comparing mean control fluorescence (at least 20 images immediately prior to stimulation) with each mean from a 10-point moving average (increment = 1). The response to the gradient stimulus was considered to have been initiated from the midpoint of the first 10-point sample at which there was a significant (and subsequently maintained) increase in fluorescence (p < 0.05) using unpaired Student’s t test.

**Assessment of the Characteristics of [Ca$^{2+}$], Oscillations—**Initial attempts to use Fourier analysis as an objective method to identify oscillating cells and to determine oscillation frequency were unsuccessful due to variability, particularly in the cycle period and in the base line upon which oscillations were superimposed. Traces that clearly incorporated repeated transitions between fluorescence levels (such as in Fig. 2a) generated noisy spectra in which the dominant frequency was often barely detectable, and oscillating cells could not be identified with any degree of reliability. Cells displaying oscillations in [Ca$^{2+}$], were therefore identified directly from time/intensity intensity plots. Only cells with cyclical changes in fluorescence, comprising repeated events of consistent characteristics, were categorized as oscillators. The period of [Ca$^{2+}$], oscillation in each cell was calculated by taking an average over 3–10 cycles.

**Flagellar Activity—**Immediately following [Ca$^{2+}$], imaging, the field of cells was observed under phase-contrast microscopy (2 Hz for 12 min). We then selected cells that were well adhered to the slide but had a freely motile and clearly visible flagellum in the phase contrast images. A scale with 1.5-μm graduations was superimposed on the image, normal to the axis of the sperm, at a point on the flagellum (typically not >10 μm beyond the mid-piece) where the position could be detected in every frame. The point where the tail crossed this scale was then recorded for each image in the phase contrast series. The square of each subframe increment in tail position was calculated (change in position in micrometers$^2$) and plotted as a 20-point moving average.

**Labeling with BODIPY FL-X Ryanodine—**Capacitated spermatozoa (6 million cells/ml) were labeled with 2.5 μM BODIPY FL-X ryanodine for 30 min at 37 °C in 5% CO$_2$, and then transferred to the imaging chamber. An additional 30-min incubation at 37 °C in 5% CO$_2$ was then carried out for the cells to adhere. The chamber was connected to the perfusion header, and 2 ml of medium was perfused through the chamber to remove excess dye.

**Assessment of Acrosomal Status—**After collection of a series of Oregon Green BAPTA-1/AM images for assessment of [Ca$^{2+}$],, signaling, the chamber was perfused with 100% methanol for 30 s and then washed with sEBSS. The field was bleached by exposure to fluorescent illumination for 30 s. (A control was carried out to confirm that this process did not affect the number of motile cells.) FITC-conjugated *Pium sativum* agglutinin (0.2 mg/ml) was introduced into the chamber and left for 45 min, and then distilled water was perfused through the chamber for 10 min. This procedure was carried out at 25 °C. An image of the field of view was captured, and the each cell was assessed for acrosomal status and related to the [Ca$^{2+}$], signaling activity from the preceding fluorescence image series. Approximately 15% of spermatozoa were not scored due to poor staining, because they were washed off during the staining stage or because we were not able to assess acrosomal status from the labeling pattern.

**Statistical Analysis—**Proportions of oscillating cells, oscillation cycle frequency, and rates of AR were compared in Microsoft Excel using paired or unpaired t tests (two-tailed) as appropriate. Percentage data were arcsine-transformed before analysis. Statistical significance was set at p < 0.05. All data are presented as means ± S.E.

**RESULTS**

**Response to Stepped Progesterone Treatment**

We previously described the biphasic [Ca$^{2+}$], response of progesterone-stimulated human sperm (27, 28). Both population and single cell responses comprise a transient [Ca$^{2+}$], increase followed by a sustained elevation in [Ca$^{2+}$], (Fig. 1a). The response is dependent upon [Ca$^{2+}$],, occurs in ~98% of viable cells, and is dose-dependent (27).

**Response to a Progesterone Gradient**

To simulate the progesterone stimulus during approach to the oocyte, we used a logarithmic progesterone gradient (0–3 μM) that started 4–5 min after commencing recording and developed over 20 min (see “Experimental Procedures”) (Fig. 1b). In contrast to the progesterone step-induced biphasic response, population responses ($R_{avg}$) showed a smooth ramped rise in [Ca$^{2+}$],, that was detectable within <1 min of progesterone introduction and that usually peaked before the end of the progesterone gradient (latency of peak = 17 ± 1 min, n = 7). [Ca$^{2+}$], then stabilized or decreased slowly. Examination of single cell responses showed a similar pattern: cells generating a gradual increase in [Ca$^{2+}$], that often peaked before completion of the progesterone gradient and fell toward the end of the recording, possibly reflecting desensitization of the response (Fig. 1c). Phasic changes in [Ca$^{2+}$],, superimposed on the raised level of [Ca$^{2+}$],, were often observed (see below), but initial [Ca$^{2+}$], transients of the type characteristic of the response to
Transient responses of human spermatozoa to a stepped progesterone stimulus are well synchronized, with 98% of the cells responding within 20 s of progesterone application (27). In contrast, only 28 ± 3% of the cells generated a significant increase in $\left[ Ca^{2+} \right]_i$ (fluorescence exceeding control levels; $p < 0.05$) within the first 20 s after initiation of a progesterone gradient, with the response rate rising to 66 ± 1 and 96 ± 0.2% after 60 s and 5 min, respectively (seven experiments, 885 cells).

**Gradient Stimulation Induces Oscillations in $\left[ Ca^{2+} \right]_i$**

In addition to causing a slow increase in sperm $\left[ Ca^{2+} \right]_i$, progesterone gradients induced slow $\left[ Ca^{2+} \right]_i$ oscillations in the caudal part of the head of more than one-third of the cells (34 ± 2%; oscillation period = 4.1 ± 0.3 min; seven experiments, 885 cells) (Fig. 1, d and e). The onset of oscillations varied between cells, but typically occurred within 3–10 min of gradient initiation, with 36 ± 5% of the cells generating the upstroke of the first oscillation within 4 min of gradient initiation (Fig. 1d), at a progesterone concentration of <10 nM according to gradient calibration. The subsequent increase in progesterone concentration was never reflected in a discernible increase in the frequency of oscillations, despite an increase in agonist concentration of ≥100-fold after oscillations commenced (Fig. 1d).

Once established, oscillations continued throughout recording, although $\left[ Ca^{2+} \right]_i$ between cycles often decayed, similar to the response in non-oscillating cells (Fig. 1d, pink trace). The amplitude of oscillations was typically a 40–100% increase in fluorescence, at least as great as the $\left[ Ca^{2+} \right]_i$ transient evoked by 3 μM progesterone (Fig. 1a), which had an amplitude of 700 nM in fluorometric measurements (27). The kinetics of $\left[ Ca^{2+} \right]_i$ oscillations varied between cells, possibly reflecting cell/cell differences in $Ca^{2+}$ mobilization and/or clearance processes.

**Progesterone Step-induced Oscillations**

Exposure of capacitated human spermatozoa to a 3 μM progesterone step induced slow oscillations in $\left[ Ca^{2+} \right]_i$, in ∼10% of the cells after the initial transient response (23). In stepped

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**Fig. 1.** a, $\left[ Ca^{2+} \right]_i$ (normalized Oregon Green BAPTA-1/AM fluorescence) in the caudal part of the head of six cells stimulated with a 3 μM progesterone step (shown above the traces) showing the simple biphasic response. b, calibration of gradient stimulation. The five tubes of the gradient maker were filled with 0, 2, 10, 20, and 30 nM FITC, and the resulting gradient was perfused through the imaging chamber while taking measurements of fluorescence intensity at 10-s intervals. FITC at each of the concentrations was also perfused separately to calibrate this gradient. During progesterone gradient experiments, the gradient maker tubes contained 0, 3 nM, 30 nM, 300 nM, and 3 μM progesterone. The arrowheads show estimated times at which these concentrations would be present in the perfusion chamber based upon the FITC calibration. The inset shows results from ELISA analysis of progesterone (micromolar) in the chamber outflow during a progesterone gradient experiment. Gradients in the fluorescence calibration and in the experiment analyzed by ELISA were initiated after 4 min of recording. Measurements correlate well with calibration and show that, between 8 and 20 min, the increase in progesterone concentration is near logarithmic. c, $\left[ Ca^{2+} \right]_i$ in the caudal part of the head of seven cells in which $\left[ Ca^{2+} \right]_i$ increased gradually (similar to the population response) during stimulation with a progesterone gradient (3 nM to 3 μM; shown above the traces). The latency of the response varied considerably between cells, and $\left[ Ca^{2+} \right]_i$ often peaked before the maximum progesterone concentration. d, $\left[ Ca^{2+} \right]_i$ in the caudal part of the head of three cells, two of which generated slow oscillations superimposed on the $\left[ Ca^{2+} \right]_i$ ramp (blue and pink traces) during stimulation with a progesterone gradient (3 nM to 3 μM; shown above the traces). In one of these cells (pink trace), oscillation began within <3 min of the gradient start (<5 nM progesterone). The gradient was initiated after 5 min of recording. The inset shows an overlay of a phase contrast image with a fluorescence image of the same cell collected at the peak of an oscillation, illustrating the localization of the fluorescence signal to the caudal part of the head/mid-piece. e, pseudo-colored images from a series (collected at 0.1 Hz) showing two cells stimulated by a progesterone gradient. Neither cell generated an initial $\left[ Ca^{2+} \right]_i$ transient upon stimulation, but the lower cell displayed a series of $\left[ Ca^{2+} \right]_i$ oscillations (arrows) superimposed on the $\left[ Ca^{2+} \right]_i$ ramp induced by the stimulus.
Oscillations were unaltered, indicating that oscillations were not an artifact of undersampling higher frequency events (aliasing). The frequency and shape of oscillations were independent of the rate at which images were collected (between 0.1 and 0.67 Hz (80 times the observed oscillation cycle frequency). The recorded rate, amplitude, and kinetics of the oscillations were done using stepped progesterone stimuli at 25 °C.

To confirm that the recorded oscillations were not affected by aliasing, we investigated the effect of image acquisition frequency. The recorded rate, amplitude, and kinetics of the oscillations were independent of the rate at which images were collected (between 0.1 and 0.67 Hz). Fig. 2a shows two cells from an experiment in which the sampling rate was changed from 0.1 to 0.67 Hz after 15 min of recording.

**Temperature Sensitivity of [Ca\(^{2+}\)], Oscillations**

In experiments at 31 and 37 °C (four experiments at each temperature; stepped progesterone stimuli), the proportions of cells displaying oscillations (5–15%) and the amplitudes of oscillations (typically a 40–50% increase in fluorescence) were similar to those observed at 25 °C. However, the kinetics (rise and fall) and oscillation rate were markedly temperature-sensitive. The oscillation period at 37 °C (0.39 ± 0.03 min; range of 0.2–0.6 min; four experiments, 298 cells) was 10 times shorter than that at 25 °C (3.4 ± 0.2 min). At 25 °C, the initial progesterone-induced [Ca\(^{2+}\)], transient often had an amplitude and kinetics similar to those of subsequent oscillations (Fig. 2a), but at 31 and 37 °C, the oscillations were much shorter than the transient (Fig. 2d), indicating that oscillations are not generated by periodic repetitions of the initial transient. This conclusion was supported by the observation that the first [Ca\(^{2+}\)], oscillation occasionally activated before decay of the initial [Ca\(^{2+}\)], transient and was superimposed upon it (data not shown). Because of the difficulty of applying drugs via the gradient maker and of maintaining stable recording conditions at temperatures above 25 °C, all subsequent studies in which saline manipulations and drug application were used for characterization of the processes underlying progesterone-induced oscillations were done using stepped progesterone stimuli at 25 °C.

**Is the Induction of Oscillations Reversible?**

After fertilization, the sperm no longer exists as a separate cell. Transformations of signaling and cell activity induced by stimuli during approach to the oocyte (such as progesterone)
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(Fig. 3, the effect of progesterone washout was essentially undetectable in the effect of manipulating Ca\(^{2+}\) oscillations in spermatozoa might be generated similarly, we examined a role in initiation (29). To investigate whether oscillations in all cells, but truncated [Ca\(^{2+}\)] transients were sometimes seen during the first few minutes of EGTA superfusion. d, addition of 1 mM La\(^{3+}\) in bicarbonate-free HEPES-buffered saline caused cessation of large well defined oscillations, but fluctuations in [Ca\(^{2+}\)], restarted in the presence of La\(^{3+}\), indicative of intracellular store release. The bars above the traces indicate the times of application of progesterone and modified salines.

could therefore be irreversible. To investigate this possibility, we observed the effect of removing the progesterone stimulus on established [Ca\(^{2+}\)], oscillations. Three patterns of response were seen. In 10% of the cells generating [Ca\(^{2+}\)], fell toward pre-stimulus levels but recovered discernibly within 1–2 min, and oscillations restarted (latency = 1.5–6 min after progesterone washout; seven experiments, 792 cells) (Fig. 3a, c). In the remaining 50% of the cells, oscillations ceased and did not restart, but these cells resumed oscillating immediately upon restoration of the progesterone stimulus (data not shown).

Ca\(^{2+}\) Influx and Generation of Oscillations

[Ca\(^{2+}\)], oscillations are usually generated by periodic mobilization of [Ca\(^{2+}\)], stores, although Ca\(^{2+}\) influx may play an important role in initiation (29). To investigate whether oscillations in spermatozoa might be generated similarly, we examined the effect of manipulating Ca\(^{2+}\) influx.

Reduction of [Ca\(^{2+}\)]. To reduce any contribution of Ca\(^{2+}\) influx, we used sEBSS with no added Ca\(^{2+}\) (low-Ca\(^{2+}\) sEBSS; measured contaminating [Ca\(^{2+}\)] < 5 μM), a reduction in [Ca\(^{2+}\)], of >200-fold. Superfusion with this medium after establishing progesterone-induced oscillations caused arrest of oscillations in <3% of the oscillating cells (five experiments, 467 cells). In most oscillating cells, the only effect was that [Ca\(^{2+}\)], between cycles fell to levels at or below that seen before the progesterone stimulus, such that oscillations were clearly enlarged (Fig. 3b). The rate of the rise in [Ca\(^{2+}\)], during the upstroke was at least as fast as in normal sEBSS. Upon return to standard sEBSS, oscillations reverted to their previous characteristics. Superfusion of cells with low-Ca\(^{2+}\) sEBSS containing 2 mM EGTA abolished [Ca\(^{2+}\)], oscillations (in some cells, a truncated [Ca\(^{2+}\)], transient was generated before complete arrest) (Fig. 3c), and they did not resume following removal of EGTA (two experiments, 191 cells). Subsequent readmission of standard sEBSS caused a large [Ca\(^{2+}\)], transient (Fig. 3c), but oscillations rarely resumed.

Effect of La\(^{3+}\).—250 μM La\(^{3+}\) strongly attenuates the Ca\(^{2+}\) influx induced by progesterone in human spermatozoa (20). To prevent precipitation, 1 mM La\(^{3+}\) was applied in bicarbonate-free HEPES-buffered medium. Bicarbonate-free medium caused a fall in [Ca\(^{2+}\)], and cessation of oscillation in ~30% of the oscillating cells, despite the continued presence of progesterone (data not shown). Application of La\(^{3+}\) then reduced [Ca\(^{2+}\)], to levels at or below the pre-stimulus concentration and caused arrest of oscillations in all cells (six experiments, 578 cells) (Fig. 3d). In ~30% of the cells, there was a slow rise in
[Ca\(^{2+}\)], on which slow [Ca\(^{2+}\)], ripples or occasional [Ca\(^{2+}\)], transients were sometimes superimposed (Fig. 3d).

The effects of progesterone washout, extracellular EGTA, and blockade of Ca\(^{2+}\) influx by La\(^{3+}\) suggest that sustained progesterone-induced Ca\(^{2+}\) influx contributes significantly to the generation and organization of the oscillations. 10 \(\mu M\) SKF-96365 (which inhibits store-operated channels in sea urchin sperm (30)) failed to inhibit oscillations in cells previously stimulated with progesterone (two experiments, 187 cells) (data not shown). An alternative phasic Ca\(^{2+}\)-entry pathway is through arachidonate-regulated calcium channels, which are activated by arachidonic acid generated by receptor-activated phospholipase (31). The phospholipase inhibitor aristolochic acid (250 \(\mu M\)) had no effect on the oscillations in progesterone-stimulated cells (three experiments, 376 cells) (data not shown).

\section*{Ca\(^{2+}\) Stores and Generation of Oscillations}

\textbf{Effects of Inhibition of Ca\(^{2+}\)-Store ATPases—}In somatic cells, it is usually possible to arrest store-mediated [Ca\(^{2+}\)], oscillations by inhibition of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPases using the inhibitors thapsigargin and cyclopiazonic acid. Thapsigargin at doses between 100 nm (a saturating but specific inhibitory dose in somatic cells (32)) and 1 \(\mu M\) had no effect on established oscillations in spermatozoa (four experiments, 410 cells) (see Supplemental Fig. 1a), and it also failed to prevent induction of oscillations by progesterone (two experiments, 338 cells) (data not shown). In contrast, thapsigargin from the same stock (100 nm to 1 \(\mu M\)) caused store emptying and immediate arrest of [Ca\(^{2+}\)], oscillations in primary rat osteoblasts.\(^2\) 10 \(\mu M\) cyclopiazonic acid had no effect on oscillations (see Supplemental Fig. 1b).

\textbf{Involvement of Phospholipase C/Inositol Triphosphate Receptors—}Treatment of human spermatozoa with progesterone is reported to induce synthesis of inositol trisphosphate (33), which often underlies the generation of [Ca\(^{2+}\)], oscillations in somatic cells (34). Neither 10 \(\mu M\) U-73122 (four experiments, 546 cells) nor 100 \(\mu M\) to 1 \(\mu M\) neomycin (three experiments, 260 cells) (both blockers of phospholipase C) had any effect on the occurrence or characteristics of progesterone-induced [Ca\(^{2+}\)], oscillations (see Supplemental Fig. 2) (2-Aminoethoxydiphenylborate (50–100 \(\mu M\)), an inhibitor of inositol trisphosphate receptors and store-operated channels (35), caused a slight fall in [Ca\(^{2+}\)], in some cells (at 100 \(\mu M\)) but failed to arrest [Ca\(^{2+}\)], oscillations (four experiments, 365 cells) (see Supplemental Fig. 3).

\textbf{Involvement of Ryanodine Receptor (RyRs)—}Treatment of cells with 50–100 \(\mu M\) ryanodine rarely induced oscillations. However, in cells generating [Ca\(^{2+}\)], ripples or irregular [Ca\(^{2+}\)], transients, we commonly observed an increase in amplitude and transition to an oscillating pattern (Fig. 4a). Ryanodine also caused a dose-dependent modulation of frequency in previously established oscillations. 50–100 \(\mu M\) ryanodine speeded up the oscillations (Supplemental Fig. 4), and 500 \(\mu M\) ryanodine reduced their frequency in >80% of the cells (four exper-

\(^2\) M. Foreman, personal communication.
progesterone and tetracaine superfusion. (38% of the cells) (Fig. 4e) at the caudal part of the head and the head/mid-piece junction with BODIPY FL-X ryanodine, with the majority being labeled (three experiments, 721 cells). 45% of the cells became stained (mean percentage (three experiments, 633 cells) (Fig. 5e). Oscillations of low amplitude occasionally persisted in the presence of the drug (c). d, after arrest of oscillations with tetracaine, the oscillations sometimes (in 26% of the cells) recovered upon washout. Similar effects were seen with a dose of 200 μM. The bars above the traces in b–d indicate the periods of progesterone and tetracaine superfusion.

Because the pharmacological experiments implicated RyRs in the generation of the progesterone-initiated [Ca2+]i oscillations, we used BODIPY FL-X ryanodine (a membrane-permeant fluorescein-tagged derivative) (36, 37) to identify RyRs (three experiments, 721 cells). 45% of the cells became stained with BODIPY FL-X ryanodine, with the majority being labeled at the caudal part of the head and the head/mid-piece junction (38%) of the cells) (Fig. 4e, colored arrows). There was also some labeling in the acrosomal area (15% of the cells including those labeled in both areas) (Fig. 4e, white arrow). Comparison with phase contrast images of the same cells showed that very heavy labeling of the mid-piece (<5% of the cells) was often associated with large cytoplasmic droplets (Fig. 4e, yellow arrows). The presence of RyRs was clearly not a result of excessive cytoplasmic retention since most of the labeled cells appeared anatomically normal (Fig. 4e, blue arrows), and some cells possessing cytoplasmic droplets showed labeling behind the nucleus, but not in the droplet itself (Fig. 4e, green arrow).

Tetracaine (25 μM to 2 mM), a potent inhibitor of Ca2+ release through RyRs, inhibited oscillations in a dose-dependent manner (six experiments, 633 cells) (Fig. 5a) and usually caused [Ca2+]i to fall below resting levels (Fig. 5b). In <10% of the oscillating cells, small oscillations persisted in the presence of tetracaine (Fig. 5b, □). Upon removal of tetracaine, oscillations restarted in 26% of the cells in which they had previously arrested (two experiments, 142 cells) (Fig. 5c).

Effect of TMB-8—To further investigate the participation of stored Ca2+ in the ability of human spermatozoa to generate [Ca2+]i oscillations, we investigated the effect of TMB-8, an inhibitor of the release of stored Ca2+ that blocks caffeine-induced Ca2+ mobilization (38). TMB-8 (200 and 300 μM) arrested oscillations in all cells, with [Ca2+]i stabilizing at the level occurring between oscillation cycles (six experiments, 551 cells) (Fig. 5d).

Effect of 2,4-Dinitrophenol—Mitochondria accumulate Ca2+ in the matrix compartment and can contribute to cellular Ca2+ buffering. To confirm that mitochondria were not responsible for the generation of [Ca2+]i oscillations, we applied the uncoupler 2,4-dinitrophenol (100–500 μM) to cells in which [Ca2+]i oscillations had previously been induced by stimulation with progesterone. [Ca2+]i oscillations persisted in the presence of 2,4-dinitrophenol in >98% of the cells (three experiments, 321 cells) (data not shown).

Flagellar Activity

The fluorescence image series collected during stepped addition of progesterone always showed a marked increase in cell movement during the initial progesterone-induced transient [Ca2+]i elevation, clearly visible as pronounced movement of the head and (when in focus) increased flagellar excursion (n >
Fig. 6. 

**Oscillations regulate flagellar activity.** 

*a* Two extracts from an image series (taken at 1.5-s intervals) showing exaggerated movement of the head in a cell that was displaying 

Martine in position to position in successive images was used for assessing flagellar excursion. c, cell showing [Ca2+] oscillations in response to stepped addition of 3 μM progesterone (blue trace). Subsequent assessment of flagellar excursion (see b and “Experimental Procedures”) in this cell showed “bursts” of increased flagellar excursion (pink trace) with a temporal pattern that matches oscillations in [Ca2+]. The scale shows the change in fluorescence and (change in position in micrometers)^2 × 5. The flagellar excursion plot is a 20-point (10-s) moving average. d, [Ca2+] oscillations (blue trace) and assessment of flagellar excursion (pink trace; assessed after collection of fluorescence image series as described for b) in a cell stimulated with a progesterone gradient. The y axis (including flagellar excursion calibration) is as described for b. The flagellar excursion plot is a 20-point (10-s) moving average. e, cell showing a simple progesterone step-induced biphasic response in [Ca2+]. The scale shows the change in fluorescence and (change in position in micrometers)^2 × 5. The flagellar excursion plot is a 20-point (10-s) moving average. f, quantification of flagellar activity reflects beat pattern. The main plot shows a 20-point (10-s) moving average of flagellar excursion ((change in position in micrometers)^2) in a cell in which [Ca2+] oscillations, induced by a progesterone gradient, were already occurring. Each of the insets shows five or six overlaid sequential images of the cell. The numbers on the trace indicate the time period at which each set of images was collected. Cyclical changes in the measurement of flagellar excursion reflect alternation between low amplitude and more extravagant beat patterns. g, simultaneous assessment of [Ca2+] and flagellar excursion using long exposures (800 ms). Images were collected at 0.1 Hz. The plot shows the change in both fluorescence (pink) and flagellar excursion (pink trace), the angle of the “V” formed by the capture of several cycles of flagellar beat during the prolonged exposure. Flagellar excursion clearly mirrors the cyclical oscillation of [Ca2+]. The arrow indicates addition of progesterone (3 μM). The insets show images collected during the control period before progesterone addition (left) and also two [Ca2+] oscillation peaks and the intervening trough.
that did not generate [Ca\(^{2+}\)], oscillations. These movements were often asymmetrical (Fig. 6c and Supplemental Material video). Increasing the rate of image acquisition from 0.1 to 0.67 Hz during recording confirmed that these movements and associated [Ca\(^{2+}\)], oscillations were not sampling/aliasing artifacts. We therefore carried out experiments in which we collected fluorescence images and then observed the same cells under phase-contrast microscopy to assess flagellar movement (lateral excursion of the proximal flagellum) over a time scale appropriate for detecting oscillation-regulated changes. Using frame-by-frame analysis (see “Experimental Procedures”) (Fig. 6b), we detected clear long-term patterns of flagellar activity. In cells showing [Ca\(^{2+}\)], oscillations, induced by stepped or gradient progesterone stimuli, we always observed cycles of flagellar activity with kinetics corresponding closely to those of the [Ca\(^{2+}\)], oscillations in that cell (correlation coefficient for period of oscillation/motility cycles = 0.94; seven experiments) (Fig. 6, c and d). Cells with a smooth [Ca\(^{2+}\)], signal following stimulation with progesterone did not show cyclical flagellar activity (Fig. 6e), and cells with an irregular sustained phase of the progesterone response showed irregular variation in flagellar activity. We examined in detail those cells in which the flagellum was in focus for most or all of its length. During periods assessed as high flagellar activity, more marked bending occurred in the proximal flagellum, and lateral excursion was greatly increased in the distal portion. Sampling at several points during a series of phase contrast images showed that quantification of flagellar activity faithfully reflected repeated transition between patterns of flagellar beating (Fig. 6f).

To reinforce the conclusion that cycling of flagellar activity was not an artifact caused by undersampling, we attempted to visualize the arc of flagellar bending during collection of long exposure fluorescence images (800–1000 ms, sufficient to capture several complete beat cycles). In a small number of cells, the magnitude of excursion of the proximal flagellum was clearly visible as a “V” marking the extremes of the arc described during the beat cycle. Fig. 6g shows a plot of fluorescence intensity and flagellar arc obtained from the same series of long exposures, showing that flagellar activity oscillates in synchrony with [Ca\(^{2+}\)], oscillations.

**Do Oscillations Induce the Acrosome Reaction?**

Repetitive [Ca\(^{2+}\)], spiking might increase the efficacy of progesterone in stimulating AR. To investigate this possibility, we assessed cells for acrosomal status after first imaging their [Ca\(^{2+}\)], response to stimulation with progesterone. In five experiments, cells were stimulated with a 3 μM stepped progesterone stimulus, imaged for 30 min at 25 °C, and then assessed for acrosomal status (see “Experimental Procedures”). Progesterone increased the frequency of AR from 13 ± 3% in controls (n = 3; imaged and assessed for AR without a progesterone stimulus) to 25 ± 2% after progesterone stimulation (p < 0.025; t test of arcsine-transformed data). Progesterone-stimulated cells were then sorted into those that oscillated after the initial [Ca\(^{2+}\)], transient and those that did not, and occurrence of AR was assessed. Fewer of the oscillating cells underwent AR (15 ± 3%) compared with the cells showing the simple biphasic response (29 ± 2%) (p < 0.05, paired t test of arcsine-transformed data; five experiments, 522 cells). A similar analysis of two progesterone gradient experiments confirmed that oscillations did not induce AR, but no significant inhibitory effect was detected.

**DISCUSSION**

A logarithmic progesterone gradient, stimulating the stimulus encountered by the sperm during approach to the egg, induced a response unlike any described previously in human or other mammalian sperm. Cells responded with a [Ca\(^{2+}\)], ramp that (in more than one-third of the cells) triggered slow [Ca\(^{2+}\)], oscillations that did not encode stimulus strength in their frequency. An initial large [Ca\(^{2+}\)], transient, which has been characteristic of all previous reports of the action of progesterone applied as a bolus, was never seen. Stimulation of cells with a 3 μM progesterone step, as reported previously (23), sometimes initiated similar oscillation of [Ca\(^{2+}\)], (after the initial [Ca\(^{2+}\)], transient) but much less frequently. The occurrence of oscillations only in some cells (up to 45%, mean of 34%) is not surprising since human spermatozoa show marked heterogeneity in their functional attributes (39). Furthermore, the calculated rates of occurrence reflect a rigid definition of oscillations (see “Experimental Procedures”). Many cells generated an irregular variation of [Ca\(^{2+}\)], after stimulation with progesterone, which could be converted to regular oscillation by ryphanodine (Fig. 4a).

[Ca\(^{2+}\)], oscillations in spermatozoa have been reported previously, but the progesterone-induced response reported here is very different. Suarez et al. (40) detected rapid oscillation of [Ca\(^{2+}\)], in synchrony with flagellar beat (3.5 Hz), in the tail of hamster sperm, an effect driven by Ca\(^{2+}\), influx. Wood et al. (41) observed rapid [Ca\(^{2+}\)], transients (duration = 0.2–1 s; period = 0.5–5 s; dose-dependent) in speract-stimulated sea urchin spermatozoa, apparently generated by a mechanism involving change in membrane potential and Ni\(^{2+}\)-sensitive Ca\(^{2+}\) influx through voltage-operated Ca\(^{2+}\) channels. In contrast, progesterone-induced oscillations in human spermatozoa are dose-independent, large, and relatively slow (period = 2–5 min at 25 °C and 0.2–0.6 min at 37 °C) and resemble those that, in somatic cells, are almost always generated by mobilization of intracellular stored Ca\(^{2+}\). Meisel et al. (42) reported very small [Ca\(^{2+}\)], ripples in human spermatozoa (duration and period = 10–20 s) superimposed on the transient induced by stepped application of 3.2 μM progesterone, which may be equivalent to the [Ca\(^{2+}\)], “noise” observed in some cells in which regular oscillations did not occur.

**Mechanisms Underlying [Ca\(^{2+}\)], Oscillations in Human Spermatozoa**—The [Ca\(^{2+}\)], oscillations described here could be generated either by periodic regulated influx of extracellular Ca\(^{2+}\) or by cyclical emptying/refilling of an intracellular Ca\(^{2+}\) store (as in somatic cells) (34). Blockade of membrane Ca\(^{2+}\) channels with 1 mM La\(^{3+}\), or use of EGTA-buffered saline (to nullify or reverse the inward Ca\(^{2+}\), gradient) both arrested the oscillations. However, under both conditions, a few cells generated occasional [Ca\(^{2+}\)], transients after abolition of Ca\(^{2+}\), influx, presumably by mobilization of stored Ca\(^{2+}\). More strikingly, oscillations persisted without any reduction in the rate of the rise or peak amplitude in low-Ca\(^{2+}\) EBSS (in which [Ca\(^{2+}\)], was <5 μM). In contrast, reduction of [Ca\(^{2+}\)], to 500 μM abolishes oscillations in sea urchin spermatozoa (41). Established oscillations were arrested by TMB-8 and by tetracaine, inhibitors of the mobilization of Ca\(^{2+}\) stores. All of these observations are compatible with a role for cyclical store emptying/refilling in the generation of [Ca\(^{2+}\)], oscillations in human spermatozoa, but clearly there remains a dependence on Ca\(^{2+}\), influx to maintain the minimum [Ca\(^{2+}\)], necessary for store refilling, as is often the case in somatic cell [Ca\(^{2+}\)], oscillations (29, 43). We have shown that store-operated and arachidonate-regulated calcium channels are unlikely to fulfill this role. Valinomycin and nifedipine (23) and nicardpine and verapamil do not affect [Ca\(^{2+}\)], oscillations in human sperm, indicating that voltage-operated Ca\(^{2+}\) channels do not participate significantly in their generation. Thus, although extracellular

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3 C. V. Harper, unpublished data.
Ca\(^{2+}\) clearly contributes to the generation of [Ca\(^{2+}\)]\(_i\), oscillations, the details of this contribution are far from clear. [Ca\(^{2+}\)]\(_i\), oscillations were highly resistant to thapsigargin, suggesting that sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPases did not contribute significantly to store refilling. The occurrence of thapsigargin-insensitive Ca\(^{2+}\) stores, dependent upon secretory pathway Ca\(^{2+}\)-ATPases, is well documented (44), and expression of these pumps in spermatozoa must be investigated.

We obtained no evidence that inositol trisphosphate receptors participate in the generation of [Ca\(^{2+}\)]\(_i\), oscillations in human sperm, but pharmacological manipulations that affect RyRs were clearly effective. Tetrodamine inhibited oscillations, and caffeine and ryanodine caused oscillations to become larger and more defined. Ryanodine also influenced oscillation frequency in a characteristic dose-dependent manner. The high doses of ryanodine that were required probably reflect (at least in part) slow permeation of ryanodine into the cells, but it is also possible that the RyR in sperm shows unusually low ryanodine sensitivity. RyRs were not detected in bovine spermatozoa (45), but Ry-R-3 has been detected in mouse sperm, and RyR-1 has been detected in immature cells (46). The ability of a temporary progesterone stimulus to switch cells to a prolonged (apparently irreversible) pattern of [Ca\(^{2+}\)]\(_i\) oscillations (Fig. 3a) is consistent with a model in which Ca\(^{2+}\)-induced Ca\(^{2+}\) release, mediated by RyRs, is sufficient for repetitive store mobilization leading to [Ca\(^{2+}\)]\(_i\) oscillation in human sperm.

During spermiogenesis in many species, including humans (47, 48) a membrane complex termed the redundant nuclear envelope is formed in the neck region of the spermatid. This structure is believed to function as a Ca\(^{2+}\) store in bovine spermatozoa and to regulate flagellar beating (see below) (12, 45). [Ca\(^{2+}\)]\(_i\), oscillations and binding of BODIPY FL-X ryanodine in human spermatozoa both appear to occur primarily in the area of the caudal head/mid-piece junction. Since oscillations were not dependent upon mitochondrial Ca\(^{2+}\) uptake (insensitivity to high doses of 2,4-dinitrophenoxy), it is possible that the redundant nuclear envelope also functions as a Ca\(^{2+}\) store in human spermatozoa and contributes to the oscillations reported here.

Functional Significance of [Ca\(^{2+}\)]\(_i\), Oscillations—The [Ca\(^{2+}\)]\(_i\) oscillations do not induce AR (and apparently suppress AR induced by progesterone), so the progesterone stimulus encountered in vivo may not induce significant levels of AR before the cells encounter the zona pellucida. However, in accordance with previous findings that flagellar beat mode is regulated primarily by changes in [Ca\(^{2+}\)]\(_i\), (4, 49), the [Ca\(^{2+}\)]\(_i\), oscillations were synchronized with movements of the sperm head driven by enhancement of flagellar activity during the periods of high [Ca\(^{2+}\)]\(_i\). The increased flagellar bend was less extravagant than hyperactivation as described in human spermatozoa (e.g. Ref. 50), although this may well reflect the restriction caused by cell attachment and/or selection of cells in which visualization of the flagellum was possible. Similar cyclical changes in flagellar beat occur in activated rabbit spermatozoa, with a cycle period of several minutes (51). Mortimer and Swan (52) reported that human spermatozoa reversibly switch between non-hyperactivated and hyperactivated motility. The mean duration of completed periods of hyperactivation observed in that study was 2 s, which is shorter than the typical duration of [Ca\(^{2+}\)]\(_i\), oscillations at 37 °C (Fig. 2d), but because the maximum length of sperm track analyzed was 8.9 s, prolonged transitions would not have been observed.

Once established, progesterone-induced [Ca\(^{2+}\)]\(_i\) oscillations did not change with the strength of the progesterone stimulus, a consequence of the unusual mechanism of their generation, so we consider that cyclical regulation of flagellar activity is likely to be their primary function. Enhancement of motility is vital for penetration of the zona (4). Its failure leads to failure to fertilize in CatSper2-null mice (53). Switching between pushing (low amplitude flagellar bend) and “rocking”/cutting movements of the head is believed to facilitate progress through the zona (54, 55), and we consider this to be a likely role for the repetitive [Ca\(^{2+}\)]\(_i\)-controlled switching of motility. The relationship of the Ca\(^{2+}\)-mobilizing mechanisms responsible for oscillations to the sperm-specific channels CatSper1 and CatSper2, both of which are required for hyperactivation (56), must be elucidated.

In summary, application of progesterone gradients to human spermatozoa initiates a novel response, comprising a [Ca\(^{2+}\)]\(_i\), ramp with superimposed slow (dose-independent) oscillations. These oscillations, which are generated by a mechanism that is most unusual compared with that typically described in somatic cells, do not induce AR but modulate flagellar beat, an effect potentially of great importance in penetration of the egg vestments prior to fertilization.

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