Encoding of progesterone stimulus intensity by intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) in human spermatozoa

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Progesterone induces a biphasic Ca^{2+} influx and consequent acrosome reaction in human spermatozoa. We have used two procedures to vary the stimulus (dosage and prior receptor desensitization) to investigate the encoding of stimulus strength by intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$). Acrosome reaction and amplitude (but not kinetics) of the transient $[Ca^{2+}]_i$ response (population measurement) showed sigmoidal dose sensitivity over the range 0.3 nM–3 μ M, saturating at $\approx$ 300 nM ($ED_{50} \approx$ 30 nM). The amplitude of the sustained response saturated at 3 μ M. Single-cell imaging showed that the amplitudes of both transient and sustained $[Ca^{2+}]_i$ responses were highly dose-dependent, but that their frequency of occurrence and kinetics were largely dose-independent. Fluorimetric measurements confirmed that progesterone-induced $[Ca^{2+}]_i$ influx was subject to desensitization, with second and subsequent applications of 3 μ M progesterone being ineffective. However, sequential additions of 3 nM, 30 nM and 3 μ M progesterone

generated transient $[Ca^{2+}]_i$ responses at each concentration, the amplitude and duration of the response to 3 μ M progesterone being reduced compared with non-pretreated cells. Single-cell imaging revealed that pretreatment had no effect on the proportion of responsive cells, but single-cell responses, similarly to population responses, were smaller and markedly reduced in duration, consistent with an effect of desensitization on a late component of the $[Ca^{2+}]_i$ transient. We conclude that the strength of the progesterone stimulus, when varied by dosage or by desensitization, is encoded by an analogue $[Ca^{2+}]_i$ signal. Dose dependency of the acrosome reaction is therefore determined not by the number of progesterone-responsive cells but by variation in the probability of exocytosis in a 'constant' responsive population.

Key words: acrosome reaction, desensitization, dose–response, imaging.

INTRODUCTION

It is well established that treatment of mammalian spermatozoa with micromolar doses of the steroid hormone progesterone *in vitro* induces Ca^{2+} influx and acrosome reaction (AR), this being one of the most studied examples of non-genomic steroid action [1]. Since progesterone is present in the cumulus oophorus at similar micromolar concentrations [2], this action of the hormone is likely to be of significance during fertilization *in vivo* [1,3]. The ability of progesterone to generate a response of intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) in human spermatozoa has been directly correlated to fertilization success *in vitro* [4], indicating that this response is biologically important.

Both fluorimetric (population) $[Ca^{2+}]_i$ assays and measurements from individual, imaged cells show that the progesterone-activated signal comprises a transient $[Ca^{2+}]_i$ 'spike' (of 30–60 s duration at 37 °C) followed by a sustained ramp or plateau [5–12]. The occurrence of the sustained phase is correlated with prior occurrence of the transient 'spike', indicating a causative link between the two components. Both the transient and sustained phases require influx of Ca^{2+} from the extracellular medium as no response is seen in Ca^{2+} -free incubations [5,13].

Remarkably little is known about the transduction machinery that links progesterone binding to activation of the initial, transient Ca^{2+} influx. Two binding sites for progesterone have been identified in human spermatozoa. One is poorly specific and has a K_d of 40 μ M but the other is specific for progesterone and has a much higher affinity (K_d in the nanomolar range

[14]). The progesterone-induced $[Ca^{2+}]_i$ response, measured fluorimetrically in populations of human spermatozoa, is dose-dependent. A 50 % effective dose (ED_{50}) for the amplitude of the progesterone-induced transient of 50–100 nM has been reported [14–17], possibly corresponding to activation of the specific receptor. Higher doses (up to 100 μ M) increase AR further (>75 % reacted cells [18]), possibly due to stimulation of the lower affinity site, but these levels of progesterone may well be non-physiological. Clearly, the micromolar doses that are believed to occur *in vivo* and are routinely used in fluorimetric and imaging studies *in vitro* are a saturating stimulus for the high-affinity specific receptor. However, the nature of submaximal responses at the single-cell level (and thus how strength of the progesterone stimulus is encoded by the high-affinity receptor and determines the rate of AR) is not known.

In many somatic cells, agonist-induced Ca^{2+} signals occur as 'all-or-nothing', 'quantal' waves or oscillations, signal strength normally being encoded by frequency [19,20]. These responses involve positive feedback through calcium-induced Ca^{2+} release, the initial trigger being an influx of extracellular Ca^{2+} (as in cardiac myocytes [21]) or mobilization of stored Ca^{2+} [19]. Alternatively, signal strength can be encoded simply by the amplitude of a 'passive' analogue $[Ca^{2+}]_i$ signal [20], such as in ATP-stimulated epithelial airway cells [22] and Muller cells [23], noradrenaline-stimulated pinealocytes [24] and bradykinin-stimulated pancreatic acinar cells [25]. Though the progesterone-induced 'spike' in human spermatozoa is normally a single event, it has the properties of a triggered wave, spreading from

Abbreviations used: AR, acrosome reaction; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; FITC-PSA, fluorescent *Pisum sativum* lectin; fura-2/AM, fura-2 acetoxymethyl ester; BAPTA, bis-(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid.

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the equatorial segment [11]. The initial Ca^{2+} influx activates generation of InsP_3 , potentially mobilizing stored Ca^{2+} as part of a regenerative all-or-nothing event [26]. Furthermore, a small proportion of human spermatozoa generate repeated $[\text{Ca}^{2+}]_i$ transients in response to stimulation with progesterone, the $[\text{Ca}^{2+}]_i$ 'spikes' being similar, in amplitude and kinetics, to the initial progesterone-induced transient (J. C. Kirkman-Brown and C. V. Harper, unpublished work). Agonist-induced AR in spermatozoa is unusual in that spermatozoa are secretory cells with a single secretory 'granule'. A quantal Ca^{2+} signal could thus encode signal strength (progesterone concentration) not by frequency but by the proportion of cells in which a response occurs. Alternatively, an analogue signal, generated in all cells capable of a response, could be converted to AR rate by the relationship between $[\text{Ca}^{2+}]_i$ and probability of successful AR.

We have employed two 'physiological' techniques to vary the amplitude of the progesterone-induced $[\text{Ca}^{2+}]_i$ transient, first characterizing the effect fluorimetrically and then investigating how this is encoded at the single-cell level. First, progesterone was applied at a range of doses from 0.3 nM to 30 μM . Second, cells were exposed to a series of 'stepped' progesterone stimuli, a procedure that results in partial or complete inhibition of the response to the second and subsequent steps, probably by desensitization of the receptor [7,13,14,16]. We report that both effects are exerted primarily, if not exclusively, at the level of the individual cell, with signal strength being encoded by the size of the $[\text{Ca}^{2+}]_i$ response.

EXPERIMENTAL

Preparation and capacitation of spermatozoa

All donors were recruited at the Birmingham Women's Hospital, Birmingham, U.K. (HFEA Centre 0119), in accordance with the Human Fertilisation and Embryology Authority Code of Practice. Approval was obtained from the Local Ethical Committee and all donors gave informed consent to the work. Semen was collected from healthy human donors by masturbation after 2–3 days of sexual abstinence and allowed to liquify for 30 min at 37 °C (95:5 air/ CO_2). Highly motile spermatozoa were selected using a direct swim-up procedure where tubes (Falcon 2054) containing 1 ml of supplemented Earle's balanced salt solution medium (with 0.3 % BSA) were underlaid with 0.3 ml of semen. Tubes were then incubated for 1 h at 37 °C and 5 % CO_2 . The top 0.7 ml was removed from each tube, pooled, counted on an improved Neubauer haemocytometer and adjusted to 6×10^6 cells $\cdot \text{ml}^{-1}$. Aliquots of 2 ml (for fluorimetry) and 200 μl (for AR assays and single-cell imaging) were left to capacitate for 6 h at 37 °C and 5 % CO_2 .

AR assays

After capacitation, 200 μl aliquots were incubated with progesterone concentrations of 0.3 nM, 3 nM, 30 nM, 300 nM, 3 μM and 30 μM (final concentrations), ionophore (A23187; 10 μM) or the appropriate solvent (0.05 % DMSO) for 1 h at 37 °C and 5 % CO_2 . Then, 10 min before the end of this incubation, propidium iodide (0.2 $\mu\text{g}/\text{ml}$) was added to each tube. Cells were centrifuged (300 g for 5 min) and smeared on to microscope slides pre-coated with 20 $\mu\text{g}/\text{ml}$ poly-L-lysine solution. Duplicate slides were prepared from each incubation. Cells were stained for acrosomal status using fluorescent *Pisum sativum* lectin (FITC-PSA) according to the methods of Cross

et al. [27]. Briefly, cells were permeabilized in 100 % methanol for 1 min, dried and stained with FITC-PSA (50 $\mu\text{g}/\text{ml}$ in PBS) in a moisture chamber for 45 min at 37 °C. Slides were washed for 15 min in running water, air-dried and mounted using hydromount. AR status was assessed using a Leitz microscope with FITC filters and a 63 $\times$ oil-immersion lens, with 400 cells per treatment being scored over two slides. Only viable cells were scored, as assessed by propidium iodide exclusion. Acrosomal status was determined as described by Cross et al. [27]: acrosome-intact cells showed a fluorescent acrosomal region and acrosome-reacted cells retained fluorescent labelling only in the equatorial region or occasionally retained no fluorescence at all. Progesterone AR was represented as the percentage of stimulation by normalizing to a maximum stimulation by ionophore A23187 (10 μM) and a minimum stimulation by DMSO treatment for each individual experiment, using the equation:

$$\text{Stimulation (\%)} = [(R - \text{DMSO}) / (\text{ionophore} - \text{DMSO})] \times 100$$

where R is the percentage AR in the experimental incubation, DMSO is the percentage AR in the parallel vehicle control and ionophore is the percentage AR in the parallel ionophore-treated incubation.

Fluorimetric measurements

For fluorimetric studies 2 ml aliquots (6×10^6 cells $\cdot \text{ml}^{-1}$) were labelled with 7.5 μM fura-2 acetoxymethyl ester (fura-2/AM) for 12 min at 37 °C and 5 % CO_2 , centrifuged (300 g for 5 min), resuspended in supplemented Earle's balanced salt solution and incubated for a further 17 min. Experiments were carried out using a Perkin-Elmer LS50B fluorescence spectrofluorimeter running Flwinlab version 2.01. The temperature of the cuvette was maintained at 37 °C. Excitation wavelengths alternated between 340 and 380 nm with emission held at 510 nm. Slit width was set at 15 nm with a sampling rate of 12.5 Hz. Dose dependence of the progesterone-induced Ca^{2+} response was assessed by measuring the amplitude of the initial calcium transient after stimulation with progesterone concentrations, ranging from 0.3 nM to 30 μM . Progesterone was added to the cuvette once a level baseline in resting $[\text{Ca}^{2+}]_i$ had been maintained for at least 100 s. For sequential additions, cumulative treatments of 3 nM, 30 nM and 3 μM progesterone were added to the cuvette with a 5–7 min delay between additions. Calibration of $[\text{Ca}^{2+}]_i$ was determined using digitonin (50 μM) to generate a maximal response and subsequent addition of EGTA (20 mM) as a minimal response. In trial experiments it was established that addition of further aliquots of EGTA caused no further reduction in fluorescence ratio. A K_d value of 224 nM was assumed, as in other relevant studies (e.g. [14,28]), and calibration was performed according to the equation of Grynkiewicz et al. [29].

Analysis of fluorimetric data

Resting $[\text{Ca}^{2+}]_i$ was calculated as the average for the 100 s period preceding progesterone addition (electronically event-marked by use of a footpedal). For each trace the transient response was measured by first identifying the absolute maximum value during the transient and then averaging the data for a 4 s period spanning this point. Amplitude of the transient was taken as the difference between the calculated peak $[\text{Ca}^{2+}]_i$ and resting $[\text{Ca}^{2+}]_i$. Time to peak was assessed by dividing the trace into eight 1 s bins (12 samples/bin) between 7 s and 15 s after the

addition of progesterone. Time to peak was taken as the interval between progesterone addition and the midpoint of the bin with the highest mean $[Ca^{2+}]_i$ value. Transient duration was measured directly from the traces as the interval between the addition of progesterone and the inflexion at the end of the decay phase. The amplitude of the sustained response was measured at 180–200 s after progesterone addition.

Single-cell imaging

200 μ l aliquots of capacitated spermatozoa at 6×10^6 cells $\cdot$ ml $^{-1}$ were loaded with Oregon Green bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA 1)-AM (12 μ M final concentration in DMSO dispersed with pluronic F-127) for 30 min at 37 °C and 5 % CO $_2$, then transferred to an imaging chamber, in which the lower surface was a poly-L-lysine-coated coverslip. A further 30 min incubation at 37 °C and 5 % CO $_2$ was then allowed for the cells to adhere. The chamber was then connected to the perfusion apparatus (peristaltic pump and header tank; perfusion rate, 0.5 ml $\cdot$ min $^{-1}$) and at least 10 ml of fresh medium was washed through. All experiments were carried out at 25 °C in a continuous flow of medium. Cells were imaged on an inverted Nikon TE200 microscope, fitted with a Zeiss 75W xenon source and an epifluorescence attachment with excitation and emission filter wheels containing a green filter set (excitation = 485 DF 15; emission = 535 DF 35). Images were taken every 10 s using a 40 $\times$ objective and a Hamamatsu Orca 1 CCD camera. There was a delay of approx. 30 s between addition of progesterone to the perfusion header and generation of the transient response. This delay reflects the travel time of progesterone through the perfusion tube, with the response to progesterone being generated within 3–5 s of exposure to the agonist [12]. The concentration of the progesterone in the superfusing saline was then maintained at a constant level unless stated otherwise.

Data acquisition and storage were controlled by a computer running the AQM Orca 2001 imaging control system (Kinetic Imaging, Bromborough, Wirral, U.K.).

Single-cell data processing

Data were processed offline using the AQM Orca 2001 software. A lasso was drawn around the rear of the head of each spermatozoon in the field of view. The image series was then replayed several times, enabling close inspection of each cell by eye. Cells were removed from analysis if the spermatozoon moved out of the lasso or if the fluorescence faded to zero within the duration of the experiment, assumed to be dye lost due to cell death. This accounted for approx. 8 % of cells. Raw intensity values were imported into Microsoft Excel and normalized using the equation:

$$R = [(F - F_{\text{rest}})/F_{\text{rest}}] \times 100 \%$$

where R is normalized fluorescence intensity, F is fluorescence intensity at time t and F_{rest} is the mean of at least 20 determinations of F taken during the control period. At each time point the normalized fluorescence intensity values (R) for each cell were compiled to generate an overall average normalized head fluorescence (R_{tot}). The total series of R_{tot} were then plotted to give the mean normalized response of head fluorescent intensity for that experiment.

Microsoft Excel was used to calculate the mean and 95 % confidence interval of fluorescence intensity calculated for (i) at least 10 images during the control period immediately prior to

stimulation with progesterone, ($C \pm c$), and (ii) the five images spanning the peak of the transient response [20–60 s after application of progesterone, ($T \pm t$)]. The transient response was considered significant if:

$$T - t > C + c$$

In experiments with sequential additions of progesterone (3 nM, 30 nM then 3 μ M), the control fluorescence ($C \pm c$) used to determine the presence or absence of a significant transient response to the second and third stimuli was obtained by averaging at least six frames immediately prior to that addition of progesterone. In these experiments the cells were sorted into each of the eight possible categories of response (see Table 3, below). Visual inspection of fluorescence/time plots for the individual cells in each of these eight categories confirmed that this technique resulted in successful sorting of the different response categories.

Amplitude of the transient response in individual cells was determined by calculating the mean normalized responses over three frames at the R_{tot} peak. Sustained response of individual cells was calculated as the normalized amplitude 6–7 min (a total of seven frames) after progesterone addition.

Statistical analysis

Dose dependency of progesterone-induced AR was analysed using Dunnett's test (Minitab) to determine difference from control (DMSO). AR percentage data were arcsine transformed prior to testing for significance. Differences between paired sets of fluorimetric experiments were analysed using paired Student's t tests (two-tailed) in Microsoft Excel. Statistical significance was set at $P < 0.05$. All data are presented as mean values $\pm$ S.E.M. ANOVA was performed where more than two data sets were compared.

Materials

Oregon Green BAPTA 1-AM and fura-2/AM were from Molecular Probes (Cambridge Bioscience, Cambridge, U.K.). FITC-PSA, DMSO, fraction V BSA (A-7906), EGTA, progesterone, PBS, Pluronic F-127, A23187 and poly-L-lysine were from Sigma-Aldrich (Poole, Dorset, U.K.). Hydromount was from BDH Merck (Poole, Dorset, U.K.). All chemicals were cell-culture-tested grade where available.

RESULTS

Progesterone-induced AR

Spontaneous AR occurred in $7.3 \pm 1 \%$ of cells ($n = 12$) and exposure to 1 % DMSO (vehicle for ionophore and progesterone) caused no increase above spontaneous levels ($6.7 \pm 1 \%$). Stimulation of cells with 10 μ M A23187 induced a 'maximal' rate of AR of $61.2 \pm 4 \%$ ($P < 5 \times 10^{-8}$ compared with DMSO; $n = 15$; paired Student's t test). Treatment with progesterone elevated AR rate to $20 \pm 2 \%$ ($P < 10^{-6}$ compared with DMSO), a value within the range reported previously by other laboratories using similar or higher doses [14,18,30,31]. Increasing progesterone to 30 μ M had little effect on AR, but at lower doses there was a clear dose–response relationship, the response saturating at 1–3 μ M (Figure 1). Application of Dunnett's test confirmed that stimulation of AR was dose-dependent, stimulation with doses ≥ 300 nM progesterone significantly increasing AR with respect to DMSO controls ($P < 0.05$). When inter-ejaculate variation was

Figure 1 Dose dependence of the action of progesterone

□ shows amplitude of the progesterone-induced $[Ca^{2+}]_i$ transient (fluorimetric measurement; left-hand y axis). ● shows amplitude of the progesterone-induced sustained $[Ca^{2+}]_i$ signal (fluorimetric measurement; left-hand y axis). For □ and ● data are means $\pm$ S.E.M. from 5–11 experiments (the errors may not exceed the size of the symbol). ■ shows progesterone-induced AR (right-hand y axis). Data are means $\pm$ S.E.M. from 9–11 measurements. Stimulation of AR was normalized to the effect of A23187 in parallel incubations (see the Experimental section).

controlled by the use of the paired Student's *t* test, the stimulatory effect of progesterone was significant even at 3 nM ($P < 0.025$ compared with DMSO; $n = 9$; paired *t* test).

Dose dependence of the $[Ca^{2+}]_i$ response

Fluorimetric measurements

Amplitude. Mean calculated resting $[Ca^{2+}]_i$ was 122 ± 7 nM ($n = 83$ experiments). Treatment with 3 μ M progesterone induced a biphasic elevation of $[Ca^{2+}]_i$. The transient phase had a peak amplitude of 649 ± 43 nM and a time to peak of 12 ± 1 s ($n = 14$; Figure 2). The transient then decayed for a period of 40–50 s (averaged transient duration was 49 ± 2 s; $n = 14$) to a point of inflexion 27 ± 5 nM above control resting $[Ca^{2+}]_i$ ($P < 5 \times 10^{-6}$; $n = 14$; paired *t* test), after which there was a secondary sustained component which took the form of a plateau or slowly developing ramp (Figure 2). The amplitude of the sustained $[Ca^{2+}]_i$ response, 3 min after stimulation with progesterone, was 131 ± 11 nM above resting $[Ca^{2+}]_i$ ($P < 5 \times 10^{-7}$; $n = 11$; Figure 2).

Dependence of the transient calcium response on progesterone concentration was assessed by measuring the response to doses over the range 0.3 nM–30 μ M (Figure 1). The maximum response, of approximately 650 nM, was seen at a progesterone concentration of ≥ 300 nM, 50% of the maximal response occurring at approx. 30 nM (which was similar to that of AR). Stimulation with 3 nM progesterone gave a $[Ca^{2+}]_i$ transient of 69 ± 11 nM ($P < 0.0005$ with respect to resting $[Ca^{2+}]_i$; $n = 7$; paired *t* test) and a slight but significant maintained elevation of resting $[Ca^{2+}]_i$ (20 ± 3 nM; $P < 0.025$; $n = 4$; paired *t* test) was seen when cells were stimulated with 0.3 nM progesterone (Figure 1).

The amplitude of the sustained response was also affected by progesterone in a dose-dependent manner. The dose–response relationship was similar to that for the transient response and for

Figure 2 Progesterone-induced biphasic $[Ca^{2+}]_i$ signal

The trace shows a typical fluorimetric (population) $[Ca^{2+}]_i$ response to 3 μ M progesterone (added at time 0). Line A shows the period used for measurement of the initial resting $[Ca^{2+}]_i$; B shows the period used for measurement of the $[Ca^{2+}]_i$ transient; C shows the period used for measurement of the amplitude of the sustained response.

Figure 3 Kinetics of the progesterone-induced $[Ca^{2+}]_i$ transient show little dose dependence

Traces are mean fluorimetric (population) responses from a series of 'parallel' experiments employing doses of 3 nM (green), 30 nM (pale blue), 300 nM (yellow), 3 μ M (dark blue) and 30 μ M (pink) progesterone. In each experiment, aliquots from a single semen sample were used to record the responses to three or more of the five different concentrations of progesterone. The data were then compiled to generate an average response for each concentration, each average including 5–11 recordings (see Table 1). Axes shows time (s) after progesterone addition and change in $[Ca^{2+}]_i$ (nM). The inset shows the traces scaled to a standard amplitude to facilitate comparison. The 3 nM response in the inset was smoothed using six point moving average.

AR. Maximum response was seen at 3 μ M progesterone, 30 μ M being slightly less effective (Figure 1).

Kinetics. For comparison of response kinetics, we compiled mean responses, using data from seven experiments in which the effects of a range of doses of progesterone were observed by 'parallel' incubations of aliquots from the same sample. Figure 3 shows the mean transients evoked by 3 nM, 30 nM, 300 nM, 3 μ M, and 30 μ M progesterone. Although amplitude was strongly dose-dependent (Figure 3; Table 1), the kinetics of the transient responses at a concentration of 30 nM progesterone and above were essentially similar (Figure 3). Duration of the transient, though showing some variation, was not dose-dependent

Table 1 Amplitude and kinetics of the fluorimetric (population) transient $[Ca^{2+}]_i$ response induced by progesterone at a range of doses

Values are means $\pm$ S.E.M. n shows the number of experiments summarized in each column. P shows the results of ANOVA analysis to determine contribution of dose to the observed variation.

	3 nM	30 nM	300 nM	3 μ M	30 μ M	P
Amplitude at peak (nM)	69 $\pm$ 11	384 $\pm$ 126	733 $\pm$ 156	689 $\pm$ 71	624 $\pm$ 147	$< 8 \times 10^{-4}$
Time to peak (s)	11 $\pm$ 1	9 $\pm$ 1	8 $\pm$ 1	12 $\pm$ 1	8 $\pm$ 1	$< 1 \times 10^{-4}$
Duration (s)	53 $\pm$ 5	44 $\pm$ 2	50 $\pm$ 3	49 $\pm$ 2	50 $\pm$ 2	> 0.25
Amplitude at inflection (nM)	11 $\pm$ 7	-9 $\pm$ 11	1 $\pm$ 9	25 $\pm$ 6	9 $\pm$ 4	< 0.05
n	7	7	8	11	5	

($P > 0.25$; ANOVA) and time to peak, despite varying significantly between doses ($P < 1 \times 10^{-4}$; ANOVA) showed no dose-dependent trend (Table 1).

Imaging of single-cell responses

Frequency of transient responses. After exclusion of cells that showed significant dye loss or excessive movement (around 8% of cells; see the Experimental section), approx. 98% of cells in control experiments responded to 3 μ M progesterone with a significant transient response (seven experiments; 1407 cells). In order to determine whether the dose dependence of the effect of progesterone reflected a reduction in the number of responsive cells, we assessed the rate of response to 3 nM and 30 nM doses of progesterone. The percentage of cells in which significant transient responses could be detected after stimulus with 3 nM progesterone was 72% ($n = 6$; 1210 cells), and after stimulus with 30 nM progesterone was 88% ($n = 3$; 553 cells).

Amplitude of transient responses. Analysis of the amplitude of single-cell transient responses, using three experiments for each dose (3 nM, 725 cells; 30 nM, 553 cells; 3 μ M, 595 cells) showed marked dose dependency (Figure 4). Mean response amplitudes at the three doses were $13 \pm 4\%$ at 3 nM ($n = 3$; 504 responding cells), $25 \pm 7\%$ at 30 nM ($n = 3$; 506 responding cells) and $43 \pm 9\%$ at 3 μ M ($n = 3$; 575 responding cells). Amplitude distributions at all doses were bell-shaped, the coefficient of variation remaining consistent despite the change in amplitude (3 nM, 0.21; 30 nM, 0.18; 3 μ M, 0.17). At 3 nM there was a distinct leftward skew to the distribution (Figure 4).

Kinetics of transient responses. The inherent noise in traces from live human spermatozoa [12] made it impossible to carry out accurate assessment of single-cell transient duration. We therefore assembled mean single-cell responses from the nine experiments (three at each dose) that were used to compile amplitude histograms. The response kinetics appeared similar at each dose (Figure 4, insets).

Sustained responses. The frequency of occurrence of significant sustained responses was lower than that for transient responses. Although there was apparently some dose dependency in the rate of occurrence [$41 \pm 15\%$ of cells at 3 nM (three experiments, $n = 499$); and $54 \pm 3\%$ at 3 μ M (three experiments, $n = 575$)], this effect was not significant ($P > 0.25$).

Since it is known that the occurrence of the sustained response is related to the amplitude of the preceding transient [12], we exploited the wide range of amplitudes provided by the various doses employed in this study to examine the relationship between

Figure 4 The amplitude of single-cell $[Ca^{2+}]_i$ transients is dose-dependent

The plot shows the amplitude distributions for single-cell transient responses (percentage increase in fluorescence) evoked by treatment with 3 nM progesterone (P; ●; three experiments; 725 cells), 30 nM progesterone (□; three experiments; 553 cells) and 3 μ M progesterone (■; four experiments; 759 cells). Data are presented as relative frequencies to facilitate comparison between the groups of experiments. Insets show mean single-cell responses at each of the three concentrations (lower panel) and responses normalized to a standard amplitude to facilitate comparison (upper panel). Note that the kinetics of the responses are not dose dependent.

the amplitudes of the transient and sustained responses. Figure 5 shows data from three experiments (one at each dose). The data for 3 nM and 3 μ M are from two aliquots from the same sample. There was a significant, positive relationship between the transient component of the $[Ca^{2+}]_i$ response and the fluorescence intensity measured 6–7 min after application of progesterone. The gradient of the fitted regression is 0.25 ($P < 5 \times 10^{-12}$). The amplitude of sustained responses was clearly dose-dependent. In those cells that showed a significant transient response, the mean increase in

Figure 5 Relationship between transient amplitude and the sustained $[Ca^{2+}]_i$ response in single-cell responses

The increase in fluorescence 3 min after application of progesterone has been plotted against the peak amplitude of the transient response for all responsive cells in three representative imaging experiments in which cells were stimulated with 3 nM (●; 71 cells), 30 nM (□; 124 cells) or 3 μ M (■; 67 cells) progesterone. The 3 nM and 3 μ M were carried out on two aliquots from the same experiment. There is a clear positive relationship between transient amplitude and the sustained $[Ca^{2+}]_i$ response; calculated regression, $y = 0.24x - 0.51$.

Figure 6 Desensitization of the response to progesterone (P)

(a) Stimulation with 3 μM progesterone abolishes the response to a second, similar stimulus. Fluorimetric (population) trace showing response of [Ca²⁺]_i to two sequential additions of 3 μM progesterone (arrows). The first addition of progesterone to the cuvette evoked the characteristic biphasic response but the second addition resulted in no measurable Ca²⁺ influx but a small, transient reduction in [Ca²⁺]_i occurred in three out of five experiments. The scale bars show 100 nM [Ca²⁺]_i and 50 s. The inset shows the responses to the first and second additions of progesterone overlaid for comparison (the second response has been smoothed using a six-point moving average to reduce noise). (b) Sub-micromolar doses of progesterone partially desensitize the cells to a subsequent, larger stimulus. The trace shows a typical fluorimetric (population) response to sequential (cumulative) additions of progesterone. Additions of 3 nM, 30 nM and 3 μM progesterone were made at the points shown by arrows. Similar results were seen in 10 other experiments. The responses to 30 nM and 3 μM progesterone were significantly smaller than those seen in samples which had received no pretreatment. The scale bar shows 50 nM [Ca²⁺]_i and 100 s.

fluorescence measured 6–7 min after progesterone addition was $4 \pm 1\%$ at 3 nM (71 cells), $7 \pm 1\%$ at 30 nM (124 cells) and $8 \pm 2\%$ at 3 μM (67 cells).

Serial (stepped) progesterone stimuli

Fluorimetric measurements

Amplitude. Responsiveness of cell populations to repeated progesterone stimuli was analysed by serial (cumulative) additions to the cuvette. After stimulation with 3 μM progesterone, cell populations were re-stimulated with a further 3 μM progesterone after a 3–6 min delay (Figure 6a). As described previously [13,14], upon application of the second stimulus

Figure 7 Desensitizing effect of progesterone (P) pretreatment on the amplitude of the calcium transient is dose-dependent

Bars show mean $\pm$ S.E.M. amplitude of the [Ca²⁺]_i transient induced by treatment with 30 nM (left-hand panel; $n = 5$) or 3 μM (right-hand panel; $n = 5$) progesterone under control conditions or after pretreatment with lower doses of progesterone. Experiments were carried out in parallel using aliquots from the same sample to assess control and desensitized responses. Axes show concentration of pretreatments (M) and amplitude of the transient response.

there was no elevation in [Ca²⁺]_i. However, in three out of five experiments, addition of the second dose of progesterone was followed by a slight reduction in [Ca²⁺]_i, lasting approx. 30 s (e.g. Figure 6a, inset), which was never seen in DMSO controls.

To investigate the dose dependence of the effect of prior stimulation, cell populations were stimulated sequentially with 3 nM, 30 nM and then 3 μM progesterone with a 5–7 min delay between additions. Transient responses were observed at each concentration (Figure 6b). The amplitude of the 3-nM-progesterone-evoked transient in these experiments was 35 ± 5 nM. Subsequent application of 30 nM progesterone evoked a second transient of 138 ± 15 nM, and the final application of 3 μM progesterone evoked a further transient of 276.5 ± 24.5 nM ($n = 11$). The amplitudes of the 30 nM and 3 μM progesterone transients were significantly reduced compared with those seen in parallel aliquots without progesterone pretreatments ($P < 0.025$; $n = 5$; parallel experiments; Figure 7). Interestingly, prior treatment with 3 nM progesterone was as effective in reducing the response to 30 nM progesterone (by 60–65%) as was 33 nM in reducing the responses to 3 μM progesterone. It was noticeable that these reduced responses had a marked ‘undershoot’ at the end of the transient, before development of the sustained response (compare the responses to 3 μM progesterone in Figures 2 and 6b). Visual inspection of the traces suggested that the sustained response to progesterone may also have been smaller in preparations that had received prior stimuli (Figure 6b). However, since the sustained response evoked by prior stimulation was present (and still slowly developing), no attempt was made to quantify this effect.

Kinetics. As well as reducing the amplitude of the progesterone-induced transient, prior stimulation also modified the kinetics of the response. Figure 8(a) shows average traces compiled from five sets of parallel experiments in which we measured responses to 3 μM progesterone, both during the pretreatment (serial addition) experiments (Figure 8a, red line) and also under control conditions (Figure 8a, blue line). In preparations pretreated with 33 nM

Figure 8 Effects of partial desensitization are similar in populations and single cells

(a) Mean fluorimetric (population) responses from five sets of parallel experiments stimulated with $3 \mu\text{M}$ progesterone (P) under control conditions (blue line) and after previous addition of 3 nM and then 30 nM progesterone (red line). The green line shows the pretreatment-sensitive component of the transient (the difference between the two traces), obtained by subtraction. The pretreatment-sensitive component has been smoothed using a 20-point moving average. (b) Responses of individual spermatozoa to sequential steps of 3 nM , 30 nM and $3 \mu\text{M}$ progesterone (final concentration, $3.033 \mu\text{M}$). The upper panel shows an image series of a single spermatozoon, pseudo-coloured to reflect fluorescence intensity (pseudo-colour scale shown to the right of the images). An increase in fluorescence (primarily localized to the post-acrosomal region) occurs after each addition of progesterone. Numbers show time in minutes from first progesterone addition (3 nM). Note that, although attached, the cell retains motility. The lower panel shows traces of two cells from the same experiment, which also responded to all three progesterone concentrations (3 nM , 30 nM and $3 \mu\text{M}$). A differing amplitude of response is seen between concentrations. Noise is partly due to movement of the sperm (see above). (c) Data from two parallel single-cell experiments showing mean traces from cells that responded to $3 \mu\text{M}$ progesterone. Blue line (162 cells) shows cells from a control experiment in which a single addition of $3 \mu\text{M}$ progesterone was used. Red line shows data from a parallel pretreatment experiment in which cells had previously been exposed to 3 nM and then 30 nM progesterone (pink line; 132 cells). Cells that failed to respond to pretreatment were excluded from the average. Green line shows the pretreatment-sensitive component of the transient (the difference between the two traces), obtained by subtraction. Compare with (a).

progesterone there was a significant reduction, not only in amplitude but also in the duration and the time to peak of the transient response (Table 2). The 'undershoot' between the end of the transient response and development of the sustained response is again clearly visible. The green line (Figure 8a) shows the difference between the two responses (pretreatment-sensitive component), showing that prior stimulation particularly affects a 'late' component of the transient calcium response.

Imaging of single-cell responses

To investigate the effect of serial progesterone treatments on single cells we used the same series of concentrations as those used for fluorimetric experiments (increments of 3 nM , 30 nM and $3 \mu\text{M}$) with a 7 min delay between additions. Records from the individual cells in six such experiments (1210 cells in total) were sorted according to whether they responded significantly to the addition of progesterone (see the Experimental section; Table 3). In these experiments 96% of the cells responded to $3 \mu\text{M}$ progesterone (similar to non-pretreated controls) and 68%

Table 2 Amplitude and kinetics of the fluorimetric (population) transient $[Ca^{2+}]_i$ response to $3 \mu\text{M}$ progesterone under control conditions and after pretreatment with 33 nM progesterone

Values are means $\pm$ S.E.M. *n* shows number of paired experiments (control and pretreated from the same sample). *P* shows results of paired *t* tests.

	Controls	Pretreated	<i>n</i>	<i>P</i>
Peak amplitude (nM)	554 ± 110	212 ± 20	5	< 0.025
Time to peak (s)	12 ± 1	8 ± 1	5	< 0.005
Peak duration (s)	48 ± 3	40 ± 2	5	< 0.0025
Amplitude at inflection (nM)	26 ± 10	-12 ± 10	5	< 0.05

of the cells generated significant $[Ca^{2+}]_i$ transients in response to each of the three progesterone stimuli in the series. Figure 8(b) shows a series of pseudo-colour images from an individual cell (upper panel; clearly showing repeated responses in the posterior sperm head) and the traces of two cells that responded to all three stimuli (lower panel).

Table 3 Responsiveness of individual cells to sequential stimulation with 3 nM, 30 nM and 3 μ M progesterone

Columns 2–9 show the percentage of cells in each of the eight possible categories of response for each of the six experiments. The final column shows the total number of cells in each experiment (1210 cells).

Experiment	Percentage of cells								Total no. of cells
	No response	3 nM only	30 nM only	3 μ M only	3 nM + 30 nM	30 nM + 3 μ M	3 nM + 3 μ M	3 nM + 30 nM + 3 μ M	
1	0.7	1.1	0	2.8	1.4	11.4	9.6	73.0	281
2	0.5	0.2	0.2	1.4	2.2	25.5	2.4	67.5	416
3	0.8	0.8	0	0.8	0	6.3	3.9	87.4	127
4	2.1	3.4	0.7	1.4	2.7	6.8	1.4	82.2	147
5	0.6	0	1.2	4.3	0.6	45.7	0	47.5	162
6	1.3	2.6	1.3	10.4	2.6	41.6	3.9	36.3	77
Mean percentage	0.68	0.71	0.30	2.15	1.79	21.75	4.01	68.61

Figure 9 Amplitude of single-cell $[Ca^{2+}]_i$ transients is reduced by pretreatment

The plot shows the amplitude distributions for single-cell transient responses (percentage increase in fluorescence) evoked by treatment with 3 μ M progesterone (P) controls (■; $n = 3$ experiments; 575 cells) and 3 μ M progesterone in series experiments after responding to 33 nM progesterone pretreatment (□; $n = 3$ experiments; 682 cells). Data are presented as relative frequencies to facilitate comparison between the groups of experiments.

Of the six single-cell imaging experiments in which cells were exposed to sequential, stepped doses of progesterone, three were carried out in parallel with control experiments in which aliquots from the same sample were treated with 3 μ M progesterone alone. The amplitudes of 3 μ M progesterone-induced transient $[Ca^{2+}]_i$ responses were calculated for cells treated with 3 μ M progesterone only (575 cells; $n = 3$ experiments) and for cells from parallel pretreatment experiments which had already responded to 3 nM and/or 30 nM progesterone (682 cells, $n = 3$). In control cells the amplitude distribution of significant transient responses was bell-shaped with a mean of $43 \pm 1\%$. After pretreatment the single-cell transient response to the same dose of progesterone (3 μ M) was shifted leftwards with a mean amplitude of $29 \pm 1\%$, significantly smaller than in parallel controls with no pretreatments ($P < 2.5 \times 10^{-59}$; unpaired t test; Figure 9).

Since the kinetics of fluorimetric responses to 3 μ M progesterone were markedly altered by pretreatment, we examined the kinetics of single-cell responses. Due to the difficulty of accurate determination of transient duration in single-cell records (see above), we assembled mean responses from those cells in which progesterone induced a significant transient response. Figure 8(c) shows traces from a pair of parallel experiments (3 μ M control and 3 μ M after pretreatment). As with the fluorimetric recordings, the duration of the mean single-

cell response to 3 μ M progesterone after 33 nM pretreatment (Figure 8c, pink line; 132 cells) was reduced compared with the response without pretreatment (Figure 8c, blue line; 162 cells) and there was an undershoot at the end of the transient (Figure 8c). The green line on Figure 8(c) shows the difference between the traces (pretreatment-sensitive component). Note that no sustained response was detectable in the single-cell average for cells pretreated with 33 nM progesterone. This pattern of response was also seen in the other two pairs of parallel experiments.

Effect of pretreatment on progesterone-induced AR

The response to 3 μ M progesterone was reduced in both amplitude and duration by pretreatment with 3 nM progesterone. We therefore investigated the effect of 3 nM pretreatment on the ability of 3 μ M progesterone to induce AR. In six experiments cells were exposed to 3 μ M progesterone, as in normal AR assays, or first exposed to 3 nM progesterone followed, after a 7 min delay, by addition of 3 μ M progesterone. AR in aliquots receiving pretreatment with 3 nM progesterone showed greatly reduced levels of progesterone-induced AR ($P < 0.01$ compared with non-pretreated controls; paired t test) (results not shown).

DISCUSSION

Progesterone, at micromolar concentrations, caused a biphasic increase in $[Ca^{2+}]_i$ and stimulation of AR as reported previously [7,8,12,15,32]. Single-cell analysis showed that nearly all of the cells, which remained viable and adhered throughout the experiment (approx. 98%), respond to 3 μ M progesterone with a $[Ca^{2+}]_i$ transient.

Progesterone dose and encoding of the progesterone stimulus

Use of different doses of progesterone showed that the amplitudes of the transient and sustained population responses were dose-dependent over the range 0.3–300 nM. The kinetics of the transient response showed no significant dose sensitivity, although the transient in fluorimetric traces did appear to activate and decay more slowly at the lowest effective doses of progesterone (Figure 3). The ED_{50} for induction of the $[Ca^{2+}]_i$ transient was approximately 30 nM, similar to the value of 50 nM reported previously by Baldi and colleagues [14,15] and consistent with activation of the high-affinity selective progesterone receptor. The sustained response had a similar (though not identical) dose sensitivity. Interestingly, spermatozoa from other mammals (such as the goat [33]) show a similar sensitivity to progesterone and doses above 15 μ M progesterone have been shown to be effective even in the octopus [34]. Data from studies on the mouse

suggest that rodent spermatozoa require much higher doses of progesterone to exert equivalent effects [3], although a recent study on rat spermatozoa found that induction of AR in these cells saturated at doses between 1 μ M and 10 μ M [35].

Data from single-cell imaging show a clear association between the occurrence of transient and sustained components of the progesterone-induced $[Ca^{2+}]_i$ response [12], suggesting that the transient response contributes to the probability of a subsequent sustained Ca^{2+} influx. Although separate transduction of the progesterone-induced transient- and sustained- $[Ca^{2+}]_i$ signals cannot be discounted, we consider it likely that their similar dose sensitivity reflects such a causative link.

We compared the single-cell transient responses with progesterone at 3 nM, 30 nM and 3 μ M, doses that generated transient $[Ca^{2+}]_i$ responses with amplitudes (assessed fluorimetrically) differing by more than an order of magnitude. Despite this large dose dependence of the population $[Ca^{2+}]_i$ response, the proportion of cells generating a significant transient increase in fluorescence at 3 nM was 72 % (compared with 98 % at 3 μ M). In contrast, the amplitude distribution of responses at 3 nM was shifted markedly to the left, the average amplitude of the fluorescence transient being only 30% of that at 3 μ M. Since (i) the negative skew of the 'spike' amplitude distribution at 3 nM progesterone (Figure 4) suggests that the smallest transient responses were not detectable and (ii) the fluorescence intensity of Oregon Green BAPTA 1 is a non-linear function of $[Ca^{2+}]$ (such that the amplitudes of the fluorescence signal will greatly underestimate the reduction in amplitude of the $[Ca^{2+}]_i$ transient [36]) it appears that, in human spermatozoa, the strength of the non-genomic progesterone stimulus is encoded primarily by the amplitude of the cellular $[Ca^{2+}]_i$ signal. Analysis of single-cell sustained responses and of the relationship between the amplitudes of transient and sustained responses shows that the sustained signal is similarly encoded, possibly due to causative linkage between the two parts of the stimulus (see above).

Effects of pretreatments on progesterone sensitivity

In agreement with previous reports, our fluorimetric recordings showed that, when spermatozoa were exposed to two sequential additions of 3 μ M progesterone (cumulative dose of 6 μ M), the second stimulus evoked no detectable $[Ca^{2+}]_i$ signal. This is believed to reflect receptor desensitization [13,14]. When sub-saturating doses of progesterone were used for the first stimulus, a subsequent higher dose was able to induce a further response. Assessment of the $[Ca^{2+}]_i$ transient amplitude showed that such responses were significantly smaller than those recorded in parallel experiments using cells not previously exposed to progesterone, there being a clear dose dependence of the effect. Desensitization induced by the steroidal sigma ligand RU 3117, which probably acts directly at the extragenomic progesterone receptor, is also dose dependent [17]. In single-cell experiments, carried out using similar protocols, the proportion of cells that responded to a 3 μ M progesterone stimulus was not reduced by prior exposure to 33 nM progesterone and most cells responded to all three sequential stimuli. We conclude that the reduction in the amplitude of the population $[Ca^{2+}]_i$ response caused by prior stimulation with progesterone was due entirely to a reduction in the amplitude of single-cell responses, not a reduction in the number of responsive cells. Intriguingly, both fluorimetric and single-cell records showed that pretreatment with progesterone inhibited primarily the later part of the transient response, having little effect on the initial rate of rise of $[Ca^{2+}]_i$ (Figures 8a and 8c). We have observed a similar 'dissection' of the transient

response after pretreatment with nifedipine [37], suggesting that the transient is composed of at least two separable components, the latter of which is nifedipine-sensitive and is strongly desensitized by pretreatment with progesterone. Assessment of the sustained response is difficult in pretreatment experiments, since the pretreatment itself induces a sustained response that is still present (and possibly still developing) during subsequent applications(s) of progesterone. However, our data suggest that the sustained response is attenuated in preparations that have been pretreated with low doses of progesterone, presumably reflecting the causative link between transient and sustained components of the response and/or an effect on a direct contribution of receptor binding to sustained Ca^{2+} influx.

A further observation in these experiments was a $[Ca^{2+}]_i$ 'undershoot', occurring between the end of the transient phase and the beginning of the sustained $[Ca^{2+}]_i$ signal. Whether this is a 'new' component of the response, caused by serial application of stimuli, or is an inhibitory action (possibly related to the effect of RU486 [7]) 'revealed' due to the truncation of the transient phase of the response, cannot be determined from our data.

Ca^{2+} signalling and AR

We have used single-cell imaging to analyse two 'physiological' mechanisms by which the amplitude of the progesterone-induced $[Ca^{2+}]_i$ response of human spermatozoa is regulated. In both cases we conclude that the progesterone-induced $[Ca^{2+}]_i$ response does not involve positive feedback leading to an all-or-nothing 'quantal' $[Ca^{2+}]_i$ response but is an analogue signal directly encoding the strength of the stimulus. For the individual spermatozoon, the 'decision' as to whether the cell will undergo AR in response to a sub-saturating dose of progesterone therefore occurs not at the point of transduction of the progesterone stimulus but at a stage downstream of Ca^{2+} mobilization.

This leads to further conclusions regarding stimulus-secretion coupling in human spermatozoa. In a 'digital' signalling system, secretion is switched on by a large increase in $[Ca^{2+}]_i$ to near micromolar levels, probably employing a low-affinity Ca^{2+} trigger, dose dependence of AR being determined 'digitally' by the proportion of cells that generate a full $[Ca^{2+}]_i$ response (Figure 10a). In contrast, in an analogue system, a higher-affinity receptor (such as calmodulin) would convert an analogue $[Ca^{2+}]_i$ signal to a probability of secretion in each spermatozoon (Figure 10b). Although our measurements of $[Ca^{2+}]_i$ do not permit assessment of the $[Ca^{2+}]$ at the site of excitation-secretion coupling, AR is induced even at doses of progesterone that cause barely detectable elevation of $[Ca^{2+}]_i$, a finding that is consistent with stimulus-secretion coupling employing a high-affinity Ca^{2+} receptor.

Interestingly, the components of the $[Ca^{2+}]_i$ response (transient and sustained) have similar dose dependence (see above). Since AR shows a similar dose sensitivity, either or both components of the $[Ca^{2+}]_i$ signal may be important. Using excess EGTA to 'freeze' Ca^{2+} influx, we have observed significant levels of AR occurring before the induction of the sustained signal indicating the importance of the $[Ca^{2+}]_i$ transient (C. V. Harper, unpublished work).

From the data presented here and by others [14–17], it appears that the micromolar doses of progesterone normally used for *in vitro* studies on human spermatozoa are saturating, at least for responses evoked through occupation of the high-affinity receptor. If progesterone is present in the cumulus and follicular fluid at such concentrations then effective, AR-inducing doses will be encountered prior to penetration of the cumulus mass.

Figure 10 Alternative models for encoding by $[Ca^{2+}]_i$ of a sub-saturating dose of progesterone (P) and determination of the rate of AR

(a) Shows a quantal model. The progesterone-induced signal is an all-or-nothing event, involving positive feedback (as in $[Ca^{2+}]_i$ waves and oscillations). At sub-saturating doses the response of the cell population is coded by the percentage of cells in which the response occurs. In individual cells the induction of AR (in AR-competent cells) is a slave relay. In the one responsive cell, the probability of exocytosis is 1, but in the two non-responsive cells it is 0. (b) Shows an analogue model. All (or most) cells generate a $[Ca^{2+}]_i$ signal in response to progesterone, even at doses well below saturation of the response. The strength of the response is coded by the amplitude of the $[Ca^{2+}]_i$ signal, which determines the probability of AR in an cell. In the example shown, the progesterone-induced $[Ca^{2+}]_i$ signal results in a probability of AR of 0.33 in all responsive cells, such that one of the three cells undergoes exocytosis. The data presented in this paper show that determination of AR rate in human spermatozoa is by an analogue system of coding.

As cells approach the cumulus they will encounter a progesterone gradient. The study of Sato et al. [38] on the action of thyrotropin-releasing hormone on GH₄C₁ cells shows that ramp and stimuli may generate a smaller peak $[Ca^{2+}]_i$ than the 'instantaneous' change in concentration used here and in parallel studies on spermatozoa. An important future target must be to investigate the response of spermatozoa to a ramp progesterone stimulus, simulating approach to the oocyte.

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