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Biphasic Elevation of $[Ca^{2+}]_i$ in Individual Human Spermatozoa Exposed to Progesterone

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Fluorimetric studies on progesterone-induced $[Ca^{2+}]_i$ signalling in mammalian spermatozoa show both the well-characterised $[Ca^{2+}]_i$ transient and a subsequent sustained phase. However, the sustained phase is thought to reflect release of the fluorochrome during the acrosome reaction and has not been subject to critical investigation. We have used single-cell imaging of $[Ca^{2+}]_i$ to analyse the progesterone-induced $[Ca^{2+}]_i$ response in large numbers (>2000) of capacitated, human spermatozoa. In 70% of cells, treatment with progesterone induced a transient increase, which typically peaked within 1 min and decayed with a similar time course. Upon rapid application of progesterone this response peaked within 5–20 s. In 35% of progesterone-treated spermatozoa a sustained elevation of $[Ca^{2+}]_i$ occurred, which became discernible during the falling phase of the transient response and persisted for at least 20 min. Both $[Ca^{2+}]_i$ responses were localised to the postacrosomal region. Averaging of large numbers of single cell responses generated traces similar to those seen in fluorimetric studies. Although the sustained response was strongly associated with the initial, transient response, a few spermatozoa generated sustained responses that were not preceded by a significant transient response (5% of cells). It is concluded that a genuine biphasic $[Ca^{2+}]_i$ signal is activated by progesterone and that the sustained response is a discrete signalling event with biological significance.

Key Words: **human spermatozoa; progesterone; calcium; acrosome reaction.**

INTRODUCTION

In all animal species studied to date, induction of the acrosome reaction (AR) by biological agonists involves influx of extracellular Ca^{2+}. In sea urchins, fucose sulphate polymers (FSPs) induce a biphasic influx, which involves activation of two discrete ion channels. The first phase is sensitive to dihydropyridines and verapamil, antagonists of L-type voltage-operated Ca^{2+} channels (VOCCs), but the second phase (activated after 5 s) is insensitive to these blockers (Guerrero and Darszon, 1989a,b; Schackman, 1989). Induction of AR, in mouse or bovine spermatozoa *in vitro*, by solubilised zona pellucida (ZP) is also achieved via activation of (at least) two Ca^{2+} influx pathways. An early event is activation of a dihydropyridine-sensitive T-type VOCC, blockade of which inhibits both the $[Ca^{2+}]_i$ response and the induction of AR (Arnoult *et al.*, 1996, 1999). Subsequent to T-channel activation a sustained elevation of $[Ca^{2+}]_i$ occurs, which must reflect release of stored Ca^{2+} or activation of another Ca^{2+} influx pathway, the identity of which is unknown (Florman *et al.*, 1998; Darszon *et al.*, 1999; Publicover and Barratt, 1999).

In mammalian spermatozoa, the steroid hormone progesterone has also been shown to induce Ca^{2+} influx and AR (Aitken, 1997). This effect is observed at micromolar concentrations of progesterone, similar to those in the cumulus oophorus (Osman *et al.*, 1989), and is therefore likely to be of significance during fertilisation *in vivo* (Fisher *et al.*, 1998; Garcia and Meizel, 1999). For human spermatozoa, progesterone is the only biological agonist for which detailed study has been undertaken. Upon application of progesterone, a rapid (within seconds) elevation of $[Ca^{2+}]_i$ is observed which initiates in the midhead region, peaks within 1 min, and decays with a similar time course (Blackmore *et al.*, 1990; Foresta *et al.*, 1993; Plant *et al.*,

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1995; Aitken *et al.*, 1996; Tesarik *et al.*, 1996; Meizel *et al.*, 1997). This $[Ca^{2+}]_i$ transient is mediated by Ca^{2+} influx since it is abolished by metal cations (such as La^{3+}, a nonselective Ca^{2+} channel blocker) or in low-Ca^{2+} medium (Blackmore *et al.*, 1990; Plant *et al.*, 1995; Aitken *et al.*, 1996). Progesterone-induced Ca^{2+} influx is accompanied by depolarisation of the membrane potential (Foresta *et al.*, 1993) but it appears that VOCCs are not involved in the immediate $[Ca^{2+}]_i$ response (Foresta *et al.*, 1993; Aitken *et al.*, 1996; Garcia and Meizel, 1999; Blackmore and Eisoldt, 1999). However, dihydropyridine VOCC agonists and antagonists are reported to modulate progesterone-induced AR in humans (O'Toole *et al.*, 1996) and mice (Shi and Roldan, 1995), suggesting that these agents might act at a stage subsequent to the initial Ca^{2+} influx.

Progesterone, similar to FSPs and ZP, may induce a secondary, sustained $[Ca^{2+}]_i$ response, which is yet to be characterised (Aitken, 1997). Though most reports detail only the initial transient response, several laboratories report a sustained elevation of $[Ca^{2+}]_i$, which occurs after the initial $[Ca^{2+}]_i$ transient, taking the form of a ramp or plateau (Yang *et al.*, 1994; Bonnacorsi *et al.*, 1995) or a discrete second transient (Tesarik *et al.*, 1996). A sustained plateau or tail of $[Ca^{2+}]_i$ following an initial spike is common in somatic cells and is often due to activation of capacitative Ca^{2+} influx (Clementi and Meldolesi, 1996). In spermatozoa, inhibition of tyrosine kinase with genistein or herbimycin A is reported to inhibit both the sustained phase of the $[Ca^{2+}]_i$ response (but not the initial transient; Bonnacorsi *et al.*, 1995; Tesarik *et al.*, 1996) and the induction of AR. These findings suggest that the sustained $[Ca^{2+}]_i$ response is vital to successful activation of AR by progesterone. However, the validity of the sustained response is open to question. The amplitude of this response in spectrofluorimetric studies is directly related to the percentage of cells undergoing the AR (Garcia and Meizel, 1996, 1999). A simple and plausible explanation of this correlation is that the secondary $[Ca^{2+}]_i$ plateau is artefactual and reflects escape of the Ca^{2+}-sensitive fluorescent dye from spermatozoa undergoing AR, giving a false indication of elevated $[Ca^{2+}]_i$ (Garcia and Meizel, 1996).

To determine the true nature of the secondary "$[Ca^{2+}]_i$" response it is necessary to undertake single-cell imaging to determine (a) whether the sustained $[Ca^{2+}]_i$ ramp/plateau is a characteristic of Ca^{2+} signalling of individual spermatozoa or reflects averaging of phasic events occurring throughout the cell population (as might be predicted if it is a result of AR) and (b) whether it is localised primarily to the area surrounding the outer acrosomal membrane (as would be predicted if it is an artefact caused by escape of the Ca^{2+}-sensitive fluorochrome). To date only one such study has been attempted. Tesarik *et al.* (1996) used confocal, single-cell imaging to observe the effects of progesterone on $[Ca^{2+}]_i$ in Fluo-3-loaded human spermatozoa. Secondary $[Ca^{2+}]_i$ responses were observed but the kinetics were fundamentally different from those observed in the spectrofluorimetric studies and the data are difficult to interpret. The secondary response occurred as a discrete peak, which initiated between 2 and 10 min after progesterone application, had a variable duration, and ended abruptly. Fluorescence then fell below the initial resting level, a response that was interpreted as loss of the fluorochrome (Fluo-3) during AR (Tesarik *et al.*, 1996). Only one cell that displayed a secondary $[Ca^{2+}]_i$ response that was not followed by an abrupt fall was reported. Furthermore, Fluo-3 fluorescence was distributed throughout the head of the cells (Tesarik *et al.*, 1996), suggesting that significant loading of fluorochrome into the acrosome may have been a factor in the observed response. It must, therefore, be considered likely that the secondary response seen in these experiments reflects influx of Ca^{2+} into the acrosome as a result of membrane rearrangements prior to AR (as has been suggested for ZP-stimulated hamster spermatozoa; Shirakawa and Miyazaki, 1999) and is thus a consequence and not a cause of AR. It is therefore impossible, on the basis of the data currently available, to assess the validity of the secondary "$[Ca^{2+}]_i$" response to progesterone.

Elevation of $[Ca^{2+}]_i$ is pivotal to induction of AR, and progesterone is the only well-characterised biological agonist of AR in the human and is potentially of significance in inducing or modulating AR in all mammals. In order to understand the action of progesterone in inducing AR it is crucial that the validity of the second $[Ca^{2+}]_i$ signal is, unequivocally, confirmed or refuted. The nature of the sustained response (if present) must be investigated at the single-cell level. We have undertaken confocal imaging in order to resolve this issue. In these experiments we have imaged large numbers of cells (100–300 per experiment; >2000 in total), we have undertaken detailed analysis of the response of each cell to progesterone, and we have used a continuous flow system to avoid accumulation of any escaped fluorochrome in the medium. We have consistently observed a secondary $[Ca^{2+}]_i$ ramp/plateau that occurs in approximately 35% of cells, primarily, but not exclusively, those that show an initial transient response. The response occurs in the posterior head and is not localised to the area surrounding the outer acrosomal membrane. We conclude that progesterone induces a genuine sustained elevation of $[Ca^{2+}]_i$ which is a potentially vital component of the $[Ca^{2+}]_i$ signal.

MATERIALS AND METHODS

Preparation and Capacitation of Spermatozoa

All donors were recruited at the Birmingham Women's Hospital (HFEA Centre 0119), in accordance with the Human Fertilisation and Embryology Authority Code of Practice. Human ejaculated spermatozoa were obtained from normal healthy donors of proven fertility by masturbation. After semen liquefaction (approximately 30 min), motile spermatozoa were harvested by swim-up (Mortimer, 1994). Briefly, 2 ml of supplemented Earle's balanced salt solution (sEBSS) + 0.3% BSA was underlayered with 1 ml of liquefied semen in a 15-ml Blue Max tube (Becton–Dickinson, U.S.A.). The tube was then incubated at an angle of 45° for 1 h at 37°C, 5% CO_2. After 1 h the upper 1.75 ml of medium (containing

the motile fraction of spermatozoa) was carefully removed using a sterile transfer pipette. The concentration of the collected spermatozoa was assessed using a Neubauer counting chamber according to WHO methods (WHO, 1999) and adjusted to 6×10^6 cells/ml with sEBSS + 0.3% BSA. Aliquots of spermatozoa were capacitated (100-μl aliquots for AR experiments; 200-μl for confocal microscopy studies of calcium influx) for 6 h at 37°C, 5% CO_2.

Confocal Imaging

Aliquots of capacitated spermatozoa (200 μl) were labelled in the incubator (5% CO_2, 37°C) with 15 μM Calcium Green-1 AM. After 30 min, the entire aliquot was gently introduced into a purpose-built, perfusable, imaging chamber (volume 200 μl), the lower surface of which consisted of a coverslip (previously coated with 10% poly-L-lysine solution and air-dried) for viewing on an inverted microscope. The chamber was then placed in the incubator for a further 30 min. This period of time allowed for further labelling and for the spermatozoa to adhere to the coverslip.

After the second labelling period the chamber was placed on the stage of the confocal microscope (Bio-Rad MRC 600 system on Nikon Diaphot inverted) and perfused with at least 10 ml of sEBSS medium at the standard perfusion rate (0.4 ml/min) to remove all traces of extracellular dye. Cells were imaged using a ×40 objective. Fluorescence excitation was from an argon ion laser filtered at 488-nm narrow bandpass (488DF10). Emitted fluorescence was filtered at 540 nm with a bandpass filter (540DF30). In order to minimise bleaching, intensity of exciting illumination was reduced to 1% of maximum by a neutral density filter. Perfusion and subsequent experiments were carried out at room temperature (24 ± 1°C).

All experiments consisted of a 5-min control period of perfusion with sEBSS followed by perfusion of the recording chamber with progesterone (3.2 μM) in sEBSS. Trial experiments were carried out to observe the time of arrival of coloured dyes at the area scanned by the microscope. Allowance was made for this delay and the time shown on the figures as the point of application indicates the estimated time of arrival of progesterone at the cell membrane. In most experiments images were captured at intervals of 15 s. For experiments to observe the rise time of the primary transient, the scanning area was reduced and images were captured at intervals of 3 s. For these experiments, preparation of spermatozoa was carried out as above but application of progesterone was by addition of 1 ml of 3.2 μM progesterone in sEBSS directly to the bath (over a period of 3 s), upstream of the cells.

Confocal Data Processing and Analysis

Data was processed offline using Lucida software (Kinetic Imaging Ltd., UK). Using the computer mouse, an ovoid was drawn around the head of each spermatozoon in the field of view. For each cell the average intensity within the head was obtained for every image in the series (typically 120 images over a 30-min recording period) and plotted against time. To exclude any cells which moved significantly during the experiment, the first and last images of each series were compared. If the cell had moved such that the defined area no longer contained the entire sperm head, the data from that cell were not included in analysis.

Raw intensity values were imported into Microsoft Excel and normalised using the equation

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

where R is normalised fluorescence intensity, F is fluorescence intensity at time t, and F_{rest} is the mean of at least 10 determinations of F taken during the control period.

At each time point the normalised fluorescence intensity values (R) for each cell were compiled to generate an overall average normalised head fluorescence (R_{tot}). The total series of R_{tot} were then plotted to give the mean normalised response of head fluorescence intensity for that experiment.

Preliminary analysis of average plots (R_{tot}) and data from our own fluorimeter studies (Punt, Barratt, and Publicover, unpublished) confirmed that our cells gave a two-component $[Ca^{2+}]_i$ response to progesterone. In order to analyse this response at the single-cell level, detailed analysis of the response of each cell was undertaken. For each cell, Excel was used to calculate the mean, and 95% confidence interval of fluorescence intensity for (i) at least 10 images during the control period ($C \pm c$), (ii) the four images spanning the time for the peak of the transient response [as assessed from R_{tot} for that experiment ($T \pm t$)], and (iii) 12 images collected during the period from 15 to 18 min after progesterone application [the sustained response ($S \pm s$)]. The transient response was considered significant if

$$T - t > C + c.$$

The sustained responses was considered significant if

$$S - s > C + c.$$

This analysis gave presence/absence of each of the two components of the response for each cell. Cells were sorted into those showing no response to progesterone, the transient response only, transient and sustained responses, or sustained response only. Visual inspection of fluorescence–time plots for the cells in each of these four categories confirmed that this technique resulted in successful sorting of the different response categories.

For analysis of the relationship between occurrence of the transient and the sustained responses, data from all experiments accepted for analysis (see below) were combined to generate total numbers for each of the four categories of response (no response, transient response only, transient and sustained responses, and sustained response only). Deviation from the null hypothesis (random association between the two responses) was tested using a χ^2 contingency table.

Criteria for Acceptance/Rejection of Data

When cells were viewed for 15–30 min without any experimental manipulation, fluorescence intensity was essentially stable. However, in a few cases there was a slow decrease or increase in fluorescence intensity (up to 10% over 30 min) which was apparent in R_{tot}. Any trend was always visible from the start of the experiment and often stabilised after 10–15 min such that there was a net change of only a few percent over 30 min. However, to avoid generation of spurious data (particularly with respect to the sustained response), data from experiments in which a trend in R_{tot} was visible during the initial control period were not used. Of a total of 15 experiments, 7 were used for analysis.

In experiments in which the population of spermatozoa gave a stable level of fluorescence there were sometimes a few cells which showed a marked trend during the control period. Dead or dying sperm do not retain dye (Tesarik *et al.*, 1996). Since cells were continuously superfused, any dye loss became apparent almost immediately. The few cells in which fluorescence intensity drifted downward, possibly indicating escape of dye, were excluded from

analysis. Most cells also retained flagellar motility (e.g., Fig. 1), confirming viability. In a few cells a marked upward drift of fluorescence, during the control period, was apparent, possibly reflecting loss of $[Ca^{2+}]_i$ homeostasis. These cells were also excluded from analysis.

Assessment of Progesterone-Induced Acrosome Reaction

After capacitation, as described above, 100-μl aliquots of spermatozoa were stimulated with either progesterone (final concentration of 3.2 μM) or A23187 (10 μM) or received solvent control (0.05% DMSO). After an incubation of 60 min, spermatozoa were centrifuged briefly (300*g* for 5 min), the supernatant was removed, and the spermatozoa were resuspended in 0.5 ml of hypo-osmotic swelling (HOS) medium (0.74% sodium citrate, 1.35% fructose in double-distilled H_2O). After 45min incubation in HOS medium, the spermatozoa were centrifuged (300*g* for 5 min) and resuspended in a minimal volume of HOS medium (20 μl), smeared on microscope slides (duplicate slides, previously coated with 10% poly-L-lysine solution), and air-dried.

Following permeabilisation by immersion in methanol (2 min), the acrosome was labelled by incubation with 50 μg/ml FITC-PSA in PBS for 45 min in a moisture chamber at 37°C. Slides were then washed in a constant flow of mains water for 15 min before air-drying and mounting with Fluoromount. Slides were stored refrigerated and kept in darkness to prevent fade.

Fluorescence microscopy was used to evaluate acrosomal status (Cross *et al.*, 1988); slides were scored blind and only viable (curly tailed) spermatozoa were scored (Aitken *et al.*, 1993). Acrosomal status was assessed as described elsewhere (Mendoza *et al.*, 1992). A total of 200 spermatozoa were scored per treatment (100 per slide).

All calculations and statistical analyses were performed using the statistics module of Microsoft Excel for Macintosh. An arcsine transformation of AR percentage data was performed prior to testing for significance between treatment groups. Paired *t* tests (two tailed) were performed to test for significance. Statistical significance was set at $P < 0.05$.

Materials

Progesterone (4-pregnene-3,20-dione), A23187 ionophore (free acid), DMSO, poly-L-lysine, *Pisum sativum* (pea) agglutinin FITC-labelled, sodium citrate, Pluronic F-127, and fructose were obtained from Sigma (Poole, Dorset); Calcium Green-1 AM was from Molecular Probes (Leiden, the Netherlands); Pentex fraction V BSA (pH 7) was from Bayer-Pentex (Newbury, Berkshire); supplemented Earle's balanced salt solution (without phenol red) was from Gibco BRL94189H (Paisley, Scotland; special order Cat No. 041); and Fluoromount was from BDH Merck (Poole, Dorset).

RESULTS

Confocal images showed that human spermatozoa labelled effectively with Calcium Green 1, fluorescence being localised primarily to the postacrosomal region (Figs. 1 and 2). Application of DMSO (vehicle) had no effect upon fluorescence intensity.

Initial (Transient) Response to Progesterone

Upon addition of progesterone (3.2 μM) to the superfusing medium, the majority of spermatozoa showed a rapid, significant increase in $[Ca^{2+}]_i$ in the posterior head, which sometimes extended into the midpiece (Figs. 1 and 2). Elevation of $[Ca^{2+}]_i$ began within seconds of progesterone application, peaked within 1–2 min, and then decreased with a similar time course (Fig. 1). This response was seen in all experiments. In the seven experiments chosen for detailed analysis the proportion of cells showing this response was 71.8 ± 7.3% ($n = 7$; Table 1).

To observe the kinetics of activation of the transient response, two further experiments were carried out in which samples were taken at intervals of 3 s (rather than 15 s), over a period of 90 s. Progesterone was applied by direct addition to the perfusing medium, upstream of the cells. In these experiments the rise time of the response (initiation to peak) was rapid but variable. In a small proportion of cells the transient response rose from 0 to >75% of peak amplitude within 3 s, but for most cells the time from initiation to peak was 10–15 s (Fig. 2). It was noticeable that there was a delay of 3–5 s between application of progesterone to the bath and initiation of the transient response (Fig. 2).

The amplitude of the significant transient $[Ca^{2+}]_i$ response was typically a 20–40% increase in fluorescence intensity over levels before application of progesterone. However, a "tail" of larger responses up to 200% was present and in a very small number of cells the transient was much greater (Fig. 4).

Second (Sustained) Response to Progesterone

In all of the seven experiments selected for detailed analysis, a proportion of the spermatozoa (mean 34.9 ± 6.9%; $n = 7$) showed a significant secondary rise in $[Ca^{2+}]_i$ (Figs. 1 and 3; Table 1). This sustained response became apparent 2–5 min after addition of progesterone to the superfusing medium, during or just after the falling phase of the initial response, and lasted until the end of recording (15–25 min after application of progesterone; Figs. 1 and 3). The secondary rise in fluorescence was clearly visible in the average trace, R_{tot}, derived by averaging the normalised responses of all spermatozoa in the field of view (Fig. 3). Images acquired during the sustained (secondary) phase of the response showed that elevation of fluorescence occurred in the postacrosomal region of the head of the spermatozoa (Fig. 1). There was no indication that the response was localised to the acrosomal area, even in those cells that showed the largest responses.

The amplitude of the sustained response varied from being a plateau, slightly elevated above control $[Ca^{2+}]_i$, to being a steeply ramped response which rose to levels similar to those occurring during the initial, transient response (Fig. 3). Analysis of the distribution of amplitudes of significant sustained increases, 15–18 min after application of progesterone, showed a pattern broadly similar to

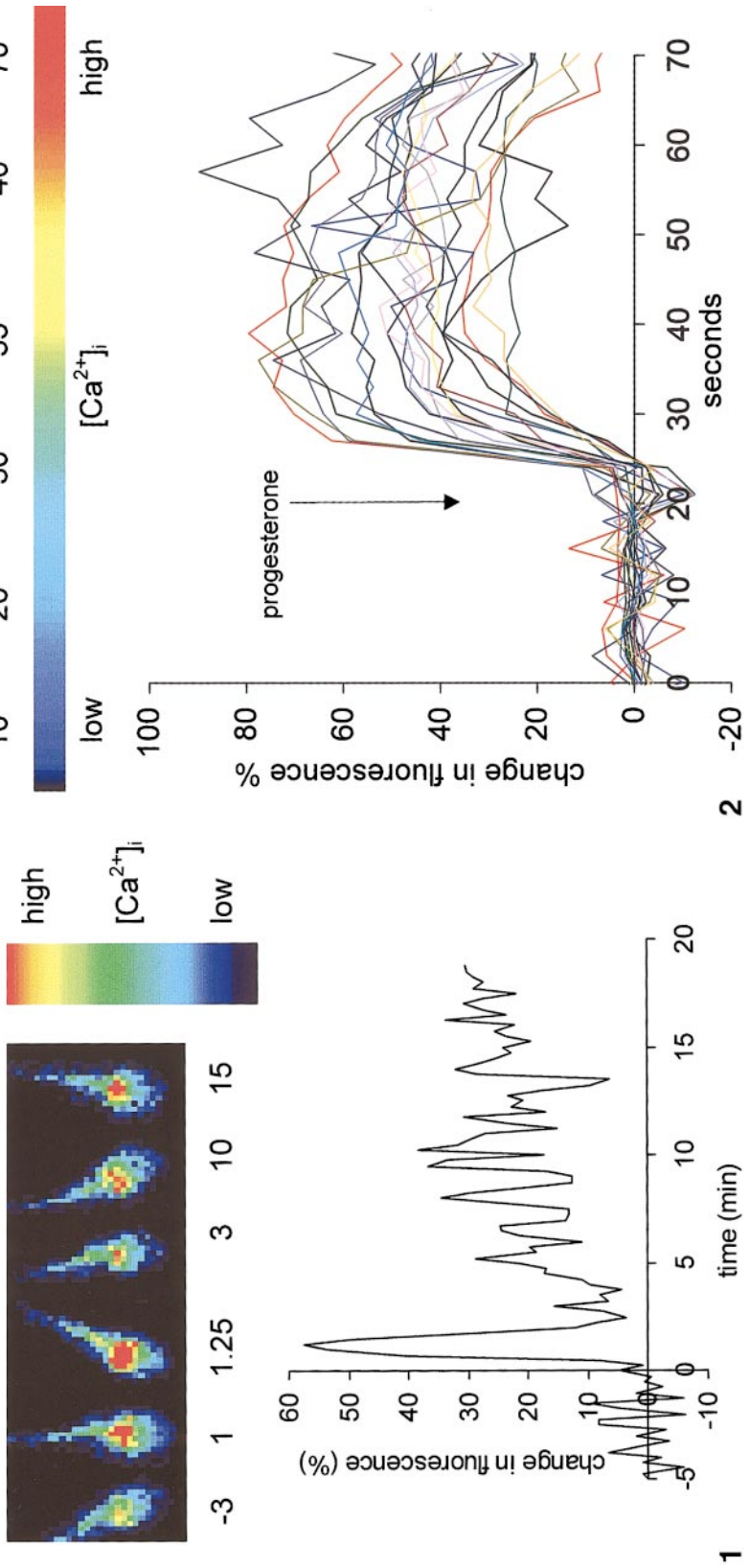

FIG. 1. Response of a single spermatozoon to progesterone (3.2 μM). Images at top are pseudo-coloured to show intensity of fluorescence. Scale is shown on right ("warm" colours show high $[Ca^{2+}]_i$). Numbers below the images indicate time in minutes relative to application of progesterone. Changes in fluorescence are primarily localised to the postacrosomal region. Graph at bottom shows a plot of normalised fluorescence for the same cell. Response occurs as a transient followed by a plateau/ramp. The noise associated with this record reflects slight movements of the sperm head due to continuing motility of the tail (note the changing position of the tail in the image series).

FIG. 2. Kinetics of the transient response. Graph shows normalised fluorescence of 20 individual spermatozoa, imaged at 3-s intervals. After direct application of 3.2 μM progesterone to the perfusion bath there is a delay of approximately 5 s after which fluorescence rises rapidly to peak. Time from initiation of the response to the peak varies between cells, from 3 to 20 s. Pseudo-colour images above the graph show a single cell, at the times indicated (seconds) from the start of recording (as on the graph). Warm colours show high $[Ca^{2+}]_i$. Changes in fluorescence occur in the postacrosomal region.

TABLE 1

Summary of Data Obtained in the Seven Experiments Used for Detailed Analysis

	No. of cells (% of total)					No. of cells (% of total)		
Experiment	T only	T and S	S only	No response	Total	Total T	Total S	Total AR
1	114 (63.0)	37 (20.5)	3 (1.7)	27 (14.9)	181	151 (83.5)	40 (22.2)	—
2	54 (47.0)	17 (14.8)	10 (8.7)	34 (29.6)	115	71 (61.8)	27 (23.5)	19.5
3	83 (41.0)	71 (35.1)	8 (4.0)	40 (19.8)	202	154 (76.2)	79 (39.1)	—
4	87 (27.0)	205 (63.6)	12 (3.7)	18 (5.6)	322	292 (90.6)	217 (67.3)	21.1
5	295 (42.8)	320 (46.5)	17 (2.5)	57 (8.2)	689	615 (89.3)	337 (49.0)	16.5
6	190 (46.9)	77 (19.0)	33 (8.1)	105 (25.9)	405	267 (65.9)	110 (27.1)	—
7	98 (27.6)	28 (7.9)	28 (7.9)	201 (56.9)	355	126 (35.3)	56 (15.8)	17.6
Total cells	921	755	111	482	2269	1676	866	
Mean %	42.2 ± 4.7	29.6 ± 7.5	5.2 ± 1.1	22.9 ± 6.5		71.8 ± 7.3	34.9 ± 6.9	

Note. Numbers of cells, with number of cells as a percentage of all cells in that experiment in parentheses. Columns 2–5 show responses sorted by category: T, transient response; S, sustained response; T and S, both transient and sustained responses. Columns for "total T" and "total S" give details of total occurrence of each of these responses. Total AR shows, for those experiments in which both $[Ca^{2+}]_i$ monitoring and AR assay were undertaken, the total percentage of AR live sperm, including both progesterone-induced and spontaneous AR.

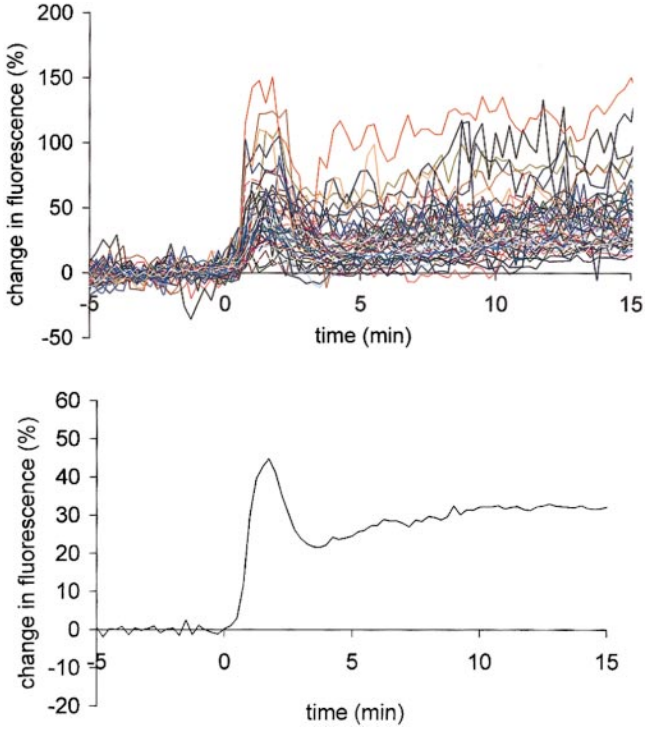

FIG. 3. Summation of individual cell responses generates a two-phase population response. Top: 40 superimposed, single-cell records from experiment 3 (all from responsive cells). Transient responses are well synchronised, the majority being an increase in fluorescence intensity of approximately 40%. Sustained responses are typically 20–40% after 15 min. Largest sustained responses have been omitted. Bottom: Mean response (R_{tot}) for all 202 cells in experiment 3. Note the smooth biphasic nature of the response, similar to that seen in fluorimetric studies.

that for the transient response. Normalised fluorescence for the majority of cells fell in the range 0–60% (modal response of 20–30%). However, the proportion of very large responses was much more marked than in the transient response (Fig. 4). Of the spermatozoa in which a significant sustained response occurred, 6.7% (58/866) had a normalised amplitude at 15–18 min after progesterone of >100%. In comparison, only 2.9% (48/1676) of significant transient responses were of this amplitude ($P < 0.000005$). For cells showing a normalised response of >200% the figures were 2.9% (25/866) for the sustained response and 0.8% (14/1676) for the transient response ($P < 0.0001$).

Relationship between Transient and Sustained Responses

The percentage of progesterone-responsive cells varied considerably between preparations (Table 1), but there was a clear correlation between the proportion of cells giving a significant transient response and the proportion in which a sustained elevation of $[Ca^{2+}]_i$ occurred ($R = 0.74$). Analysis of the occurrence of transient and sustained responses at the level of individual cells showed that the association between the two responses was highly nonrandom ($P < 10^{-30}$; χ^2), the occurrence of the sustained response being heavily biased towards those cells in which a transient response occurred (Table 1; Fig. 3). Assessment of the amplitude of transient responses in the seven experiments selected for detailed analysis showed no consistent difference between those cells which also gave a sustained response and those in which the transient response occurred in isolation (Table 2). The mean transient response was larger in cells which also gave sustained response in only three of the seven experiments ($P = 0.60$; paired t test) and this difference was significant ($P < 0.05$) in only one case.

In all experiments a small proportion of cells (mean 5.2 ±

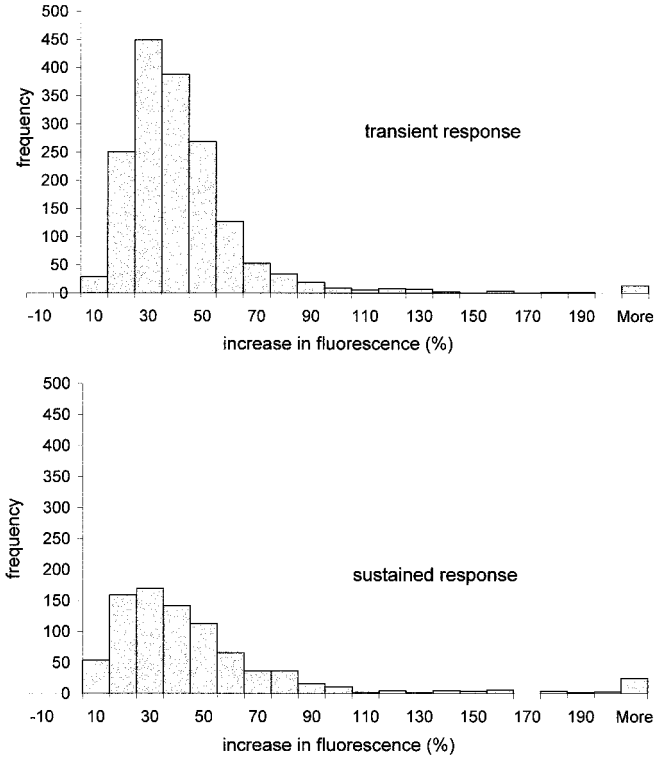

FIG. 4. Summary amplitude distributions for all significant transient and sustained responses. Top: Transient responses—normalised amplitude at time of peak for R_{tot}. Bottom: Sustained responses—normalised amplitude after 15–18 min. Note that amplitude distribution for sustained response has a slightly lower modal value but a more pronounced tail.

1.1% of the total population; $n = 7$) showed a sustained increase in fluorescence which was not preceded by a significant transient response. Detailed examination of the responses of these cells showed that, in many cases (approximately 50%) there was a small (5–15%), nonsignificant, transient increase in fluorescence, followed by a much larger secondary response. However, in the remaining cells no transient was detectable. These cells showed only a sustained increase in fluorescence, which commenced 4–6 min after progesterone application and persisted to the end of the recording period (Fig. 5).

Induction of Acrosome Reaction

Assessment of AR by staining with FITC-PSA showed that, in control experiments (incubated with DMSO in the absence of progesterone), acrosome reaction occurred in 9.6 + 2.1% of spermatozoa (mean ± SEM; $n = 11$). Treatment of samples with progesterone increased the percentage of acrosome-reacted spermatozoa to 19.3 + 2.7% ($n = 11$; $P < 0.001$). In four of the experiments used for detailed analysis of progesterone-induced $[Ca^{2+}]_i$ signalling, parallel assessment of progesterone-induced AR was undertaken. In every case the percentage of cells showing a sustained response was greater than the level of agonist-induced AR and in three of these experiments the percentage of cells showing a sustained response exceeded the total of spontaneous and agonist-induced, acrosome-reacted, live cells (Table 1).

TABLE 2

Relationship between Occurrence of the Sustained Response and Amplitude of the Transient Response

Experiment	T only	T+S	*P*
1	25.9 ± 1.2 (114)	33.8 ± 2.6 (37)	<0.005
2	56.2 ± 1.2 (54)	31.7 ± 4.0 (17)	0.066
3	44.5 ± 3.7 (83)	39.0 ± 4.8 (71)	0.24
4	35.1 ± 4.0 (87)	39.8 ± 1.5 (205)	0.27
5	37.8 ± 1.4 (295)	41.4 ± 1.5 (320)	0.08
6	40.7 ± 3.3 (190)	38.0 ± 1.7 (77)	0.61
7	35.6 ± 6.8 (98)	35.0 ± 7.5 (28)	0.95

Note. For each of the seven experiments used for detailed analysis, the mean amplitude (±SEM) of the transient response (as percentage increase over control fluorescence) was calculated for those cells that showed a transient response only (T only—column 2) and for those that gave a transient response which was followed by a sustained response (T+S; column 3). Numbers in parentheses show number of cells in each group. *P* shows level of significance for the difference between mean values for each experiment (*t* test).

DISCUSSION

This study is the first detailed analysis of progesterone-induced $[Ca^{2+}]_i$ signalling in large numbers of individual human spermatozoa. We have found the response to pro-

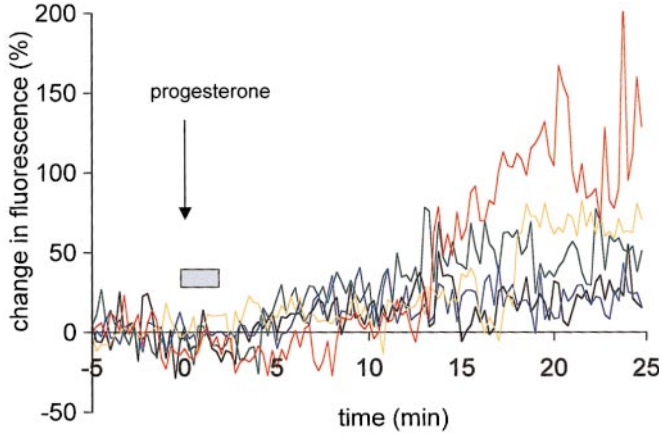

FIG. 5. Occurrence of sustained response without prior transient. Responses from five cells in experiment 7, all of which gave a significant sustained increase but no discernible transient response. Shaded box shows expected time and typical amplitude (25–35%) for transient response.

gesterone, at the single-cell level, to be biphasic, consisting of a transient response, which peaks within 1–2 min followed by a sustained ramp or plateau. The dose of progesterone that we have used (3.2 μM) is similar to those used by other groups studying this response (e.g., Blackmore *et al.*, 1990; Yang *et al.*, 1994; Aitken *et al.*, 1996; Garcia and Meizel, 1996, 1999) and is consistent with the concentration of progesterone within the cumulus oophorus (Osman *et al.*, 1989). Our observations are therefore biologically relevant.

The initial, transient component of the single-cell response resembles the $[Ca^{2+}]_i$ transient described by other groups using single-cell imaging (Plant *et al.*, 1995; Aitken *et al.*, 1996) and fluorimetry (e.g., Bonaccorsi *et al.*, 1995; Garcia and Meizel, 1999). The percentage of cells in which a significant transient rise in $[Ca^{2+}]_i$ was observed ranged from 35 to 91%. Since capacitation and experimental incubation were maintained as constant as possible, this variability may reflect differences in responsiveness between samples. The mean response (71.1 $\pm$ 7.7%) was lower than has previously been reported (90–93%; Plant *et al.*, 1995; Aitken *et al.*, 1996). The reason for this discrepancy is open to speculation but it is likely that capacitation status is responsible for at least part of this difference (Baldi *et al.*, 1991; Mendoza and Tesarik, 1993; Garcia and Meizel, 1999). When we induced the transient response by rapid superfusion with progesterone we found that the time from initiation to peak of individual cell responses could be reduced to 5–15 s. There was a noticeable delay of 3–5 s between progesterone application and initiation of the response. Though part of this delay may reflect latency of arrival of progesterone at the cell membrane, it seems likely that there is a significant interval between progesterone binding and initiation of Ca^{2+} influx.

As the transient response decays, a subset of spermatozoa (mean 35%—Table 1) shows a sustained ramp or plateau response which persists for the duration of recording (up to 20 min). Though the amplitude of this response varied between cells, it is clear that the shape and time course of these events at the single-cell level resembles those observed in fluorimetry experiments. Furthermore, reassembly of large numbers of single-cell events into an average generates a trace very like those observed by fluorimetry. We believe that the progesterone-induced sustained $[Ca^{2+}]_i$ response is a genuine signalling event, for the following reasons. First, the kinetics of the response at the single-cell level are a sustained plateau or ramp (as occurs in many somatic cells), not a phasic event as might be expected were it an artefact of AR. Second, single-cell imaging shows that the secondary response is localised to the postacrosomal area of sperm heads and does not occur in the area around the outer acrosomal membrane, where Calcium green leakage might occur during AR. Finally, the mean proportion of cells showing a secondary response to progesterone (35%) significantly exceeds the mean percentage of live cells undergoing AR (both spontaneous and progesterone-induced; 19%, $P < 0.05$; two-tailed t test; arcsine-converted data). When both $[Ca^{2+}]_i$ and AR were monitored for the same sample, the proportion of cells showing the sustained response exceeded (in three of four cases) the sum of spontaneous and agonist-induced AR.

It is noteworthy that, though progesterone reliably induced AR in our studies (as assessed by FITC-PSA), we did not routinely observe the rapid loss of fluorescence described by others and attributed to AR (Tesarik *et al.*, 1995; Shirakawa and Miyazaki, 1999). Furthermore, we did not observe the unusual kinetics of the secondary $[Ca^{2+}]_i$ responses described by Tesarik *et al.* (1995), composed of the initial transient response and a second, large, discrete response which terminated in an abrupt fall in fluorescence. The simplest interpretation of these differences is that our loading procedure did not result in significant accumulation of dye into the acrosome, such that we were able to monitor, accurately, the progesterone-induced $[Ca^{2+}]_i$ signal (including the sustained response) but did not detect AR. Our observation that the fluorescent signal was localised primarily in the postacrosomal region is in accord with this view and is consistent with the findings of Shirakawa and Miyazaki (1999) on hamster spermatozoa. These authors observed a $[Ca^{2+}]_i$ response upon application of solubilised zona pellucida, which was localised to the postacrosomal region. After a delay of 20–30 s, loss of fluorescence occurred in the acrosomal region, possibly reflecting AR, but there were no coincident changes in the postacrosomal region. If significant acrosomal labelling occurs in Fura-2 AM-labelled spermatozoa, it is likely that part of the large sustained response seen in some fluorimetric experiments does reflect release of fluorochrome during AR.

There is a very strong association between the transient and the sustained responses. However the sustained response does occur in a small number of cells (5% of total) which appear to show only a small nonsignificant transient response or, in some cases, no discernible transient response. It therefore appears that the transient is not an absolute prerequisite for generation of the sustained response. Furthermore, the transient response in those cells that also generate a sustained response is not significantly larger than in those that do not. At least two models might be consistent with these observations.

(i) The primary response may act synergistically with a second pathway to facilitate generation of the sustained response. For example, one of the candidate mechanisms for sustained Ca^{2+} influx into mammalian spermatozoa is activation of capacitative calcium entry (CCE; Publicover and Barratt, 1999). Progesterone-induced production of IP_3 in human spermatozoa (a prerequisite for activation of CCE) is dependent on influx of extracellular Ca^{2+} and occurs during the first 10–20 s after exposure to the agonist, during the transient response (Thomas and Meizel, 1989). A possible reason for the strong (but not absolute) association between the two phases of progesterone-induced $[Ca^{2+}]_i$ signalling might therefore be that the secondary response involves generation of IP_3 which is mediated by a combination of receptor activation and Ca^{2+} influx during the transient response. In those cells that do not generate a

major transient Ca^{2+} influx a secondary response would be considerably less likely but might still occur at low frequency, as observed here.

(ii) There may be more than one type of progesterone receptor expressed on spermatozoa. Luconi *et al.* (1998) detected two binding sites (K_d = 0.06 and 26 μM). If two separate receptors are responsible for the two components of the $[Ca^{2+}]_i$ signal, a high association between their expression would generate the pattern of responses described here and would also be consistent with our observation that occurrence of the sustained response is not correlated with the amplitude of the transient response.

The biphasic nature of the response to progesterone superficially resembles the responses of mammalian spermatozoa to ZP and sea urchin spermatozoa to AR-inducing egg-derived FSP (see Introduction). However, these three "similar" responses do not reflect activation of equivalent signalling pathways. The initial Ca^{2+} influx events induced by ZP and FSP are both sensitive to dihydropyridines (Guerrero and Darszon, 1989b; Arnoult *et al.*, 1996, 1999), whereas the initial transient response to progesterone shows little if any dihydropyridine-sensitivity (Foresta *et al.*, 1993; Aitken *et al.*, 1996; Garcia and Meizel, 1999; Blackmore and Eisoldt, 1999). Furthermore, though the second phase of the sea urchin spermatozoon response to FSP is dihydropyridine-insensitive (Guerrero and Darszon, 1989b), the prolonged Ca^{2+} signal in hamster spermatozoa stimulated by solubilised ZP is at least partly blocked by 10 μM nifedipine (Shirakawa and Miyazaki, 1999). Our own preliminary data indicate that the sustained phase of the response of human spermatozoa to progesterone is partially inhibited by 10 μM nifedipine, such that the proportion of cells in which a significant sustained response occurs is reduced. However, this effect is variable and difficult to interpret. It appears that at least one more mechanism must contribute to the sustained phase. Whether the nifedipine-insensitive component of the sustained response is capacitative Ca^{2+} influx is yet to be established. The ability of inhibitors of sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPases to induce Ca^{2+} influx in mammalian spermatozoa and spermatogenic cells suggests that such an influx pathway may be available (Blackmore, 1993; Santi *et al.*, 1998). Pretreatment of human spermatozoa with thapsigargin does not occlude the sustained response to progesterone (as might be expected if thapsigargin and progesterone activate the same influx pathway) and in fact appears to enhance it (Blackmore, 1993). Since our studies indicate that only a proportion of cells respond to progesterone, it is possible that the combined effect of thapsigargin and progesterone may induce a capacitative Ca^{2+}-influx in cells that do not show a response to one of the agents alone. Clearly, further investigation is required.

An important question, with respect to interpretation of our findings, regards the significance of the sustained response for AR. Since it is not yet possible to monitor reliably the kinetics of the AR of human spermatozoa in real time, this is not a simple question to address. In the experiments of Tesarik *et al.* (1995), a discrete, secondary increase in Fluo-3 fluorescence, followed by a sudden decrease below control levels, was observed in progesterone-stimulated human spermatozoa. This phenomenon probably indicates occurrence of AR (see above). In most instances this event was observed several minutes after exposure to the hormone, during the sustained response described here. In a few instances (6 of a total of 27 cells) loss of dye occurred during the initial $[Ca^{2+}]_i$ transient. Meizel *et al.* (1990) detected AR of capacitated spermatozoa within 30 s of progesterone application, during the transient response. We consider it possible that AR is distributed between an early phase induced by the $[Ca^{2+}]_i$ transient and a later phase associated with the sustained response. With regard to the relative effectiveness of the transient and sustained responses in inducing AR, it may be of significance that the sustained response, though occurring in fewer cells, generates very high $[Ca^{2+}]_i$ levels in larger numbers of cells. We have found that 10 μM nifedipine, which may partially inhibit the sustained response to progesterone (see above), significantly reduces progesterone-induced AR in human spermatozoa, as has been reported previously (Shi and Roldan, 1995; O'Toole *et al.*, 1996). An important technical development will be the ability to achieve reliable, simultaneous assessment of the AR (initiation and full development) in conjunction with a physiological response (e.g., $[Ca^{2+}]_i$) in human spermatozoa.

In conclusion, we have used confocal imaging to study $[Ca^{2+}]_i$ signalling in large numbers of progesterone-stimulated human spermatozoa. We have demonstrated, unequivocally, that the single-cell response is biphasic, consisting of the well-characterised transient response followed by a sustained ramp or plateau. Characterisation of this response is a key objective for advancing our understanding of human spermatozoa and induction of the AR.

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