Ca²⁺-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay

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Chlortetracycline (CTC) fluorescence patterns were used to assess Ca²⁺-related changes in the capacitation state of human spermatozoa incubated under conditions that would affect their intracellular Ca²⁺ levels. Initial experiments were designed to identify consistently occurring patterns and to correlate these with acrosomal status. Incubation for up to 1 h with the ionophore A23187 (10 µmol l⁻¹), known to promote capacitation and acrosomal exocytosis, allowed the identification of three different CTC staining patterns which were very similar to those described for mouse spermatozoa. For this reason, they were given the same nomenclature: 'F' – characteristic of uncapacitated, acrosome-intact cells; 'B' – characteristic of capacitated, acrosome-intact cells; and 'AR' – characteristic of capacitated, acrosome-reacted cells. The distribution of the three patterns in the ionophore-treated suspensions was very different from that in control suspensions treated with dimethylsulfoxide only, with a significantly higher proportion of cells displaying the B and AR patterns and a significantly lower number of cells displaying the F pattern in the ionophore-treated group at all times. A strong concordance was found between the acrosomal status of cells determined using both CTC and fluorescein-conjugated *Pisum sativum* agglutinin (PSA) staining methods on the same cells. Verification of PSA staining patterns with acrosomal status was obtained by means of transmission electron microscopy. The proportion of cells with uniform fluorescence in the acrosomal region correlated with acrosome-intact cells; those with only equatorial segment staining correlated with fully-reacted cells, and those exhibiting equatorial fluorescence and patchy fluorescence over the rest of the acrosomal region correlated with cells in intermediate stages of exocytosis. Having established and verified the morphological basis for the CTC staining patterns, we then incubated cells in medium containing standard (1.80 mmol l⁻¹) and high (3.60 mmol l⁻¹) CaCl₂. In both media the proportion of F cells decreased with time, whereas the B and AR patterns increased, but the high Ca²⁺ treatment significantly accelerated the change from F to B to AR at all time points. In contrast, when spermatozoa were incubated in a Ca²⁺-deficient medium for up to 22 h, the majority of cells displayed the uncapacitated F pattern. The introduction of millimolar Ca²⁺ during the final 15 min of incubation failed to alter the CTC patterns, thus confirming the fact that human spermatozoa require the continuous presence of extracellular Ca²⁺ to undergo capacitation and the acrosome reaction. These results suggest that changes in CTC fluorescence patterns indicate Ca²⁺-related changes in the functional state of human spermatozoa and therefore that CTC assessment may prove useful in clinical assessment of human sperm fertilizing potential.

Introduction

When mammalian spermatozoa are first released from the male reproductive tract they are unable to fertilize oocytes immediately, despite being morphologically mature and independently motile. The spermatozoa must undergo a post-release maturation phase termed capacitation which is obligatory for mammalian spermatozoa to attain full fertilizing potential (Austin, 1951; Chang, 1951). Upon completion of capacitation, the spermatozoa

have the capacity to undergo the acrosome reaction, resulting in the release of lytic enzymes and membrane alterations necessary for sperm–egg fusion (reviewed by Fraser, 1987a).

The importance of extracellular Ca²⁺ in sperm function has been widely reported. As early as 1915 Loeb demonstrated that Ca²⁺ was necessary for successful fertilization in invertebrates, and in 1971 its importance for mammalian fertilization was reported by Iwamatsu and Chang (1971). Early experiments suggested that Ca²⁺ was required only at the end of the capacitation process with an influx of Ca²⁺ required to initiate acrosomal exocytosis. This conclusion was based on the observation

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that incubation of guinea-pig spermatozoa in a Ca^{2+} -deficient medium for sufficient time to permit capacitation in complete medium and then addition of millimolar concentrations of Ca^{2+} triggered acrosomal exocytosis (Yanagimachi and Usui, 1974). However, subsequent studies on other species demonstrated that the continuous presence of at least some extracellular Ca^{2+} was required to complete capacitation and to attain full fertilizing potential (hamster – Yanagimachi, 1982; mouse – Fraser, 1982, 1987b; human – Stock and Fraser, 1989).

Interest in analysing the fertilizing potential of human spermatozoa has increased with the advent of *in vitro* fertilization; one approach has been to analyse the incidence of spontaneous acrosome reactions. Unfortunately, this is fairly low and variable, even in samples from fertile men (e.g. Byrd and Wolf, 1989; Stock and Fraser, 1987); this makes discrimination between fertile and infertile individuals on the basis of spontaneous acrosome loss unlikely. It would therefore be more useful if the proportion of capacitated cells, i.e. that population of acrosome-intact cells with the potential to undergo acrosomal exocytosis upon interaction with oocytes, could be quantified. We investigated the possibility of using the fluorescent probe chlortetracycline (CTC) to assess capacitation in human spermatozoa. The CTC fluorescence technique was first used by Ward and Storey (1984) to assess the functional status of mouse spermatozoa. The major advantage of CTC is that it not only allows discrimination between acrosome-intact cells and acrosome-reacted ones, but also divides acrosome-intact cells into two further, functionally different, categories, i.e. uncapacitated and capacitated. Such a distinction is not possible with current assessment techniques. A previous study indicated that CTC analysis might be applied to human spermatozoa (Lee *et al.*, 1987). We investigated the possibility of using CTC to assess the capacitation state of human spermatozoa by incubating them under various conditions known to affect the intracellular Ca^{2+} concentrations of mammalian sperm cells. A preliminary report of some of these experiments has been published (DasGupta and Fraser, 1991).

Materials and Methods

Media

The standard medium used was Earle's medium with added benzylpenicillin (Glaxo Laboratories, Greenford) which contained $1.80 \text{ mmol CaCl}_2 \text{ l}^{-1}$. Modifications to the medium included alterations to the Ca^{2+} concentration: Ca^{2+} -deficient medium (CaCl_2 omitted) and high Ca^{2+} medium ($3.60 \text{ mmol CaCl}_2 \text{ l}^{-1}$). A concentrated Ca^{2+} stock solution (22.5 mmol l^{-1}) was prepared by the addition of $11.5 \times$ the normal amount of CaCl_2 to standard Earle's medium; $20 \mu\text{l}$ of this was added to each $230 \mu\text{l}$ of Ca^{2+} -deficient medium or standard Ca^{2+} medium to produce 1.80 mmol l^{-1} or 3.60 mmol l^{-1} CaCl_2 -containing medium, respectively. All media contained human serum albumin (Sigma, Poole, Dorset) at 4 mg ml^{-1} .

A 5 mmol l^{-1} stock solution of ionophore A23187 (Sigma) was prepared in dimethylsulfoxide (DMSO) and stored as $10 \mu\text{l}$ aliquots at -20°C ; before use an aliquot was thawed and $90 \mu\text{l}$ of protein-free Earle's medium was added. This was used at $1/50$ to give a final concentration of $10 \mu\text{mol A23187 l}^{-1}$. A

DMSO solution, prepared by adding $10 \mu\text{l}$ of DMSO to $90 \mu\text{l}$ of protein-free Earle's medium, was used at $1/50$ in control suspensions.

Sperm preparation

Semen was obtained from both fertile donors and individuals undergoing assessment at the Diagnostic Andrology Service, The Hallam Medical Centre. Motile cells were prepared by using mini-Percoll (Ord *et al.*, 1990) gradients ($300 \mu\text{l}$ each of 85.5%, 63% and 45% Percoll (95%, 70%, 50% v/v solutions prepared from Percoll supplied as a 90% solution; Pharmacia LKB, Biotechnology AB, Uppsala)). After centrifugation at $600 g$ for 5 min, the supernatant was removed; pelleted cells were resuspended in Earle's medium, centrifuged again and resuspended in fresh medium. The sperm concentration was determined using a haemocytometer and then adjusted to $5 \times 10^6 \text{ cells ml}^{-1}$; suspensions were transferred to 15 ml centrifuge tubes (Greiner, Phillip Harris Scientific, London). A small drop of the suspension was placed on a microscope slide and the proportion of cells exhibiting progressive motility was estimated; this was usually $>90\%$. The suspensions were then gassed with $5\% \text{ CO}_2$ – $5\% \text{ O}_2$ – $90\% \text{ N}_2$ and incubated at 37°C .

Cell viability

The vital dye Hoechst bis-benzamide 33258 (Sigma) was used to assess cell viability. A 100 mg ml^{-1} stock solution was made by dissolving the dye in AnalaR water (BDH, Lutterworth, Leics) and this was stored in a foil-wrapped bottle at 4°C for a maximum of one month. Before use, the stock solution was diluted $1/1000$ in protein-free Earle's medium and further diluted $1/100$ in the sperm suspensions (final concentration of the dye was $1 \mu\text{g ml}^{-1}$). The suspensions were incubated for 2 min and then washed by centrifugation at $900 g$ for 5 min through 4 ml of 2% polyvinylpyrrolidone (PVP 40, Sigma) solution. The resulting pellet was resuspended in culture medium and cells were then stained with one of the fluorescent dyes as detailed below. Cells were assessed on a Leitz 100Z microscope equipped with phase contrast and epifluorescent optics using filter block A (ultraviolet excitation range). The Hg excitation beam was passed through a 340 – 380 nm band pass filter and fluorescence emission was observed through an RKP 400 beam-splitting mirror.

The samples used had high proportions of motile cells and the numbers of Hoechst-positive (dead) cells closely corresponded to the numbers of immotile cells. Because the number of dead cells was low and because they were distributed randomly among the assessment categories, all our data are for Hoechst-negative (live) cells only. Two hundred cells were counted in each replicate.

CTC assessment

The method used was a modification of that described by Ward and Storey (1984). The chlortetracycline (CTC) solution was prepared fresh each day and contained $750 \mu\text{mol CTC l}^{-1}$ (Sigma) in a buffer of $130 \text{ mmol NaCl l}^{-1}$, $5 \text{ mmol cysteine l}^{-1}$, $20 \text{ mmol Tris-HCl l}^{-1}$ (final pH 7.8). The solution was kept wrapped in foil to prevent the entry of light and stored at 4°C .

Cells were fixed in suspension (Fraser and McDermott, 1992), a procedure that allowed spermatozoa to be fixed under different incubation conditions at essentially the same time. The method involved adding 100 μ l of sperm suspension to a small (1.5 ml) foil-wrapped microcentrifuge tube, then adding 100 μ l of CTC solution and mixing thoroughly. Cells were then fixed by adding 8 μ l of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris-HCl l⁻¹ buffer (final pH 7.4) and thoroughly mixing.

Slides were prepared by placing 10 μ l of the suspension onto a clean microscope slide. A drop of 0.22 mol 1,4-diazabicyclo[2,2,2]octane l⁻¹ (DABCO; Sigma) in glycerol plus phosphate-buffered saline (9:1) was mixed in carefully to retard fading of fluorescence. A coverslip was placed on top and the slide was gently but firmly compressed between two tissues. Any excess fluid was thus removed and the number of spermatozoa lying flat on the slide was maximized, an orientation crucial for accurate assessment. The slide was then sealed along the edges with colourless nail varnish, and stored in a light-proof container in the cold. Although slides retained fluorescence for 4–5 days, in general they were assessed on the same or the following day. Cells were assessed using filter block D (ultraviolet plus violet excitation range). The Hg excitation beam was passed through a 355–425 nm band pass filter and CTC fluorescence emission was observed through an RKP 455 beam-splitting mirror.

PSA assessment

A stock solution of fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin (PSA) was made up with AnalaR water at 0.1 mg ml⁻¹ and stored in aliquots in foil wrapped microcentrifuge tubes at -20°C. Suspensions to be assessed with PSA were centrifuged at 600 g for 5 min and the pelleted cells were resuspended in 50 μ l of cold ethanol. This suspension was left for a minimum of 30 min at 4°C and 10 μ l was then placed on a clean microscope slide and allowed to air dry. On top of this, 5 μ l of PSA solution was spread and the slide was placed in a humidified chamber at 4°C in the dark. When dry, the slides were gently immersed several times in AnalaR water to wash off any excess stain and again placed in a humidified chamber at 4°C in the dark. A drop of DABCO was then placed on top of the dried slide and a coverslip was placed on top. The edges were sealed with colourless nail varnish. Cells were assessed using filter block I₂ (blue excitation range). The Hg excitation beam was passed through a 450–490 nm band pass filter and FITC fluorescence emission was observed through an RKP 510 beam-splitting mirror.

Statistical analysis

Data were analysed using Cochran's test for modification of 2 $\times$ 2 contingency tables (Snedecor and Cochran, 1980).

Experimental Details and Results

Series I: comparison of CTC patterns in control and ionophore-treated suspensions

Ionophores are used frequently to manipulate the intracellular concentrations of specific ions. The ionophore A23187, which

Fig. 1. Three patterns of chlortetracycline fluorescent staining observed on human spermatozoa: (a) F, with fluorescence over entire head; (b) B, with a fluorescence-free band in the postacrosomal region; (c) AR, with dull fluorescence over the entire head. Bright staining in the midpiece is seen at all times. Scale bar represents 2 μ m.

has a specificity for Ca²⁺ over Mg²⁺, was used to study possible changes in CTC staining patterns in human spermatozoa following introduction of the drug. Spermatozoa were prepared in standard medium and samples were divided into two, with one receiving 10 μ mol A23187 l⁻¹ in DMSO and the other DMSO only. After incubation for 1 h, cells were stained with CTC, fixed and examined under epifluorescent illumination. Three distinct patterns on the sperm head were observed consistently, although the relative proportions differed significantly between DMSO and ionophore-treated samples. Unlike an earlier study (Lee *et al.*, 1987), the patterns were found to be similar to those described for mouse spermatozoa and so we used the same nomenclature: 'F', with uniform fluorescence in the head, which is characteristic of uncapacitated, acrosome-intact cells; 'B', with a fluorescence-free band in the post-acrosomal region, which is characteristic of capacitated, acrosome-intact cells; 'AR', with dull or absent fluorescence, which is characteristic of acrosome-reacted cells. Bright fluorescence in the midpiece was seen on all cells. Typical examples of these are shown (Fig. 1). Having identified these three patterns, we evaluated

Fig. 2. Distribution of chlortetracycline fluorescence patterns in human sperm suspensions incubated for 1 h in the absence (DMSO control) or presence of $10 \mu\text{mol A23187 l}^{-1}$ ($n = 7$). Data are presented as means $\pm$ SEM. ▨ uncapacitated, acrosome-intact spermatozoa F; ■ capacitated, acrosome-intact spermatozoa B; ▩ capacitated, acrosome-reacted spermatozoa AR. * $P < 0.05$, *** $P < 0.001$ compared with DMSO control suspensions.

Fig. 3. Changes in chlortetracycline fluorescence patterns in human sperm suspensions incubated for 60 min in the absence (DMSO control; open symbols) or presence of $10 \mu\text{mol A23187 l}^{-1}$ (filled symbols) and sampled at 10 min intervals ($n = 10$). Data are presented as mean values. (○, ●) F; (△, ▲) B; (□, ■) AR.

their distribution in the two treatment groups. Seven replicate experiments were carried out.

Cells which had been incubated with ionophore primarily exhibited the B and AR patterns (30% and 68%, respectively; Fig. 2). These proportions were significantly higher ($P < 0.05$ and $P < 0.001$) than values for the corresponding control suspensions, in which 78% of the cells still exhibited the F pattern. Thus, incubation with A23187 promoted the transition from the F to the B and AR patterns of CTC fluorescence.

Series II: kinetics of change in CTC patterns in ionophore-treated suspensions

To examine the kinetics of the response to A23187, we used the same procedure as for Series I, but removed, stained and fixed aliquots at intervals of 10 min over 1 h ($n = 10$). In the ionophore-treated group there was a marked decrease in F

Fig. 4. Human sperm cells exhibiting the three patterns of fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin staining. a: unreacted, acrosome-intact; b: intermediate, undergoing acrosomal exocytosis; c: reacted, acrosome-reacted. Scale bar represents $2 \mu\text{m}$.

pattern cells: within 30 min, only about 50% of the cells exhibited the F pattern and by 60 min this had fallen to 20% (Fig. 3). This decline was accompanied by steady increases in B and AR pattern cells throughout the 60 min. By comparing the treated and control values, it can be seen that at 10 min, the greatest effect of A23187 was an increase in B cells; very few AR cells were present. By 20 min, however, it was evident that the rate of increase in AR cells was greater than that for B cells and by 50 min approximately equal numbers of B and AR cells were observed. These data suggest that the ionophore treatment initially promotes the F to B transition, followed by rapid acrosomal exocytosis. In marked contrast, the DMSO-treated controls showed relatively little change over 60 min: there was a small decrease in F, a small increase in B and essentially negligible increase in AR pattern cells.

Series III: correspondence of acrosomal status between CTC and FITC-PSA assessment of cells

FITC-PSA was used successfully to assess the acrosomal status of human spermatozoa (Cross *et al.*, 1986). Three distinct patterns were described: fluorescence on the entire acrosome characteristic of acrosome-intact cells; fluorescence in the equatorial segment only, characteristic of acrosome-reacted cells; patchy fluorescence on the acrosome, which probably indicates cells undergoing exocytosis. To determine whether cells assessed to be acrosome-intact or acrosome-reacted using CTC fluorescence would have the acrosomal status when also stained with PSA, since the latter is now used by many different laboratories, we stained the suspension sequentially with Hoechst bis-benzamide to verify live/dead status, then CTC and finally PSA.

Suspensions were first incubated with Hoechst bis-benzamide and the stain was washed out in the same manner as described above. The resultant pellet was then resuspended in culture medium and stained with CTC. The cells were not fixed at this stage but were centrifuged at $600 g$ for 5 min and the pelleted cells were resuspended in cold ethanol. After a minimum of 30 min, PSA staining was performed and slides were prepared. Cells were first assessed for their CTC staining pattern, then for their PSA staining pattern, and finally for their live/dead status

Fig. 5. Comparison of chlortetracycline and fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) assessments made on the same human spermatozoa ($n = 20$). CTC patterns: ▨ F; ■ B; ▩ AR.

($n = 20$, with incubation times ranging from 1 to 22 h). The typical appearance of PSA-stained cells is shown (Fig. 4).

All cells exhibiting the F pattern had intact acrosomes according to PSA assessment (Fig. 5). The majority of B pattern cells also had intact acrosomes, although a minority exhibited the intermediate PSA staining pattern. With very few exceptions, cells with the AR pattern had fully reacted acrosomes. These results suggest that the CTC patterns F and B are found on cells with intact acrosomes, although some of the B pattern cells may have initiated exocytosis, and that the AR pattern is seen on cells with fully reacted acrosomes.

Series IV: ultrastructural and PSA assessment of ionophore-treated cells

Samples from donors were prepared in Earle's medium and incubated at 37°C. After 1 h, 10 $\mu\text{mol A23187 l}^{-1}$ was added to the suspension which was incubated for 30 min and washed at 600 g for 5 min. The resulting pellet was resuspended in 2 ml Earle's medium and 200 μl of this was transferred to a microcentrifuge tube. An equivalent amount of fixative (3% glutaraldehyde in 0.1 mol cacodylate buffer l^{-1} , pH 7.4, with 2.5 mmol $\text{CaCl}_2 \text{l}^{-1}$) was added to the larger aliquot and thoroughly mixed. After 1 h at room temperature, the suspension was centrifuged at 600 g for 5 min and the pellet was resuspended in 1 ml cacodylate buffer and this was centrifuged again. The pellet was gently overlaid with 1 ml of buffer and stored at 4°C until processed. The smaller aliquot was centrifuged at 600 g for 5 min and the pellet was resuspended in 50 μl of cold ethanol. A PSA slide preparation was then made (see above) and the distribution of the three PSA patterns was evaluated ($n = 4$).

The glutaraldehyde-fixed samples were post-fixed in osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100 CX electron microscope.

The PSA-labelled samples were evaluated for proportions of cells exhibiting the intact, intermediate and reacted patterns of

Fig. 6. Ultrastructural appearance of human spermatozoa: (a) acrosome-intact; (b), (c) undergoing acrosomal exocytosis, with differing amounts of acrosomal matrix still present; (d) acrosome-reacted. The bar represents 0.5 μm .

staining (200 cells per samples). The sectioned material from the same original suspensions was counted for proportions of cells that appeared ultrastructurally to be acrosome-intact, undergoing acrosomal exocytosis and acrosome-reacted (100 cells in each sample). Typical examples of the ultrastructural categories are shown (Fig. 6).

The results (Fig. 7) indicated that there were no significant differences between PSA and ultrastructural assessment in any of the three categories. Of particular interest is the correlation between the proportion of cells exhibiting the intermediate PSA fluorescence pattern and the cells that have lost some, but not all, of the acrosomal matrix when viewed with transmission

Fig. 7. Correlation between acrosomal status of human sperm cells incubated for 30 min in $10 \mu\text{mol A23187 l}^{-1}$ and then evaluated with fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) and transmission electron microscopy. □ PSA, ■ TEM.

electron microscopy. The ultrastructural appearance of these intermediate stages was similar to that described by Nagae *et al.* (1986) and Stock and Fraser (1987). The apparent correlation between the presence of small clumps of acrosomal matrix and the often patchy nature of PSA staining in the intermediate stage is consistent with the report by Cross *et al.* (1986) that PSA binds to acrosomal material.

Series V: capacitation state in standard and high Ca^{2+} medium

Previous studies have demonstrated that incubation of human spermatozoa in culture medium containing high concentrations of Ca^{2+} promotes the acrosome reaction when compared with cells incubated in standard medium (Stock and Fraser, 1989). The effect of increasing the Ca^{2+} concentration on CTC patterns was next investigated in human spermatozoa. Sperm suspensions were prepared in standard medium ($1.80 \text{ mmol CaCl}_2 \text{ l}^{-1}$) and divided into two groups. To one half of the sample concentrated Ca^{2+} stock solution was added to give a final CaCl_2 concentration of 3.60 mmol l^{-1} . After 1, 4–5 and 18–22 h, aliquots were removed and the cells were stained with CTC ($n = 22$).

As the incubation progressed, the proportion of F pattern cells decreased and the proportions of B and AR cells increased in both media (Fig. 8). However, at all three assessment times there were significantly fewer ($P < 0.001$ at all times) F pattern cells and significantly more ($P < 0.001$ at 1 and 4–5 h; $P < 0.01$ at 18–22 h) B pattern cells in the high Ca^{2+} group. In standard medium, the mean values $\pm$ SEM for F cells were $82.4 \pm 4.3\%$ at 1 h, $65.7 \pm 7.7\%$ at 4–5 h and $46.9 \pm 7.8\%$ at 18–22 h; in high Ca^{2+} medium, the corresponding values were $65.8 \pm 7.3\%$, $46.6 \pm 7.4\%$ and $30.5 \pm 7.2\%$. In standard medium the mean values $\pm$ SEM for B cells were $15.6 \pm 4.1\%$ at 1 h, $29.5 \pm 7.3\%$ at 4–5 h and $44.5 \pm 6.6\%$ at 18–22 h; in high Ca^{2+} medium, the corresponding values were $31.2 \pm 6.1\%$, $47.5 \pm 6.2\%$ and $53.4 \pm 6.6\%$. After incubation for 4–5 h in high Ca^{2+} , approximately equal proportions of F and B cells were observed. This position was not seen in the standard Ca^{2+} medium until 18–22 h. The rate of change in

Fig. 8. Changes in chlortetracycline patterns during incubation for 22 h in standard Ca^{2+} (1.80 mmol l^{-1} ; open symbols) and high Ca^{2+} (3.60 mmol l^{-1} ; closed symbols) media. Data are presented as mean values; the SEM values are given in the text. (○, ●) F; (△, ▲) B; (□, ■) AR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with standard Ca^{2+} medium.

these two patterns was essentially the same in both conditions, but within 1 h incubation in high Ca^{2+} there were fewer F and more B cells and this differential was maintained throughout the duration of the experiment. The proportion of AR cells also increased as the incubation continued, but the values were quite low. In standard medium, the mean values $\pm$ SEM were $1.2 \pm 0.6\%$ at 1 h, $4.2 \pm 2.1\%$ at 4–5 h, and $8.6 \pm 2.3\%$ at 18–22 h. In high Ca^{2+} medium, the corresponding values were $3.0 \pm 1.7\%$, $5.9 \pm 2.1\%$ and $16.0 \pm 2.6\%$. These were significantly higher ($P < 0.05$ at 1 and 4–5 h; $P < 0.01$ at 18–22 h) than values from the standard Ca^{2+} counterparts.

Examination of the data indicated that, in the high Ca^{2+} suspensions, distribution of CTC patterns observed at 1 h was essentially that seen in the standard suspension at 4–5 h and the distribution at 4–5 h was that seen in standard Ca^{2+} at 18–22 h. Thus, incubation in high Ca^{2+} accelerated the transition from the uncapacitated to the capacitated state.

Series VI: capacitation state in a Ca^{2+} -deficient medium as assessed by CTC

Sperm suspensions were prepared in Ca^{2+} -deficient medium and samples were divided into two. High Ca^{2+} stock solution was added to one half to give a final concentration of $1.80 \text{ mmol CaCl}_2 \text{ l}^{-1}$. An equivalent amount of Ca^{2+} -deficient medium was added to the other half. After 4 h 45 min, and 21 h 45 min, an aliquot of the Ca^{2+} -deficient suspension was transferred to a microcentrifuge tube and high Ca^{2+} stock was added to give a final concentration of $1.80 \text{ mmol CaCl}_2 \text{ l}^{-1}$. After 15 min all three suspensions were stained with CTC ($n = 10$).

Even after 22 h incubation in Ca^{2+} -deficient medium, $>95\%$ of cells exhibited the F pattern and the introduction of millimolar Ca^{2+} did not alter the distribution of CTC patterns (Fig. 9). In marked contrast, and consistent with the distribution observed in earlier experiments (Fig. 8), there was a time-dependent decrease in F pattern cells and a corresponding increase in B and AR pattern cells in the standard Ca^{2+} medium. At 22 h, the percentages were: $55.0 \pm 2.4\%$ F, $37.8 \pm 1.7\%$ B,

Fig. 9. Chlortetracycline patterns in human sperm suspensions incubated for 22 h in either Ca²⁺-deficient (-Ca²⁺) or standard Ca²⁺ (+Ca²⁺) media. Fifteen minutes before assessment, 1.80 mmol Ca²⁺ was added to an aliquot of -Ca²⁺ suspension (- → +Ca²⁺) ($n = 10$). Data are presented as means $\pm$ SEM. ▨ F; ■ B; ▤ AR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with -Ca²⁺ suspensions.

$7.3 \pm 1.2\%$ AR. All values were significantly different from those of the Ca²⁺-deficient group ($P < 0.05$ – $P < 0.001$).

Discussion

In this study we investigated the possibility that CTC fluorescence can be used to assess capacitation in human spermatozoa. Because current evidence suggests that capacitation involves a rise in intracellular Ca²⁺ concentration (reviewed by Fraser, 1987a, b; White and Aitken, 1989; Fraser and McDermott, 1992), we incubated human sperm cells under various conditions known to affect intracellular Ca²⁺ concentrations and sperm function in other mammalian species. In the presence of extracellular Ca²⁺, divalent cation ionophores such as A23187 cause an increase in intracellular Ca²⁺ in mammalian spermatozoa (Babcock *et al.*, 1976). Furthermore, Fraser and McDermott (1992), using CTC to assess responses, demonstrated that A23187 treatment of uncapacitated mouse sperm cells accelerated capacitation and then triggered acrosomal exocytosis, as indicated by a rapid change in the distribution of CTC patterns. We therefore treated motile suspensions of human spermatozoa with $10 \mu\text{mol A23187 l}^{-1}$ for 1 h; this concentration of ionophore has been used in a number of studies on human spermatozoa (for example Cummins *et al.*, 1991; Lee *et al.*, 1987) and had no marked effect on viability. We then stained cells with CTC, and compared the fluorescence patterns with those observed in control (DMSO) samples. We observed three distinct patterns which were remarkably like those seen in mouse spermatozoa. For this reason, we used the same nomenclature for the patterns observed on human cells: F – uncapacitated, acrosome-intact cells; B – capacitated, acrosome-intact cells; AR – capacitated, acrosome-reacted cells. Incubation for 1 h in A23187 promoted a marked change in the distribution of CTC patterns, with few F and many B and AR pattern cells, compared with controls, which had a majority of F pattern cells. To examine kinetics of the ionophore-induced changes, we

treated the cells with A23187 and sampled at 10 min intervals up to 60 min. The rapid, time-dependent decrease in F and increase initially in B, followed closely by AR pattern cells was similar to that reported by Fraser and McDermott (1992) for mouse spermatozoa. In contrast, the control samples showed only slight changes in distribution of CTC patterns. Thus, a treatment known to increase intracellular Ca²⁺ concentrations could promote a significant change in the CTC patterns reflecting changes in intracellular Ca²⁺ in human spermatozoa in the same manner as they do in mouse spermatozoa (Fraser and McDermott, 1992).

To validate our conclusions about the presence or absence of the acrosome in CTC-stained cells, we stained cells with both CTC and PSA. PSA binds to acrosomal matrix (Cross *et al.*, 1986) and is now used quite commonly to evaluate acrosomal status in human spermatozoa (e.g. Cummins *et al.*, 1991; Mendoza *et al.*, 1992). With PSA, it is possible to distinguish acrosome-intact, acrosome-reacting (probably) and acrosome-reacted cells, but it is not possible to divide acrosome-intact cells into uncapacitated and capacitated groups. CTC does allow this latter distinction to be made. We would predict that F and B cells are acrosome-intact. Our results indicated that all the F pattern (intact) cells were also classified as intact by PSA assessment. Furthermore, the majority of B pattern cells also fell into this same PSA category. A minority of B cells were classed as intermediate with PSA. Thus these results are consistent with our predictions.

To further validate our conclusions, we compared the distribution of PSA staining patterns with ultrastructural patterns in the same samples of sperm cells treated with A23187. We chose PSA because the three PSA patterns appear to be related to changes in acrosomal integrity that ought to be correlated with detectable ultrastructural alterations. In contrast, CTC binds to the cell surface and only correlates with two structural states, acrosome intact and acrosome reacted. Ultrastructural assessment revealed that a reasonable proportion of cells was exhibiting the intermediate stages of the acrosome reaction described by Stock and Fraser (1987), the remainder being either acrosome-intact or reacted. The proportions of cells in the intermediate stages, ultrastructurally, were very similar to the proportions of cells exhibiting the intermediate PSA staining pattern. Thus, our data are consistent with the report by Cross *et al.* (1986) that PSA binds to the acrosomal matrix. They also suggest that the intermediate PSA pattern, often with patchy fluorescence in the acrosomal region, corresponds to cells undergoing acrosomal exocytosis. In this study, as in earlier ones (e.g. Stock and Fraser, 1987), cells at this stage ultrastructurally have reasonably intact plasma and outer acrosomal membranes, although they must have at least small perforations, as the acrosomal matrix is considerably diminished. This retention of much of the plasma membrane would explain why some of the cells showing the B pattern with the surface-binding CTC coincided with the intermediate PSA pattern.

In the first report that CTC might prove useful for monitoring capacitation and the acrosome reaction in human spermatozoa, Lee *et al.* (1987) described four distinct CTC patterns which differed from those described for the mouse. The temporal changes in pattern distribution suggested transition from the 'early fresh', with a bright band in the post-acrosomal region, to the 'dark posterior' with bright anterior and dark posterior head

regions, to the 'clear perimeter', with fluorescence over the head and a bright perimeter, and finally to the 'acrosome-reacted', with no fluorescence. Kholkute *et al.* (1992) reported that they observed these same patterns. While the 'dark posterior' is similar to the B pattern, we have never observed this sequence of patterns. Very infrequently we saw cells with a bright perimeter, but our interpretation of this is a cell being viewed side-on, i.e. with a broad face of the cell at 90° to the face being viewed. One of the reasons that we looked more closely at the kinetics of changes promoted by A23187 was to determine whether the patterns described by Lee *et al.* (1987) were intermediates that we had overlooked or somehow missed. However, we saw only F, B and AR patterns. The close correlation between CTC and PSA assessments on the same cells gives strong support to our interpretation of the observed patterns. With CTC, the method of slide preparation is important: the squash technique used here maximizes the proportion of flat and hence analysable cells. Other individuals examining our own slides have observed only the F, B and AR patterns, and other researchers, working independently, have reported seeing these same three patterns (Kay *et al.*, 1993).

We now feel confident that CTC patterns do allow accurate determination of acrosomal status. The more important question is whether the CTC patterns also allow determination of capacitation state. Certainly the results obtained with A23187 would suggest that this is so. The responses of human cells closely parallel those observed in mouse cells and we know that A23187 treatment of mouse cells promotes not only rapid capacitation and acrosomal exocytosis, but also demonstrable fertilizing ability *in vitro* (Fraser, 1982). Furthermore, ionophore treatment of human sperm suspensions enhances their ability to penetrate zona-free hamster oocytes *in vitro* (Aitken *et al.*, 1984).

Earlier studies have shown that incubating mouse spermatozoa in high Ca^{2+} medium also accelerates capacitation and acrosomal exocytosis (Fraser and McDermott, 1992) and promotes rapid fertilization *in vitro* (Fraser, 1987b). Similar treatment of human spermatozoa significantly stimulated the incidence of spontaneous acrosomal exocytosis, as determined by transmission electron microscopy, and hence presumably accelerated capacitation (Stock and Fraser, 1989). We investigated this possibility using CTC and were able to confirm those earlier conclusions: at each of the three assessment times (up to 18–22 h), significantly fewer uncapacitated F and significantly more capacitated B and AR pattern cells were observed in the high (3.60 mmol l^{-1}) Ca^{2+} suspensions. Our results therefore suggest that the accelerated transition to the capacitated B and AR CTC patterns is a reflection of more rapid increases in intracellular Ca^{2+} in the high Ca^{2+} suspensions. This presumably occurs because the ability of the cell to maintain low (nanomolar) intracellular Ca^{2+} concentrations becomes less effective in the presence of raised extracellular Ca^{2+} . This interpretation is consistent with the marked changes that occur in response to A23187 treatment seen in the study reported here.

Finally, we compared the effect of incubating cells in Ca^{2+} -deficient and standard Ca^{2+} medium, for up to 22 h, on CTC patterns. As in the above experiment, a time-dependent change towards the capacitated patterns was observed in the Ca^{2+} -containing medium. However, even after 22 h almost all of the cells in Ca^{2+} -deficient medium were still in the uncapacitated F

pattern, whether or not millimolar Ca^{2+} was added at the end of incubation. From this we conclude that human spermatozoa, like mouse cells, require extracellular Ca^{2+} to complete capacitation. If they had completed capacitation in the absence of added Ca^{2+} , the majority of cells should have been in the B pattern and the addition of Ca^{2+} should have caused a shift to the AR pattern. These results confirm the earlier report by Stock and Fraser (1989), based on TEM assessment, that Ca^{2+} -deficient medium did not support spontaneous acrosomal exocytosis and, therefore, did not appear to support capacitation. In contrast to our findings, White *et al.* (1990) reported that differences were not observed in incidence of spontaneous acrosome loss between Ca^{2+} -containing and Ca^{2+} -deficient media. We cannot explain the discrepancies between our studies and that of White, except to note that all other mammalian spermatozoa have been shown to require millimolar Ca^{2+} for acrosomal exocytosis, spontaneous or otherwise.

In conclusion, we demonstrated that the CTC fluorescence patterns observed in human sperm suspensions and the time-dependent sequence of change in the presence of extracellular Ca^{2+} , from F to B to AR, are similar to those described in mouse sperm suspensions. F and B patterns are observed on acrosome-intact cells and the AR pattern, on acrosome-reacted cells. Given that the transition from F to B in mouse cells coincides with completion of capacitation and the acquisition of demonstrable functional ability, we propose that the transition from F to B in human cells also represents a change from the uncapacitated to the capacitated state. We therefore suggest that CTC assessment may prove to be useful in both predicting human sperm functional potential and revealing treatments that might be used therapeutically for individuals where capacitation is somehow defective.

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