A possible role for Ca^{2+} -ATPase in human sperm capacitation

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Mammalian spermatozoa require extracellular Ca^{2+} , some of which must be internalized, to undergo capacitation and acrosomal exocytosis. The mechanisms controlling the intracellular Ca^{2+} concentration are unclear, but current evidence suggests that a Ca^{2+} -ATPase may be involved. Using treatments that potentially modulate enzyme activity, we investigated this possibility in human spermatozoa; the capacitation state and acrosomal integrity were monitored by chlortetracycline fluorescence. Incubation of cells in the presence of quercetin, a Ca^{2+} -ATPase inhibitor, significantly accelerated the transition from the uncapacitated F pattern of chlortetracycline fluorescence to the capacitated, acrosome-intact B pattern within 1 h. This was followed by an increase in the number of cells displaying the capacitated, acrosome-reacted AR pattern. Since most Ca^{2+} -ATPases in somatic cells are sensitive to calmodulin, we also investigated the effect of the calmodulin antagonist W-7 on chlortetracycline patterns. At $1\text{--}125\ \mu\text{mol l}^{-1}$, W-7 significantly stimulated capacitation and acrosomal exocytosis. Furthermore, W-7 at $1\ \mu\text{mol l}^{-1}$ proved to be more effective than W-5, a less potent antagonist, suggesting that the observed responses in human spermatozoa did reflect a calmodulin-sensitive mechanism. When the glucose concentration in the culture medium was varied (from 0 to $5.56\ \text{mmol l}^{-1}$) to alter the availability of ATP for enzyme activity, it was found that a reduced concentration of glucose promoted capacitation more rapidly than did the standard concentration of $5.56\ \text{mmol glucose l}^{-1}$. However, maximal changes, particularly in promoting the shift from the B to the AR pattern of chlortetracycline fluorescence, required millimolar concentrations of glucose during the last few minutes before assessment. Finally, the addition of partially purified mouse sperm decapacitation factor (proposed to activate a Ca^{2+} -ATPase and thus maintain a low intracellular Ca^{2+} concentration) to capacitated human sperm suspensions caused a significant reversal in the capacitation state of cells (from the B to the F pattern). The F pattern of chlortetracycline fluorescence predominates in conditions favouring low concentrations of intracellular Ca^{2+} . From these results, we suggest that a Ca^{2+} -ATPase may play an important role during human sperm capacitation. A time-dependent decrease in endogenous enzyme activity would allow the intracellular concentration of Ca^{2+} to rise to a critical value necessary for initiation acrosomal exocytosis and subsequent successful fertilization.

Introduction

Upon release from the male reproductive tract mammalian spermatozoa are unable to fertilize oocytes immediately, despite being morphologically mature and independently motile. The changes involved in the acquisition of fertilizing potential are collectively called capacitation (Austin, 1951; Chang, 1951), and once this has been completed the spermatozoa have the ability to undergo acrosomal exocytosis upon contact with the oocyte. The presence or absence of various ions has been shown to affect capacitation and acrosomal exocytosis. For example, it is well established that Ca^{2+} plays a

crucial role in sperm function. As long ago as 1915, Loeb demonstrated that extracellular Ca^{2+} is necessary to obtain fertilization in invertebrates; a similar requirement has been found in mammalian spermatozoa, as first demonstrated by Iwamatsu and Chang (1971) in the mouse.

Initially, it was thought that Ca^{2+} is required only towards the end of capacitation: guinea-pig spermatozoa preincubated in Ca^{2+} -deficient medium can express hyperactivation and acrosomal exocytosis within minutes of introducing extracellular Ca^{2+} (Yanagimachi and Usui, 1974). However, further studies demonstrated that similar treatment of hamster (Yanagimachi, 1982), mouse (Fraser, 1982, 1987b) and human (Stock and Fraser, 1989; DasGupta *et al.*, 1993a) spermatozoa does not trigger a marked increase in acrosome loss. Furthermore, such spermatozoa are less fertile than cells incubated

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continuously in Ca^{2+} -containing medium (Fraser, 1982; Yanagimachi, 1982). It is generally accepted that internalization of extracellular Ca^{2+} , causing a rise in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), is required for both capacitation and acrosomal exocytosis. This would explain why cells incubated in Ca^{2+} -deficient medium fail to complete capacitation and undergo the acrosome reaction. Studies on mouse spermatozoa have shown that different concentrations of extracellular Ca^{2+} are required to support these two phases (Fraser, 1987b), with relatively little Ca^{2+} sufficing during capacitation but much more being required for exocytosis and fertilization. This suggests that different mechanisms may regulate $[\text{Ca}^{2+}]_i$ during the acquisition of functional ability.

In somatic cells, a Ca^{2+} -ATPase located in the plasma membrane helps to maintain low $[\text{Ca}^{2+}]_i$ by pumping Ca^{2+} out of the cell (Schatzmann, 1982). Such an ATPase has been identified in mammalian spermatozoa, but its importance in the regulation of $[\text{Ca}^{2+}]_i$ is the subject of considerable debate (Fraser, 1987a; Roldan and Fleming, 1989). However, it has been shown that incubating guinea-pig (Roldan and Fleming, 1989) and mouse (Fraser and McDermott, 1992) spermatozoa in the presence of compounds known to inhibit Ca^{2+} -ATPase activity in somatic cells shortens capacitation time, indicating that the enzyme may play a role in regulating the events of capacitation. It has been suggested that a decapacitation factor present in mouse spermatozoa at the time of release from the male reproductive tract may stimulate Ca^{2+} -ATPase activity; as the factor is lost during capacitation, enzyme activity would decline and $[\text{Ca}^{2+}]_i$ would rise (Fraser and McDermott, 1992; Adeoya-Osiguwa and Fraser, 1993).

Ca^{2+} -ATPases in somatic cells are generally sensitive to calmodulin (e.g. Carafoli, 1987). Recent studies have demonstrated that treating mammalian spermatozoa with calmodulin antagonists can significantly accelerate the transition to the capacitated state (Adeoya-Osiguwa and Fraser, 1993; Fraser *et al.*, 1993), suggesting that the Ca^{2+} -ATPase in mammalian spermatozoa may be sensitive to calmodulin. In the first of those studies, restricting the availability of a glycolysable substrate, which would limit the concentration of ATP (required for enzyme activity), promoted an increase in the rate of $^{45}\text{Ca}^{2+}$ uptake and accelerated both capacitation and fertilization *in vitro*.

Recent experiments (DasGupta *et al.*, 1993a) have suggested that completion of capacitation in human spermatozoa, as observed by the transition from the uncapacitated F pattern of chlortetracycline (CTC) fluorescence to the capacitated B and AR patterns, requires an increase in $[\text{Ca}^{2+}]_i$. In the present study we investigated the possibility that a Ca^{2+} -ATPase plays a role in modulating this transition. A preliminary report of these experiments has been published (DasGupta *et al.*, 1993b).

Materials and Methods

Media

The standard medium used was Earle's medium containing 5.56 mmol glucose l^{-1} , 0.1 mmol pyruvate l^{-1} and 100 U benzylpenicillin ml^{-1} (Glaxo Laboratories, Greenford). A concentrated glucose stock solution was prepared by adding 50 ×

the normal amount of glucose to glucose-free Earle's medium. From this a low glucose stock solution was prepared; when used, this gave a concentration of 5.56 $\mu\text{mol glucose l}^{-1}$. All media contained 4 mg human serum albumin ml^{-1} (Sigma Chemical Co., Poole).

Sperm preparation

Semen was obtained from both fertile research donors and from individuals undergoing assessment at the Diagnostic Andrology Service, London. Motile cells were prepared using mini-Percoll gradients (Ord *et al.*, 1990) [300 μl each of 85.5%, 63% and 45% (95%, 70% and 50% v/v solutions) prepared from a 90% substock solution of Percoll, made by adding nine parts of 100% Percoll (Pharmacia, Uppsala) to one part of a solution containing 10 ml 10 × Earle's concentrate (ICN Flow, High Wycombe, Bucks), 9 ml Albuminar (Armour Pharmaceutical Co. Ltd, Eastbourne), 0.37 ml sodium lactate (BDH, Poole), 2 ml Hepes buffer (Sigma Chemical Co.) and 3 mg sodium pyruvate]. After centrifugation at 600 g for 5 min at room temperature, the supernatant was removed; pelleted cells were resuspended in fresh medium, centrifuged again and resuspended in fresh medium. The sperm concentration was determined using a haemocytometer and then adjusted to 5×10^6 cells ml^{-1} ; suspensions were transferred to 15 ml centrifuge tubes (Greiner, Phillip Harris Scientific, London). A small drop of each suspension was placed on a microscope slide and the proportion of cells exhibiting progressive motility was estimated: this was usually > 90%. The suspensions were treated as detailed below, gassed with 5% CO_2 –5% O_2 –90% N_2 and incubated at 37°C.

Cell viability

To assess cell viability we used the vital dye Hoechst bis-benzimide 33258 (Sigma Chemical Co.). A 100 mg ml^{-1} stock solution was made in AnalaR water (BDH) and stored in a foil-wrapped bottle at 4°C for a maximum of 1 month. Before use the stock was diluted 1/1000 in protein-free Earle's medium and further diluted 1/100 in the sperm suspensions to a final concentration of 1 $\mu\text{g ml}^{-1}$. The suspensions were incubated at 37°C for 2 min and then washed by centrifugation at 900 g for 5 min at room temperature through 4 ml of 2% polyvinylpyrrolidone (PVP 40, Sigma Chemical Co.) solution. The resulting pellet was resuspended in culture medium and then stained with CTC 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) to determine whether they were alive or dead. 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 number of dead cells was low and they were distributed randomly among the CTC categories. All results are therefore for Hoechst-negative (live) cells only.

CTC assessment

The method used for CTC assessment was a modification of that described by Fraser and McDermott (1992). The CTC

solution, containing 750 $\mu\text{mol CTC l}^{-1}$ (Sigma Chemical Co.) in a buffer of 130 mmol NaCl l^{-1} , 5 mmol cysteine l^{-1} and 20 mmol Tris-HCl l^{-1} (final pH 7.8), was prepared daily and kept at 4°C in the dark. Cells were fixed in suspension so that cells incubated under different conditions could be fixed at essentially the same time; 100 μl of sperm suspension was added to a small (1.5 ml) foil-wrapped microcentrifuge tube followed by 100 μl of CTC solution, and these were mixed thoroughly. Cells were then fixed by adding 8 μl of 12.5% (w/v) paraformaldehyde in 0.5 mmol Tris-HCl l^{-1} buffer (final pH 7.4) and thoroughly mixing.

Slides were prepared by placing 10 μl of the suspension on to a clean microscope slide. A drop of 0.22 mmol 1,4-diazabicyclo [2.2.2]-octane l^{-1} (Sigma Chemical Co.) dissolved in glycerol:PBS (9:1, v/v) 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. This removed any excess fluid and ensured that 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 the slides retained fluorescence for 4–5 days, in general they were assessed on the same day or on the following day. Cells were assessed for CTC patterns using filter block D (ultraviolet and violet range). The Hg excitation beam was passed through a 355–425 nm band-pass filter and the CTC fluorescence emission was observed through an RKP 455 beam-splitting mirror.

It was first determined whether the cells were alive or dead, and they were then assessed for CTC staining patterns. Each of 200 live cells was classified as expressing one of the following three CTC patterns: 'F', uniform fluorescence over the head, which is characteristic of uncapacitated, acrosome-intact cells; 'B', a fluorescence-free band in the postacrosomal region, which is characteristic of capacitated, acrosome-intact cells; and 'AR', dull or absent fluorescence in the head region, characteristic of capacitated, acrosome-reacted cells (DasGupta *et al.*, 1993a).

Statistical analysis

Data were analysed using Cochran's test for the modification of 2×2 contingency tables (Snedecor and Cochran, 1980). This test compares responses *within* replicates, so that a significant response overall requires individual responses to be both consistent and of a sufficient magnitude. Each treatment sample was compared with the appropriate control sample.

Experimental Details and Results

Series 1: capacitation in the presence of quercetin, a Ca²⁺-ATPase inhibitor

A stock solution of 20 mmol quercetin (Sigma Chemical Co.) l^{-1} in dimethylsulfoxide (DMSO; Sigma Chemical Co.) was prepared, diluted to 10 mmol l^{-1} using DMSO:0.9% NaCl (1:1), and frozen in aliquots. For each experiment, substocks at concentrations of 5 and 2.5 mmol l^{-1} were prepared using the

same DMSO:NaCl diluent. These were added (1/50 dilution) to suspensions to give final quercetin concentrations of 200, 100 and 50 $\mu\text{mol l}^{-1}$; DMSO was added to the control suspensions (to a final concentration of 1%). After 1, 3, 5 and 22 h, aliquots were removed and stained with Hoechst and CTC ($n = 10$).

In all groups, the rate of change for each CTC pattern was greater over the first 5 h than during the subsequent interval to 22 h (Fig. 1). The effect of quercetin was noted from the first time of assessment after 1 h; subsequent changes then paralleled those observed in the control suspensions. Overall, there were significantly fewer cells ($P < 0.01$ – 0.001) displaying the F pattern and more cells ($P < 0.05$ – 0.001) displaying the B and AR patterns in the quercetin-treated suspensions (with the greatest response in the group treated with 200 $\mu\text{mol quercetin l}^{-1}$) than in the DMSO-treated control suspensions. After 1 h, the most pronounced change was an acceleration of the transition from the F to the B pattern. Although increases in AR pattern cells were noted, the greatest rate of change in this category was noted later, at 3–5 h. Thus, there appeared to be an initial response leading to accelerated capacitation and a subsequent occurrence of the acrosome reaction.

Quercetin proved to be fluorescent under the violet light used to observe CTC fluorescence, but the slightly orange fluorescence of quercetin was distinguishable from CTC, which is yellow. The motility of quercetin-treated samples did not diminish noticeably. There was an initial increase in the vigour of motility compared with control suspensions, but from 5 h onwards the motility began to deteriorate and by 22 h the majority of the cells were immotile in the groups treated with 100 and 200 $\mu\text{mol quercetin l}^{-1}$. The motility of the control suspensions remained relatively stable over the period evaluated, with the majority (70%) of cells still exhibiting progressive motility at 22 h.

Series 2: capacitation in the presence of W-7, a calmodulin inhibitor

A stock solution of 6.25 mmol N-(6-aminohexyl)-1-naphthalenesulfonamide l^{-1} (W-7; Sigma Chemical Co.) was made in AnalaR water (BDH) daily and stored at 4°C in the dark. Substocks were prepared and added (1/50 dilution) to suspensions to give final concentrations of 125, 25, 5 and 1 $\mu\text{mol l}^{-1}$. These concentrations were chosen because the IC_{50} value for W-7 in various calmodulin-dependent enzymatic systems in somatic cells is about 25–50 $\mu\text{mol l}^{-1}$ (Hidaka *et al.*, 1980; Hidaka and Tanaka, 1983). Aliquots were removed after 1 and 3 h and stained with Hoechst and CTC ($n = 8$).

After 1 h and 3 h the proportions of uncapacitated F pattern cells in all W-7-treated suspensions were markedly and significantly lower ($P < 0.001$) than those in control suspensions (Fig. 2). There was no evidence for a concentration-dependent response to W-7; all concentrations were equally effective. After 1 h, 92% of the cells in the control suspensions displayed an F pattern, while only 51–56% displayed this pattern in the W-7-treated suspensions; after 3 h, these values were 74% in control and 31–40% in treated suspensions. There were significantly more ($P < 0.001$) capacitated B and AR

Fig. 1. Changes in the distribution of (a) F, (b) B and (c) AR patterns of chlortetracycline (CTC) fluorescence in human spermatozoa over 22 h of incubation in either standard medium ($\circ$) or 50 ($\triangle$), 100 ($\blacksquare$) and 200 ($\blacklozenge$) $\mu\text{mol quercetin l}^{-1}$. Data are presented as means $\pm$ SEM ($n=10$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with controls (standard medium).

pattern cells in the W-7-treated suspensions, with about 26% B pattern cells and 19% AR pattern cells compared with about 7% B cells and 2% AR cells in controls after 1 h (Fig. 2a); after 3 h, the values were about 31% for B cells and 34% for AR cells in W-7-treated suspensions compared with about 21% B cells and 5% AR cells in controls (Fig. 2b).

The highest concentration of W-7 ($125 \mu\text{mol l}^{-1}$) severely affected sperm motility, the majority of the cells being immotile within 1 h. The other three concentrations stimulated sperm motility, with the cells moving much more vigorously in W-7-treated suspensions compared with controls at 1 h; cells were still motile at 3 h.

Fig. 2. Changes in chlortetracycline (CTC) patterns in human spermatozoa during incubation for (a) 1 h and (b) 3 h in 1–125 $\mu\text{mol W-7 l}^{-1}$. Data are presented as means $\pm$ SEM ($n=8$). ($\square$) F pattern, ($\blacksquare$) B pattern, (hatched) AR pattern. At both time points, all values in the treated groups were significantly different ($***P < 0.001$) from control values (no W-7).

Series 3: comparison of the calmodulin antagonists W-7 and W-5

The responses to W-7 observed in Series 2 suggest that a calmodulin-sensitive mechanism may be involved in human sperm capacitation. To investigate this further, we compared responses of these cells to W-7 and W-5, another but less effective calmodulin antagonist (Hidaka and Tanaka, 1983). Stock solutions of W-7 and W-5 (Sigma Chemical Co.) were prepared in AnalaR water and stored at 4°C in the dark. Suspensions were prepared and divided into three aliquots; W-7 and W-5 at 1 $\mu\text{mol l}^{-1}$ were added to two of these and the third served as the untreated control ($n=5$).

As in Series 2, W-7 had a significant effect on the CTC patterns, with a large decrease ($P < 0.001$) in F and large increases in B ($P < 0.01$) and AR ($P < 0.001$) pattern cells (Fig. 3). W-5 also had an obvious, but less pronounced, effect than W-7 on CTC patterns. There were significantly more ($P < 0.01$) F pattern cells and significantly fewer ($P < 0.05$) B and AR pattern cells in the W-5-treated suspensions than in the W-7-treated suspensions. These responses are consistent with a role for a calmodulin-sensitive mechanism in modulating human sperm capacitation.

Series 4: effect of various glucose concentrations

Sperm suspensions were prepared in glucose-free medium and divided into three parts. To one of these, a stock solution with a high concentration of glucose was added to give a final concentration of 5.56 mmol l^{-1} ; to another aliquot a stock solution with a low concentration of glucose was added to give a final concentration of 5.56 $\mu\text{mol l}^{-1}$. Fifteen minutes before these aliquots were assessed at 1, 5 and 22 h, the glucose concentration in the aliquot containing the low glucose concentration was raised to 5.56 mmol l^{-1} .

As noted in Series 1, the most rapid changes in all groups occurred during the first 5 h, with a decrease in the proportion of uncapacitated F pattern cells and an increase in the proportions of capacitated B and AR pattern cells. The stimulatory effect of reduced glucose was apparent after 1 h and subsequent changes in general paralleled those observed in control suspensions. At all times of assessment there were significantly fewer ($P < 0.01$ – 0.001) F pattern cells in the suspensions incubated in a reduced glucose concentration than in the suspensions incubated in the millimolar glucose concentration (Fig. 4a). Among the experimental groups, the response was

Fig. 3. Changes in chlortetracycline (CTC) patterns in human spermatozoa following incubation for 1 h in either W-7 or W-5 at $1 \mu\text{mol l}^{-1}$. Data are presented as means $\pm$ SEM ($n = 5$). (□) F pattern, (■) B pattern, (▨) AR pattern. ** $P < 0.01$ and *** $P < 0.001$ compared with controls.

least in the glucose-deficient medium and greatest in the medium containing the micromolar concentration of glucose that was raised to the millimolar concentration; exposure to the standard glucose concentration for 15 min had a marked effect on the suspensions with the low glucose concentration causing the proportion of F pattern cells to decrease further.

There was more uniformity among the experimental groups in the proportions of B pattern cells (Fig. 4b), with the values at any single time point being similar to each other, and all significantly higher ($P < 0.01$ – 0.001) than the value for control suspensions. With respect to AR cells, there were no significant differences between the groups containing millimolar glucose and no glucose, and only a slightly higher but non-significant response in the group with a continuous low concentration of glucose (Fig. 4c). In contrast, exposure to a millimolar concentration of glucose for 15 min caused a marked increase in AR pattern cells in suspensions with the low concentration of glucose; the values for suspensions in which the glucose concentration was raised were significantly higher ($P < 0.01$ – 0.001) than those in the control suspensions with millimolar glucose at all times assessed.

The motility in the glucose-deficient suspensions decreased slightly, with cells being rather sluggish and non-progressive. In all other treatment groups the cells remained progressively motile up to 22 h, with $> 75\%$ of the cells still motile.

Series 5: effect of mouse decapacitation factor on capacitated human spermatozoa

Earlier studies on mouse spermatozoa demonstrated the presence of surface-associated decapacitation factor molecules that modulate fertilizing ability (Fraser, 1984). The addition of either a crude or partially purified preparation of mouse sperm decapacitation factor to capacitated mouse sperm suspensions inhibits fertilizing ability; it also inhibits acrosomal exocytosis while causing a marked decrease in the number of capacitated B pattern cells and an increase in the F pattern cells (Fraser *et al.*, 1990). In the present study

we investigated whether adding partially purified mouse decapacitation factor to human spermatozoa, capacitated by incubating overnight, had any effect on the distribution of CTC patterns.

In these experiments partially purified mouse decapacitation factor was prepared as described by Fraser *et al.* (1990). Briefly, epididymal sperm suspensions were prepared in modified Tyrodes's medium supplemented with $0.3 \text{ mg BSA ml}^{-1}$ (crystalline, Sigma Chemical Co.). After incubation for 30 min at 37°C in $5\%\text{CO}_2$ – $5\%\text{O}_2$ – $90\%\text{N}_2$, suspensions were centrifuged at $11\,600 \text{ g}$ for 4 min at room temperature. Supernatants containing the factor were removed, frozen in liquid N_2 , lyophilized overnight and stored at -20°C . Partial purification was achieved by dissolving the lyophilized supernatants in distilled water, centrifuging at $10\,000 \text{ g}$ for 4 min at 4°C and subjecting the supernatant to gel filtration on Ultrogel AcA44 (Jones Chromatography, Hengoed, Glamorgan) using 0.4% (w/v) ammonium bicarbonate for elution. On the basis of the elution profile with four peaks, fractions were pooled to produce Fraction A (most of peak 2) and Fraction B (back of peak 2, and the trough and front half of peak 3). A bioassay of these fractions had previously shown that Fraction A contains little inhibitory decapacitation factor activity, while Fraction B contains the majority of activity (Fraser *et al.*, 1990). Fractions were frozen in liquid nitrogen and lyophilized. For experimental use, the material was reconstituted in $200 \mu\text{l}$ standard culture medium.

Human sperm suspensions were prepared and then incubated at 37°C . After 1 h an aliquot was removed and stained with CTC; this served as an early control sample. After 24 h a second aliquot was stained to serve as the pretreatment control. From the remaining suspensions, three $200 \mu\text{l}$ aliquots were transferred to small (1.5 ml) microcentrifuge tubes and centrifuged at 600 g for 5 min at room temperature. The supernatant was removed; one of the aliquots was resuspended in $200 \mu\text{l}$ of standard culture medium, one in Fraction A (negative control) and the remaining aliquot in Fraction B containing the decapacitation factor. After 30 min and 1 h, samples from

Fig. 4. Changes in the distribution of (a) F, (b) B and (c) AR patterns of chlortetracycline (CTC) fluorescence in human spermatozoa over a 22 h period of incubation in (●) a standard glucose concentration (5.56 mmol l^{-1}), (△) a glucose-deficient medium, (□) a low concentration of glucose ($5.56 \mu\text{mol l}^{-1}$) or (■) a medium with a low glucose concentration that was raised to a high concentration of glucose for the final 15 min of the experiment. Data are presented as means $\pm$ SEM ($n = 10$). ** $P < 0.01$ and *** $P < 0.001$ compared with controls (standard glucose concentration).

all three aliquots were stained with Hoechst and CTC ($n = 8$).

As in the other series, after 1 h in standard medium most cells exhibited the uncapacitated F pattern (only 5% of the

cells displayed the acrosome-intact B pattern), but after 24 h the majority of cells were capacitated, exhibiting either the B (35%) or AR (20%) pattern of CTC fluorescence (Fig. 5). When these suspensions were incubated in the presence

Fig. 5. Changes in chlortetracycline (CTC) patterns in human sperm suspensions incubated in standard medium for 24 h followed by exposure for 1 h to either partially purified mouse decapacitation factor (DF) known to inhibit capacitation and fertilization in the mouse, or material from the inactive fraction (Fr A) after chromatography. Control samples were assessed after 1 h (Con-1), 24 h (Con-24) and 25 h (Con-25). Data are presented as means $\pm$ SEM ($n = 8$). (□) F pattern, (■) B pattern, (▨) AR pattern. ** $P < 0.01$ and *** $P < 0.001$ compared with controls (Con-24).

of decapacitation factor for 30 min and 1 h, significantly fewer ($P < 0.01$ – 0.001) cells exhibited the B pattern and significantly more ($P < 0.01$ – 0.001) cells exhibited the uncapacitated F pattern than in the control suspensions at the same point in time. The responses were greater in the group treated for 1 h; these are the results shown in Fig. 5. Fraction A, the negative control, did not significantly alter the distribution of CTC patterns from that seen in controls. The motility of the cells did not appear to be affected by the decapacitation factor or by Fraction A, being similar to that in the control group.

Discussion

Current evidence indicates that the internalization of Ca^{2+} leading to a rise in $[\text{Ca}^{2+}]_i$ is required during both capacitation and acrosomal exocytosis in mammalian spermatozoa (e.g. Fraser and McDermott, 1992; DasGupta *et al.*, 1993a). Such a requirement is consistent with the inability of mammalian spermatozoa to complete capacitation and to undergo acrosomal exocytosis in the absence of extracellular Ca^{2+} (Fraser, 1982, 1987b; DasGupta *et al.*, 1993a). In somatic cells, a Ca^{2+} -ATPase that is located in the plasma membrane is important in maintaining low $[\text{Ca}^{2+}]_i$ by pumping Ca^{2+} out of the cell (Schatzmann, 1982). Recent studies on guinea-pig (Roldan and Fleming, 1989) and mouse (Fraser and McDermott, 1992; Adeoya-Osiguwa and Fraser, 1993) spermatozoa have provided evidence, albeit indirect, that Ca^{2+} -ATPase plays a role during capacitation, its activity declining, and thus permitting a rise in $[\text{Ca}^{2+}]_i$. In the study reported here, we used treatments with the potential to modulate the activity of a Ca^{2+} -ATPase to investigate whether this enzyme might play a role in human sperm capacitation monitored by changes in CTC fluorescence patterns on the sperm head.

When human spermatozoa were incubated in the presence of the Ca^{2+} -ATPase inhibitor quercetin (Wuthrich and Schatzmann, 1980) at 50 – $200 \mu\text{mol l}^{-1}$, all concentrations accelerated the transition from the uncapacitated to the capacitated state, as shown by a decrease in the proportion of F pattern cells and an increase in the proportion of B pattern cells compared with controls. Significant responses were observed within the first hour of treatment and then maintained throughout the period of assessment. A significant rise in the proportion of AR pattern cells was also observed, but there was a lag, with the greatest rate of change noted at 3–5 h. Inhibition of Ca^{2+} -ATPase activity by quercetin would allow $[\text{Ca}^{2+}]_i$ to rise, thereby promoting capacitation; with sufficiently high $[\text{Ca}^{2+}]_i$, acrosomal exocytosis would be triggered. These results with human sperm cells are consistent with those reported for guinea-pig, mouse and bull spermatozoa treated with quercetin (Roldan and Fleming, 1989; Fraser and McDermott, 1992; Fraser *et al.*, in press). Our observations provide indirect evidence that a decrease in Ca^{2+} -ATPase activity may occur during human sperm capacitation, enabling a rise in $[\text{Ca}^{2+}]_i$ to the threshold required for capacitation. We also observed that quercetin, which was itself fluorescent, was localized particularly in the postacrosomal region; a similar observation was made with quercetin-treated mouse spermatozoa (Fraser and McDermott, 1992). If the quercetin is bound to a Ca^{2+} -ATPase, then the Ca^{2+} -ATPase may be abundant in this region.

In all somatic cells except skeletal muscle, Ca^{2+} -ATPase has been shown to be sensitive to calmodulin (Schatzmann, 1982; Carafoli, 1987). Calmodulin has been identified in mammalian spermatozoa (e.g. Jones *et al.*, 1980; Feinberg *et al.*, 1981; Camatini *et al.*, 1986), and immunofluorescence studies have shown that calmodulin is found in both the sperm head and flagellum. Furthermore, there is evidence suggesting that calmodulin can activate a Ca^{2+} -ATPase in ram spermatozoa

(Forrester and Bradley, 1980). When human sperm suspensions were treated with the calmodulin antagonist W-7 (Hidaka and Tanaka, 1983) at 1–125 $\mu\text{mol l}^{-1}$, there was a very significant decrease in the proportion of uncapacitated cells and a concomitant rise in capacitated cells, both acrosome-intact and acrosome-reacted. Furthermore, the more potent antagonist W-7 had a more pronounced effect on CTC patterns than did W-5, a weaker antagonist (Hidaka and Tanaka, 1983), a result consistent with the involvement of a calmodulin-dependent mechanism. These data therefore suggest that calmodulin could play a role in modulating human sperm Ca²⁺-ATPase activity, a finding consistent with that of Adeoya-Osiguwa and Fraser (1993). These authors incubated mouse spermatozoa in 20 μmol trifluoperazine (TFP) l^{-1} , another calmodulin antagonist, and noted that the uptake of ⁴⁵Ca²⁺ early in capacitation was significantly stimulated, as was the transition from the F to the B and AR CTC patterns; more recently, TFP-treated mouse sperm suspensions were found to be significantly more fertile *in vitro* than untreated controls (L. R. Fraser, unpublished). These results further support the hypothesis that a decrease in calmodulin-sensitive Ca²⁺-ATPase activity plays an important role in capacitation. The recent report that the intracellular concentration of calmodulin decreases during capacitation of bull spermatozoa *in vitro* (Leclerc *et al.*, 1992) is consistent with this hypothesis. The present study has focused on W-7-related changes in the sperm head, but effects of W-7 on the flagellum were also noted. Although the highest concentration of W-7 used markedly inhibited motility, as did TFP at concentrations > 20 $\mu\text{mol l}^{-1}$ (Aitken *et al.*, 1988), all three lower concentrations tested stimulated motility. Whether these effects were exerted via the same pathways as those in the head is not yet known.

Since the Ca²⁺ pump requires ATP to maintain activity, we investigated the effect of reducing the glucose concentration in the culture medium to limit the availability of endogenous ATP. All treatments evaluated (omission of glucose, micromolar glucose, and raising micromolar concentrations of glucose to millimolar concentrations for the final 15 min) significantly stimulated the transition from the uncapacitated to the capacitated state when compared with controls containing a continuous millimolar concentration of glucose, the last of these experimental treatments being the most effective. Significant responses in all groups with reduced concentrations of glucose were detected by the end of the first hour of incubation; the differences, relative to the controls, were then maintained throughout the incubation and assessment period. Our observations that approximately the same proportions of AR cells were present in the control and glucose-deficient groups are consistent with those of Stock and Fraser (1991). Using transmission electron microscopy for assessment, they found approximately equal proportions of acrosome-reacted human sperm cells whether the exogenous substrates were glucose alone, glucose plus pyruvate or pyruvate plus lactate. Since all media in the present study contained pyruvate, irrespective of the glucose concentrations, these two sets of data are in agreement with each other.

The fact that introducing glucose during the final 15 min of the experiment produced the greatest decrease in F pattern cells and the greatest increase in AR pattern cells is similar to observations by Fraser and Herod (1989) that these changes in

CTC patterns in mouse spermatozoa require the presence of a glycolysable substrate. The fact that the patterns altered rapidly from F to B and AR upon the introduction of glucose was consistent with the demonstrably rapid, high fertilizing ability of similarly treated mouse sperm suspensions *in vitro*. Fraser and Herod (1989) surmised that the changes in CTC-binding patterns reflected energy-dependent changes in the cell surface components to which CTC binds.

Finally, studies have shown that uncapacitated epididymal mouse spermatozoa have a decapacitation factor that is lost or inactivated during capacitation (Fraser, 1984). When either crude or partially purified decapacitation factor is added to capacitated suspensions, there is a significant inhibition of fertilizing ability and a concomitant significant reversion from B to F in CTC staining patterns (Fraser, 1984; Fraser *et al.*, 1990). Thus, CTC fluorescence patterns detected in mouse spermatozoa are strongly correlated with the absence or presence of this factor. When partially purified mouse sperm decapacitation factor was added to capacitated human spermatozoa, a similar significant reversion from the B to the F pattern of CTC fluorescence was observed. These results therefore suggest that there could be similarities in the underlying physiological mechanisms that control the functional state of spermatozoa in the two species.

In conclusion, our data indicate that a Ca²⁺-ATPase may play a role during capacitation in human spermatozoa. Fraser and McDermott (1992) suggested that upon leaving the epididymis mouse spermatozoa are coated with decapacitation factor, which maintains a high level of Ca²⁺-ATPase activity; during capacitation this factor is lost, resulting in decreased Ca²⁺-ATPase activity and a consequent rise in [Ca²⁺]_i. Recent experiments have indicated that adding partially purified decapacitation factor to mouse sperm suspensions, washed to remove the factor, consistently reduces the accumulation of ⁴⁵Ca²⁺. This result is consistent with stimulation of Ca²⁺-ATPase activity by decapacitation factor (S. Adeoya-Osiguwa and L. R. Fraser, unpublished). Although an analogous decapacitation factor has yet to be identified in human spermatozoa, the results from our experiments suggest that a similar mechanism could be involved.

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