Ca²⁺-related changes in the mouse sperm capacitation state: a possible role for Ca²⁺-ATPase

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Summary. Mammalian spermatozoa require extracellular Ca²⁺, some of which must be internalized, to undergo complete capacitation. At a critical threshold, a rise in intracellular Ca²⁺ will trigger acrosomal exocytosis. We used chlortetracycline (CTC) fluorescence patterns to assess changes in the capacitation state of mouse spermatozoa after incubation under various conditions that would affect their intracellular Ca²⁺ concentrations. Under standard conditions with 1.80 mmol CaCl₂ l⁻¹ known to support capacitation within 120 min and subsequent fertilization *in vitro*, a rise in the number of capacitated, acrosome-intact cells (B pattern) was observed over the first 60 min, followed by a decline. A detectable increase in capacitated, acrosome-reacted cells (AR pattern) coincided with the maximum of B pattern cells and a continued rise was observed over the following 60 min. With incubation in 3.60 mmol Ca²⁺ l⁻¹, the rise in AR cells began at 30 min, suggesting that this treatment accelerates capacitation. Introduction of ionophore A23187 at 15 min to cells in standard Ca²⁺ produced a similar but even more rapid response, with a maximum in B pattern cells and a noticeable rise in AR cells within 10 min. Thus ionophore-treated cells proceed through capacitation, but do so very quickly. However, ionophore in the presence of 90 µmol Ca²⁺ l⁻¹ could promote transition from the uncapacitated F pattern to the capacitated B pattern, but could not trigger acrosomal exocytosis, indicating that the latter requires high extracellular Ca²⁺. After preincubation in Ca²⁺-deficient medium, most cells exhibited the uncapacitated F pattern and the introduction of millimolar Ca²⁺ altered this distribution only slowly, over a period of 50 min. In contrast, preincubation in 90 µmol Ca²⁺ l⁻¹ resulted in a minority of F pattern cells and, within 10 min of millimolar Ca²⁺ introduction, a significant increase in AR cells was observed. These results suggest that changes in intracellular Ca²⁺ are important both for transition from the uncapacitated to the capacitated state, and for triggering of acrosomal exocytosis; furthermore, changes in CTC fluorescence patterns reflect alterations in Ca²⁺ within the cells. Both quercetin and ethacrynic acid, compounds that inhibit Ca²⁺-ATPase in somatic cells, caused a significant increase in the proportion of B pattern capacitated cells, suggesting that intrinsic Ca²⁺-ATPase activity may play a role during capacitation. The limited effect on acrosomal exocytosis *per se* suggests that another mechanism, possibly involving calcium channels, may control this latter event.

Keywords: mouse; ionophore; Ca²⁺-ATPase; quercetin; ethacrynic acid; decapacitation factors; spermatozoa

Introduction

Experimental evidence has indicated that extracellular Ca²⁺ plays a fundamental role in sperm function. As long ago as 1915, Loeb demonstrated that extracellular Ca²⁺ was necessary to obtain fertilization in invertebrates; a similar requirement for mammals exists, as first demonstrated by

Iwamatsu & Chang (1971) in the mouse. In all species examined, an influx of Ca^{2+} from the extracellular compartment is required to initiate the acrosome reaction, thereby releasing lytic enzymes and promoting membrane changes that are important for sperm-oocyte interaction (reviewed by Fraser, 1987a). Hyperactivated motility, which appears to facilitate sperm movement through the viscous oviductal environment (Suarez *et al.*, 1990) and is obligatory for penetration of the zona pellucida (Fraser, 1981), is also Ca^{2+} dependent (guinea-pig: Yanagimachi & Usui, 1974; mouse: Fraser, 1977; hamster: Yanagimachi, 1982). Temporally, hyperactivated motility and the acrosome reaction are late events, relative to the time of mammalian sperm release from the male reproductive tract. As has been known since 1951 (Austin; Chang) mammalian spermatozoa must undergo a post-release final maturation, termed capacitation, which prepares them for fertilization. Hyperactivated motility, which appears to be a component of the capacitation process, occurs towards the end and the acrosome reaction occurs only in fully capacitated cells.

Ca^{2+} was initially considered to be required by mammalian spermatozoa only at the end of capacitation: guinea-pig spermatozoa, preincubated in Ca^{2+} -deficient medium (no added Ca^{2+} salt but containing traces of Ca^{2+} contributed by other constituent salts) for sufficient time to permit capacitation in complete medium, could express hyperactivation and undergo acrosomal exocytosis within minutes of extracellular Ca^{2+} introduction (Yanagimachi & Usui, 1974). However, more recent studies have demonstrated that similar treatment applied to hamster (Yanagimachi, 1982), human (Stock & Fraser, 1989) and mouse spermatozoa (Fraser, 1982, 1987b) did not trigger a marked increase in acrosome loss. Furthermore, such spermatozoa were less fertile than cells incubated continuously in Ca^{2+} -containing medium (Fraser, 1982; Yanagimachi, 1982). Functionally, they appeared to be only partially capacitated, requiring Ca^{2+} to complete capacitation and hence acquire full fertilizing ability. Mouse sperm suspensions typically require 2 h to complete capacitation in the presence of Ca^{2+} . If incubated in Ca^{2+} -deficient medium ($\pm$ EGTA), they require approximately 1 h after the addition of Ca^{2+} to reach full functional potential (Fraser, 1982, 1987b). The minimum extracellular Ca^{2+} concentration that is required to support mouse sperm capacitation *per se* as well as the terminal events of hyperactivation and acrosomal exocytosis to achieve fertilization, was determined. Quite low concentrations of Ca^{2+} will promote capacitation (minimum of $90 \mu\text{mol l}^{-1}$), but millimolar concentrations ($1\text{--}80 \text{ mmol l}^{-1}$) are required for maximal fertilization (Fraser, 1987b). In a recent study, it has been reported that the inclusion of EGTA in Ca^{2+} -deficient medium used for incubation of guinea-pig spermatozoa abolished the rapid response to added extracellular Ca^{2+} (Roldan & Fleming, 1989), suggesting that these cells also require Ca^{2+} , albeit at low concentrations, during capacitation.

Most studies that have evaluated Ca^{2+} -related changes in sperm function have assessed fertilizing ability *in vitro*, or spontaneous acrosome loss or both. In this study we used the chlortetracycline fluorescence technique (Ward & Storey, 1984) which provides information on the functional state of sperm cells. It is particularly useful because it allows discrimination between uncapacitated and capacitated acrosome-intact cells, a distinction not possible with conventional assessment techniques. Using this approach we have investigated the kinetics of Ca^{2+} -related changes in the capacitation state of sperm populations. A preliminary report of some of this work has been made (McDermott & Fraser, 1990).

Materials and Methods

Media

A modified Tyrode's solution (Fraser, 1983) lacking pyruvate and lactate and containing $5\text{--}56 \text{ mmol glucose l}^{-1}$, $1\text{--}80 \text{ mmol CaCl}_2 \text{ l}^{-1}$ and crystalline BSA (4 mg ml^{-1} ; Sigma, Poole, Dorset, UK) was the standard medium. Calcium-deficient medium was prepared by omitting CaCl_2 ; although this contains trace amounts of free Ca^{2+} ($< 20 \mu\text{mol l}^{-1}$ when measured with a Ca^{2+} electrode; Fraser, 1987b), it supports neither complete capacitation nor fertilization *in vitro* (Fraser, 1982, 1987b). To introduce $1\text{--}80 \text{ mmol CaCl}_2 \text{ l}^{-1}$, a stock of Tyrode's containing $22\text{--}5 \text{ mmol CaCl}_2 \text{ l}^{-1}$ was prepared (high Ca^{2+} stock); $20 \mu\text{l}$ of this was added to each $230 \mu\text{l}$ Ca^{2+} -deficient medium

or suspension. Appropriate substocks of the 22.5 mmol CaCl₂ l⁻¹ solution were prepared and used in the same ratio to produce low Ca²⁺ (90 µmol l⁻¹) medium.

Sperm suspension preparation

The contents of the cauda epididymides from one mature (>8 weeks) TO male mouse (Harlan OLAC, Bicester, Oxon, UK) were released into 1 ml medium. When two different Ca²⁺ concentrations were being compared, spermatozoa were released into medium containing the lower Ca²⁺ concentration for the experiment to ensure homogeneity of the various treatment groups. After 5 min for dispersal, the suspension was gently agitated, 460 µl was removed to a fresh dish and the appropriate Ca²⁺ stock was added to achieve the desired concentration. Suspensions were incubated at 37°C in an atmosphere of 5% CO₂-5% O₂-90% N₂; duration of incubation depended on experimental design. Plastic culture dishes, 30 mm diameter (Sterilin, Teddington, Middx, UK), were used and all media were overlaid with autoclaved liquid paraffin (Boots, Nottingham, UK).

Chlortetracycline assessment

The method used is a modification of that described by Ward & Storey (1984). The chlortetracycline (CTC) solution, containing 750 µmol CTC l⁻¹ (Sigma) in a buffer of 130 mmol NaCl l⁻¹, 5 mmol cysteine l⁻¹, 20 mmol Tris-HCl l⁻¹ (final pH 7.8), was prepared fresh and kept wrapped in foil to exclude light. To obtain precise timings for the kinetic studies, we stained and fixed the cells in suspension; this enabled us to fix spermatozoa, incubated under different conditions, at essentially the same time. The method involved adding 45 µl sperm suspension to a small (0.5 ml) microcentrifuge tube wrapped in foil, then 45 µl CTC solution. After mixing well, 4 µl of 12.5% (w/v) para-formaldehyde in 0.5 mol Tris-HCl l⁻¹ buffer (final pH 7.4) was added with mixing. Slides were prepared by placing 10 µl of this suspension on a clean slide. One drop of 0.22 mol l,4-diazabicyclo[2.2.2]octane l⁻¹ (Sigma) in glycerol was mixed in, carefully, to retard fading of fluorescence. A coverslip was added and the slide was gently but firmly compressed between tissues to remove excess fluid. This helped to maximize the number of spermatozoa lying flat on the slide, an orientation necessary for accurate assessment. Slides were sealed with colourless nail varnish and stored in a light-proof box in the cold. In general, they were assessed on either the same day or the next; it was evident, however, that storage under these conditions maintained fluorescence for at least 4–5 days. Cells were assessed on an Olympus BHS microscope equipped with phase-contrast and epifluorescent optics. The Hg excitation beam was passed through a 405 nm band pass filter and CTC fluorescence emission was observed through a DM 455 dichroic mirror.

In each sample, 100 spermatozoa were classified as expressing one of three CTC staining patterns: 'F', with uniform head fluorescence, which is characteristic of uncapacitated, acrosome-intact cells; 'B', with a fluorescence-free band in the postacrosomal region, which is characteristic of capacitated, acrosome-intact cells, or 'AR', with dull or absent head fluorescence, which is characteristic of acrosome-reacted cells. The presence or absence of the acrosomal cap on each cell was verified under phase-contrast illumination (Fraser, 1987b).

Statistical analysis

Data were analysed using Cochran's test for combination of 2 × 2 contingency tables (Snedecor & Cochran, 1980).

Experimental Details and Results

Series I: capacitation state in standard and high Ca²⁺

In these experiments, a suspension was prepared in standard Tyrode's solution containing 1.80 mmol Ca²⁺ l⁻¹ (1X Ca). After 5 min, 460 µl was transferred to a new culture dish and 40 µl of high Ca²⁺ stock (see Materials and Methods) was added to raise the Ca²⁺ concentration to 3.60 mmol Ca²⁺ l⁻¹ (2X Ca). Suspensions were incubated for a total of 120 min, with aliquots being removed, stained and fixed at 15, 30, 60 and 120 min. Three replicate experiments were carried out (*n* = 3).

Because we were focusing on capacitated cells, only values for B and AR patterns have been given; the remainder of the cells, to make each time-point up to 100%, were exhibiting the uncapacitated F pattern. Considering the 1X Ca suspensions first, over 120 min the proportion of B pattern cells rose to a maximum at 60 min and then declined (Fig. 1). From 60 min onward, the

proportion of AR cells increased, i.e. as B patterns declined in number the ARs increased. Such a time-dependent increase in spontaneously acrosome-reacted cells is a common feature of *in vitro* capacitation systems (e.g. Fraser, 1982, 1987b; Yanagimachi, 1982; Roldan & Fleming, 1989).

Fig. 1. CTC fluorescence patterns in mouse sperm suspensions incubated in media containing either $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ (1X Ca^{2+}) or $3.60 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ (2X Ca^{2+}). Only data (mean $\pm$ SEM) for the capacitated patterns, B and AR, are shown ($n = 4$). ($\blacktriangle$) B, 1X Ca^{2+} ; ($\triangle$) B, 2X Ca^{2+} ; ($\bullet$) AR, 1X Ca^{2+} ; ($\circ$) AR, 2X Ca^{2+} . * $P < 0.05$ compared with corresponding 1X Ca^{2+} suspensions.

In the 2X Ca suspensions, a similar pattern was observed, although the time of maximum B values was considerably earlier, at 30 min. At this same point, a marked rise in the proportion of AR pattern cells occurred; at both 30 and 60 min, a significantly higher ($P < 0.05$) proportion of cells were exhibiting the AR pattern in the 2X Ca medium than in the 1X Ca . By 120 min, the values in the two groups were similar. These results therefore suggest that capacitation is accelerated in the presence of elevated extracellular Ca^{2+} , but that the pattern of response is similar: the detectable increase in AR cells coincides with the maximum number of cells exhibiting the B pattern.

Series II: capacitation state following exposure to a Ca^{2+} ionophore

Ionophores are frequently used to manipulate the intracellular concentration of specific ions. We used the ionophore A23187, with specificity for Ca^{2+} over Mg^{2+} , to study CTC patterns following introduction of the drug. Since spermatozoa, like most eukaryotic cells, have low intracellular Ca^{2+} (in the nanomolar range), ionophore treatment in the presence of extracellular Ca^{2+} results in increased intracellular Ca^{2+} (Babcock *et al.*, 1976). This in turn will trigger the acrosomal exocytosis after a short time, even if the cells are not fully capacitated at the time of treatment (e.g. guinea-pig and hamster: Talbot *et al.*, 1976; boar: Peterson *et al.*, 1978; mouse: Fraser, 1982; human: Aitken *et al.*, 1984; Cross *et al.*, 1986; Stock & Fraser, 1989). The rapidity of the response has suggested that capacitation is 'bypassed'. We have used CTC assessment to determine whether this is the case.

A stock solution of $3 \text{ mmol A23187 l}^{-1}$ (free acid, Sigma) stock was prepared in DMSO and used at a final concentration of $15 \mu\text{mol l}^{-1}$. A preliminary investigation indicated that the DMSO present (0.5%) had no detectable effect on CTC pattern distribution (data not shown). In the first experiments, sperm suspensions were prepared in standard Tyrode's with $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$; after 15 min, an aliquot was removed and A23187 was added. Samples were taken from the treated suspensions, stained and fixed at 20, 25, 35 and 55 min (i.e. 5, 10, 20 and 40 min after ionophore addition); samples from the untreated suspensions were assessed at 20 and 55 min only ($n = 4$).

The general pattern of changes after ionophore introduction is essentially that observed in Series I, with a maximum of B cells (10 min after A23187 addition) coinciding with the first detectable rise in AR cells (Fig. 2). The proportion of cells exhibiting the B pattern then declined, while the number of AR cells increased markedly. In the untreated suspension, the second (and final) assessment point in this series essentially coincided with the maximum number of B cells (see Fig. 1). At this point, the proportion of AR cells was significantly higher ($P < 0.05$) in the ionophore-treated group than in the control group. These results suggest that cells exposed to ionophores do go through the stages of capacitation, but very quickly. Furthermore, they suggest that the changes in CTC fluorescence patterns reflect changes in intracellular Ca²⁺ levels.

Fig. 2. Changes in CTC fluorescence patterns in mouse sperm suspensions incubated in medium containing 1.80 mmol Ca²⁺ l⁻¹ following the introduction of 15 µmol A23187 l⁻¹ at 15 min (arrow). Only data (mean ± SEM) for the capacitated patterns, B and AR, are shown ($n = 4$). (▲) B, A23187; (△) B, control; (●) AR, A23187; (○) AR, control. * $P < 0.05$ compared with corresponding ionophore-free suspensions.

We then investigated the effect of A23187 on spermatozoa incubated in low Ca²⁺ (90 µmol l⁻¹) medium which supports capacitation but not the acrosome reaction. Since ionophore-induced responses require extracellular Ca²⁺, we therefore investigated whether low Ca²⁺ would provide sufficient Ca²⁺ to trigger acrosomal exocytosis. After incubation for 15 min, an aliquot was removed and A23187 was added; both suspensions were incubated for a further 30 min and assessed ($n = 4$). After a 45 min incubation in low Ca²⁺ medium, most cells expressed the uncapacitated F pattern (Fig. 3); this is consistent with data in Figs 1 and 2. In suspensions treated with A23187, a significant reduction ($P < 0.025$) in the proportion of F cells and an equally significant increase in number of B pattern cells were observed, relative to the untreated suspensions. Unlike the earlier experiments in this series, however, there was essentially no change in the proportion of AR cells. Thus, in low Ca²⁺ medium A23187 treatment promotes a sufficient rise in intracellular Ca²⁺ to significantly accelerate the transition from the uncapacitated to the capacitated, acrosome-intact state. The next step, which triggers acrosomal exocytosis, requires higher concentrations of extracellular Ca²⁺ to raise the intracellular Ca²⁺ above the threshold for the exocytotic event.

Series III: capacitation state in Ca²⁺-deficient medium

Earlier studies showed that if mouse spermatozoa were incubated for 120 min in Ca²⁺-deficient medium and then received 1.80 mmol Ca²⁺ l⁻¹, the suspensions were of low fertility and essentially

Fig. 3. Changes in CTC fluorescence patterns in mouse sperm suspensions incubated in medium containing $90 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$ and receiving $15 \mu\text{mol A23187 l}^{-1}$ at 15 min. Cells were assessed after a further 30 min ($n = 4$). Data are presented as mean $\pm$ SEM. (□) F; (■) B; (▨) AR. ** $P < 0.025$ compared with corresponding ionophore-free suspensions.

could not undergo rapid acrosomal exocytosis in response to high Ca^{2+} (Fraser, 1982, 1987b). We evaluated the capacitation state of such spermatozoa to determine whether the distribution of CTC patterns is consistent with these functional abilities.

In the first experiments, suspensions in Ca^{2+} -deficient and Ca^{2+} -containing media were assessed at 30 and 120 min ($n = 3$). A significant increase ($P < 0.025$) in number of AR cells occurred in the complete medium, but no changes with time were observed in the other group (Fig. 4). We then assessed the effect of adding Ca^{2+} after 120 min to such Ca^{2+} -deficient suspensions; cells were prepared for analysis after 10 min in $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ ($n = 4$). There was essentially no change in the proportion of cells in any of the three categories after the introduction of Ca^{2+} : most cells continued to express the uncapacitated F pattern (Fig. 5). In contrast, the suspensions incubated continuously in $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ had significantly fewer ($P < 0.01$) F cells and significantly more ($P < 0.01$) AR cells.

Fig. 4. CTC fluorescence patterns in mouse sperm suspensions incubated for 120 min in media without ($-\text{Ca}^{2+}$) and with ($+\text{Ca}^{2+}$) $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$. Only data (mean $\pm$ SEM) for the capacitated patterns, B and AR, are shown ($n = 3$). (▲) B, $+\text{Ca}^{2+}$; (△) B, $-\text{Ca}^{2+}$; (●) AR, $+\text{Ca}^{2+}$; (○) AR, $-\text{Ca}^{2+}$. *** $P < 0.01$ compared with $-\text{Ca}^{2+}$ suspensions.

Fig. 5. CTC fluorescence patterns in mouse sperm suspensions incubated for 120 min in media without ($-Ca^{2+}$) and with ($+Ca^{2+}$) $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ for 120 min; aliquots of $-Ca^{2+}$ suspensions received $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ ($- \rightarrow +Ca^{2+}$) and all treatment groups were assessed 10 min later ($n = 3$). Data are presented as mean $\pm$ SEM. ($\square$) F; ($\blacksquare$) B; ($\boxtimes$) AR. **** $P < 0.001$ compared with $+Ca^{2+}$ suspensions.

In the next experiments, suspensions were incubated for 120 min in Ca^{2+} -deficient medium and millimolar Ca^{2+} was added, as above. The kinetics of change in the capacitation state were assessed by taking samples 15, 20, 25, 30, 40 and 50 min after Ca^{2+} addition. For comparison, samples were taken at 15, 30 and 50 min from the continuous Ca^{2+} suspensions ($n = 3$). A maximum of B pattern cells occurred 20 min after addition of Ca^{2+} , the proportion of B cells then declining (Fig. 6). At the same point, an obvious rise in the proportion of AR cells began and continued throughout the assessment until, at 50 min, the value was close to that in the continuous Ca^{2+} group. This pattern of change is again similar to that seen in Fig. 1, the main difference being that the maximum of B cells occurred at 60 min in the continuous $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ cells (Fig. 1) and at 20 min after introduction of Ca^{2+} to suspensions preincubated in Ca^{2+} -deficient medium (Fig. 6). These observations are consistent with functional studies indicating that the latter treatment permits partial capacitation to occur but requires extracellular Ca^{2+} for completion.

In the final experiment in this series, the CTC patterns in suspensions incubated in low Ca^{2+} medium and then given $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ were evaluated. Suspensions in low and standard Ca^{2+} were prepared and incubated for 120 min; an aliquot of the former was removed and high Ca^{2+} stock added to increase the Ca^{2+} concentration. After 10 min, cells were prepared for assessment ($n = 4$).

Increasing the Ca^{2+} concentration in the low Ca^{2+} suspensions caused a marked alteration in the distribution of the three categories of CTC staining: a significant decline ($P < 0.025$) in the proportion of B cells and a significant increase ($P < 0.025$) in the proportion of AR cells occurred (Fig. 7). Within 10 min of Ca^{2+} increase, the CTC pattern distribution was essentially that seen in the cells incubated continuously in $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$. It should also be noted that only a few cells expressed the F pattern after 120 min incubation in low Ca^{2+} medium, in sharp contrast to the majority of F cells after incubation in Ca^{2+} -deficient medium (Fig. 5). These responses to micromolar and millimolar Ca^{2+} , in terms of CTC assessment of capacitation state, are consistent with the functional studies: given sufficient extracellular Ca^{2+} , such cells are immediately highly fertile (Fraser, 1987b).

Series IV: capacitation state in the presence of Ca^{2+} -ATPase inhibitors

Results from the earlier series suggested that completion of capacitation, as evidenced by transition from the F pattern to the B and AR patterns of CTC fluorescence, requires an increase in

Fig. 6. Changes in CTC fluorescence patterns in mouse sperm suspensions preincubated for 120 min in Ca^{2+} -deficient medium and then receiving $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ ($- \rightarrow +\text{Ca}^{2+}$). Cells were incubated for a further 50 min, for a total of 170 min, with assessments at the times shown ($n = 3$). Suspensions incubated continuously in Ca^{2+} ($+\text{Ca}^{2+}$) were also assessed for comparison. Only data (mean $\pm$ SEM) for the capacitated patterns, B and AR, are presented. (▲) B, $- \rightarrow +\text{Ca}^{2+}$; (△) B, $+\text{Ca}^{2+}$; (●) AR, $- \rightarrow +\text{Ca}^{2+}$; (○) AR, $+\text{Ca}^{2+}$.

Fig. 7. CTC fluorescence patterns in mouse sperm suspensions incubated for 120 min in media with $90 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$ (low Ca^{2+}) and $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ ($+\text{Ca}^{2+}$); millimolar Ca^{2+} was then added to aliquots of low Ca^{2+} (low $\rightarrow +\text{Ca}^{2+}$) suspensions and all treatment groups were assessed 10 min later ($n = 4$). Data are presented as mean $\pm$ SEM. (□) F; (■) B; (▨) AR. * $P < 0.05$, *** $P < 0.01$ compared with $+\text{Ca}^{2+}$ suspensions.

intracellular Ca^{2+} . In many somatic cells, low intracellular Ca^{2+} concentrations are maintained by a Ca^{2+} -ATPase which pumps Ca^{2+} out of the cells. Since mammalian spermatozoa are known to have such a Ca^{2+} pump (see Roldan & Fleming, 1989), treatment of cells to inhibit the pump should result in an increase in intracellular Ca^{2+} . We therefore investigated the effects of two Ca^{2+} -ATPase inhibitors, quercetin and ethacrynic acid, both of which stimulated the acrosome reaction in guinea-pig spermatozoa (Roldan & Fleming, 1989).

Quercetin (Sigma) was difficult to dissolve and maintain in solution: neither ethanol nor acetone was satisfactory. A stock solution of $20 \text{ mmol quercetin l}^{-1}$ in dimethyl sulfoxide (DMSO) (Sigma) was prepared and a diluent consisting of 1:1, DMSO:0.9% NaCl, was used to dilute the stock to

10 mmol l⁻¹. This was frozen in aliquots which were thawed and used as needed. For each experiment, the 10 mmol l⁻¹ stock was diluted further, using the DMSO: NaCl diluent, to provide 5 and 2.5 mmol l⁻¹ substocks. These were added to suspensions at a 1/50 dilution, giving final quercetin concentrations of 200, 100 and 50 µmol l⁻¹: sperm suspensions in standard medium were prepared and after 5 min to allow dispersal, 4 µl of each stock was added to 196 µl sperm suspension. Since the final concentration of DMSO in these suspensions was 1%, 4 µl of diluent was added to 196 µl sperm suspension as the control group. After 40 min, samples were prepared and assessed (*n* = 3).

Fig. 8. Changes in CTC fluorescence patterns in mouse sperm suspensions incubated for 40 min in increasing concentrations of quercetin (*n* = 3). Data are presented as mean ± SEM. (□) F; (■) B; (▨) AR. **P* < 0.05, ***P* < 0.025 compared with untreated control suspensions.

All three quercetin concentrations tested significantly increased (*P* < 0.025) the proportion of B cells, resulting in a majority of cells with this pattern (Fig. 8); at 100 and 200 µmol l⁻¹ there were also significant increases in the AR cells (*P* < 0.05, *P* < 0.025, respectively). In contrast, most cells treated with DMSO only exhibited the uncapacitated F pattern. This distribution is like that seen at similar times in other experiments (e.g. Figs 1, 3), indicating that the DMSO *per se* is not affecting the CTC patterns. Quercetin itself was fluorescent under the violet light used for CTC assessment. In the head, fluorescence was greatest in the postacrosomal region, although some staining could also be detected in the acrosomal cap; in the tail, the midpiece was strongly fluorescent. The colour of the quercetin was slightly orange and hence distinguishable from the CTC which is yellow. The pattern of quercetin fluorescence in an acrosome-intact cell is shown (Fig. 9a); the same cell visualized under phase contrast is shown (Fig. 9b), with the acrosomal cap indicated by an arrow.

In the final experiments, ethacrynic acid (Sigma) was dissolved in DMSO and this was diluted with the DMSO: 0.9% NaCl diluent to give stocks of 5, 2.5 and 1.25 mmol l⁻¹. Five minutes after preparation of sperm suspensions, 196 µl aliquots were removed and 4 µl of each drug stock was added to give final concentrations of 100, 50 and 25 µmol l⁻¹. Because the 1% DMSO had not affected the CTC staining in earlier experiments, DMSO was not added to control suspensions. After 40 min, samples were prepared and assessed (*n* = 3).

Ethacrynic acid, like quercetin, caused a significant increase in the proportion of B cells (at 50 µmol l⁻¹, *P* < 0.05; at 100 µmol l⁻¹, *P* < 0.025). There were also increases in the proportion of AR cells, although these were not statistically significant (Fig. 10).

Although motility was not formally assessed, neither quercetin nor ethacrynic acid noticeably reduced sperm motility. Thus there was no evidence to suggest that these compounds were causing the cells to die and undergo degenerative changes.

Fig. 9. (a) Fluorescence pattern exhibited by an acrosome-intact mouse sperm cell incubated in the presence of quercetin. In the head, fluorescence is particularly visible in the postacrosomal region (arrow). (b) Phase contrast micrograph of the cell shown in (a); the acrosomal cap is indicated by an arrow. $\times 1600$.

Fig. 10. Changes in CTC fluorescence patterns in mouse sperm suspensions incubated for 40 min in increasing concentrations of ethacrynic acid ($n = 3$). Data are presented as mean $\pm$ SEM. ($\square$) F; ($\blacksquare$) B; ($\boxtimes$) AR. * $P < 0.05$, ** $P < 0.025$ compared with untreated control suspensions.

Discussion

In this study we evaluated changes in the capacitation state, as monitored by CTC fluorescence, of epididymal mouse sperm cells incubated under various conditions known to affect sperm function. The treatments chosen included those capable of altering intracellular Ca²⁺ levels. Over a period of 120 min in standard Tyrode's medium containing 1.80 mmol Ca²⁺ l⁻¹, the proportion of cells exhibiting the B pattern (capacitated, acrosome-intact) increased, reaching a maximum at 60 min, and then declined. From that point onwards, a sustained rise in the proportion of AR cells was also observed. When spermatozoa from the same initial suspensions were incubated in medium with 3.60 mmol Ca²⁺ l⁻¹ (2 × standard), the same pattern of change was observed: the maximum number of B pattern cells coincided with the first obvious increase in AR cells; the number of B pattern cells then declined and the number of AR cells continued to rise. The difference lay in the timing, with the maximum occurring at the earlier time of 30 min. Thus high extracellular Ca²⁺ promoted more rapid capacitation as determined by CTC assessment, a finding consistent with fertilization studies *in vitro* where similarly-treated suspensions were functionally superior to those incubated in 1.80 mmol Ca²⁺ l⁻¹ (Fraser, 1987b).

A possible explanation for this response to 2 × Ca²⁺ is that such treatment causes an increase in intracellular Ca²⁺ which in turn promotes the events of capacitation. As spermatozoa exist in an environment with millimolar concentrations of extracellular Ca²⁺, yet maintain intracellular Ca²⁺ in the nanomolar range, they must have efficient mechanisms that can remove excess intracellular Ca²⁺. Both Ca²⁺-ATPases and Ca²⁺-Na⁺ exchangers have been suggested to serve this function (reviewed by Fraser, 1987a; Roldan & Fleming, 1989). It is probable that spermatozoa become more permeable to Ca²⁺ during capacitation, allowing intracellular Ca²⁺ to rise (e.g. White & Aitken, 1989) until a critical threshold is reached; a further increase would then trigger acrosomal exocytosis (see Harrison, 1982; Yanagimachi, 1988; Florman *et al.*, 1989). From this and earlier studies it would appear that in the presence of high extracellular Ca²⁺, the rate of Ca²⁺ influx exceeded that of efflux at an earlier time and consequently promoted more rapid transition from the uncapacitated F to the capacitated B pattern of CTC fluorescence. A steady rise in the proportion of cells undergoing acrosomal exocytosis then followed. We suggest that the changes in CTC distribution on the surface of the cell provide an indirect indication of changes in intracellular Ca²⁺ concentrations within the head. Thus, cells with the F pattern would have lower intracellular Ca²⁺ than those with the B pattern.

It should perhaps be noted here that in the original description of the CTC technique, Ward & Storey (1984) reported a time-dependent increase in the proportion of B pattern cells, reaching peak values of about 80%. We have never observed such high proportions in sperm cells incubated under standard conditions which support capacitation and fertilization *in vitro* (Fraser & Herod, 1990). It is uncertain whether this is a reflection of strain differences. We have also consistently seen significant increases in the proportion of cells classified as 'acrosome-reacted' in suspensions with demonstrable fertilizing ability, whether assessed by electron microscopy (Fraser, 1982), phase-contrast light microscopy (e.g. Fraser, 1984, 1987b) or fluorescence microscopy (Fraser & Herod, 1990). The majority of these would have undergone spontaneous exocytosis, but some would be dead, degenerating cells. For most light microscope assessments we have filtered suspensions to select for motile and hence living cells; this works effectively, producing suspensions with about 90% motile cells. Under conditions that inhibit acrosomal exocytosis and fertilization, approximately 10% of filtered cells lack an acrosomal cap (Fraser & Quinn, 1981; Fraser, 1982) and probably represent non-motile, degenerating cells. In the present study, we have not filtered suspensions because this reduces the number of cells and makes analysis much more difficult. Some of the spermatozoa classified as AR will therefore have lost the acrosomal cap because of degeneration; we estimate this to account for about 20% of the cells. For example, about 10% of spermatozoa incubated for 120 min in Ca²⁺-deficient medium, filtered and then assessed lacked an acrosomal cap (Fraser, 1987b); in this study, the value for similarly incubated but unfiltered cells

was about 30% (Fig. 5). This means that values for only motile cells would show increases in the F and B categories and a decrease in the AR. Even then, the proportion of B pattern cells would not reach about 80% and the proportion of AR cells would still increase significantly with time. Since Ward & Storey (1984) used unselected suspensions, as we have done, these discrepancies are puzzling. However, it is the overall change or otherwise in CTC pattern distribution within each experiment that is most relevant.

Using an alternative technique to increase intracellular Ca^{2+} , we investigated the kinetics of change in CTC pattern distribution after addition of the Ca^{2+} ionophore A23187 to freshly prepared suspensions (15 min incubation) in standard Tyrode's with $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$. Rapid responses were observed: there was a rise in B pattern cells, reaching maximum numbers within 10 min of ionophore addition, and then declining. From this time onwards, there was a continual rise in proportion of AR cells, in marked contrast to the control suspensions where modest changes were observed during the same period, as in Series I. Thus, the treated cells do undergo the same capacitation-dependent transition (from F to B) observed under standard conditions, but they do so much more rapidly and then continue on to undergo acrosomal exocytosis. Spermatozoa did not have to undergo capacitation before the ionophore could evoke a response; rather, the ionophore promoted rapid transition to the capacitated state. It has been suggested in some studies that only capacitated cells can respond to ionophore treatment (e.g. Lee *et al.*, 1987; Byrd *et al.*, 1989), but many others have demonstrated rapid responses by uncapacitated suspensions from several species (e.g. human: Aitken *et al.*, 1984; Cross *et al.*, 1986; Stock & Fraser, 1989; mouse: Fraser, 1982; ram: Shams-Borhan & Harrison, 1981). Our present results are consistent with those of the latter group.

The conclusion drawn from the above experiment was further strengthened when cells were treated with ionophore in the presence of $90 \mu\text{mol Ca}^{2+} \text{ l}^{-1}$, a concentration that supports capacitation *per se* but not acrosomal exocytosis (Fraser, 1987b). Under these conditions, A23187 rapidly promoted a significant shift from a majority of F to a majority of B pattern cells (Fig. 3), but could not elicit acrosomal exocytosis. This then confirms the ability of the ionophore to promote capacitation in initially uncapacitated cells. It also supports the hypotheses that extracellular Ca^{2+} is internalized during the events that comprise capacitation and that changes in the distribution of CTC fluorescence on the sperm surface reflect changes in intracellular Ca^{2+} , with the transition to the acrosome-intact capacitated B pattern requiring an increase. These observations are consistent with those drawn by Singh *et al.* (1978) who demonstrated that high extracellular Ca^{2+} was necessary to obtain a secondary Ca^{2+} influx into guinea-pig spermatozoa, treated with A23187, which would trigger acrosomal exocytosis.

Such a requirement for internalization of Ca^{2+} during capacitation could explain the inability of spermatozoa to complete capacitation in the absence of extracellular Ca^{2+} (Fraser, 1982, 1987b; Fig. 5) and hence their inability to undergo rapid acrosomal exocytosis following the introduction of millimolar Ca^{2+} . However, they do respond if given sufficient time. The kinetics of this response indicate a more rapid shift from uncapacitated to capacitated CTC patterns (Fig. 6), compared with values obtained under standard conditions (Fig. 1). This is consistent with the conclusion, based on data relating to changes in fertilizing ability, that mouse spermatozoa incubated in Ca^{2+} -deficient medium undergo partial but not complete capacitation and require approximately 1 h in the presence of extracellular Ca^{2+} to acquire full functional ability (Fraser, 1982, 1987b). In the present study, within 50 min the proportions of B and AR cells were similar in treated suspension and those incubated continuously in the presence of Ca^{2+} . In contrast, the incubation of spermatozoa in micromolar Ca^{2+} medium for 2 h prepared them to respond rapidly to added Ca^{2+} : within 10 min the distribution of CTC patterns on these cells could not be distinguished from those incubated continuously in $1.80 \text{ mmol Ca}^{2+} \text{ l}^{-1}$ (Fig. 7). Such quick changes are consistent with the demonstrated fertilizing ability of similarly-treated spermatozoa (Fraser, 1987b).

Since our results suggested that increases in intracellular Ca^{2+} are necessary for complete capacitation, we investigated possible mechanisms involved in controlling Ca^{2+} fluxes. In somatic

cells, a Ca²⁺-ATPase located in the plasma membrane is important in maintaining low intracellular Ca²⁺ by pumping Ca²⁺ out (Schatzmann, 1985). Such an ATPase has been identified in mammalian spermatozoa, although its importance in regulating intracellular Ca²⁺ has been the subject of some debate (reviewed by Fraser, 1987a; Roldan & Fleming, 1989). However, Roldan & Fleming (1989) have provided evidence that incubating guinea-pig spermatozoa in the presence of compounds known to inhibit Ca²⁺-ATPases in somatic cell systems shortened capacitation time, on the basis of assessment of spontaneous acrosome reactions. Thus, activity of Ca²⁺-ATPase may play a role in regulating the events of capacitation.

We therefore investigated the effects of two Ca²⁺-ATPase inhibitors, quercetin and ethacrynic acid, on capacitation in mouse spermatozoa. At concentrations of 50 µmol l⁻¹ and higher, both compounds promoted transition from the uncapacitated to the capacitated state, as evidenced by a significant increase in the proportion of cells expressing the B pattern of CTC fluorescence (Figs 8 and 10). This would be consistent with our proposal that such cells have increased intracellular Ca²⁺: inhibition of Ca²⁺-ATPase would reduce the rate at which Ca²⁺ is pumped out of the cells and hence intracellular Ca²⁺ would rise. Quercetin also caused a significant increase in the proportion of AR cells at 100 and 200 µmol l⁻¹; there was an increase in AR cells following incubation with ethacrynic acid, although this did not reach statistical significance. These results indicate that treatments that can inhibit Ca²⁺-ATPase activity in somatic cells (e.g. Fewtrell & Gomperts, 1977; Wüthrich & Schatzmann, 1980) significantly accelerated capacitation, the effect being primarily on transition to the capacitated state rather than on triggering acrosomal exocytosis. Our conclusions are consistent with those of Roldan & Fleming (1989) who obtained a significant increase in acrosome loss with both compounds, but only at concentrations of 100–200 µmol l⁻¹. The CTC technique permits subdivision of the acrosome-intact group into two on the basis of function, and hence allows detection of more subtle changes. These results therefore suggest that a mechanism causing a decrease in Ca²⁺-ATPase activity during capacitation could be responsible for an increase in intracellular Ca²⁺ to the point where cells could respond to the physiological initiator of acrosomal exocytosis.

In earlier studies, uncapacitated epididymal mouse spermatozoa have been shown to have a decapacitation factor that is lost or inactivated during capacitation (Fraser, 1984). More recently, we have demonstrated that when decapacitation factor is known to be present, the F pattern of CTC fluorescence predominates. Removal of the factor from uncapacitated cells by gentle washing significantly increases both fertilizing ability (Fraser, 1984) and the proportion of cells exhibiting the B pattern (Fraser *et al.*, 1990). Conversely, the re-introduction of factor, either in a crude or partially purified preparation, to capacitated suspensions significantly decreases fertilizing ability (Fraser, 1984; Fraser *et al.*, 1990) and increases the proportion of F pattern cells, while decreasing those with B and AR patterns. Thus the CTC fluorescence pattern is strongly correlated with the presence or absence of the decapacitation factor. Of particular interest to the present study is the demonstration that removal of the factor from sperm suspensions incubated in Ca²⁺-deficient medium significantly increased their fertilizing ability *in vitro* when millimolar Ca²⁺ was added, compared with unwashed counterparts also receiving Ca²⁺ (Fraser, 1984). In the light of results detailed here, one possible mechanism of action for the decapacitation factor is regulation of Ca²⁺-ATPase activity: when present, the factor would maintain a high level of enzyme activity and hence low intracellular Ca²⁺; when absent, being lost either relatively slowly over the normal capacitation period or rapidly as occurs with washing, enzyme activity would fall and intracellular Ca²⁺ would rise. This hypothesis is currently being tested.

In somatic cells CTC has been used as a probe to visualize membrane-associated Ca²⁺ (Caswell & Hutchison, 1971), with results indicating that CTC may provide information on Ca²⁺ concentrations associated with the inner surface of membranes (Caswell, 1972; Hallett *et al.*, 1972). This would be consistent with a CTC distribution reflecting the presence of decapacitation factor and Ca²⁺-ATPase activity: this is a membrane-bound enzyme (e.g. Schatzmann, 1985) and in its role as a Ca²⁺ pump, the Ca²⁺ concentration in its immediate vicinity would be greater at times of high

activity. Quercetin, shown to inhibit Ca^{2+} -ATPase irreversibly (Wüthrich & Schatzmann, 1980), proved to be fluorescent and in the mouse sperm head it was localized particularly in the post-acrosomal region, the region where CTC fluorescence is absent in B pattern cells. Thus apparent distribution of the Ca^{2+} -ATPase and capacitation-related changes in CTC patterns are consistent with a role for this enzyme during capacitation. The facts that quercetin bound strongly to the midpiece and that bright CTC fluorescence is observed on the midpiece at all times (Ward & Storey, 1984; Fraser *et al.*, 1990) suggest that activity of Ca^{2+} -ATPase in the tail may be maintained throughout capacitation, thereby ensuring low intracellular Ca^{2+} in that compartment. Certainly, treatments known to cause increases in intracellular Ca^{2+} can, in addition to stimulating acrosomal exocytosis, inhibit sperm motility, e.g. A23187 (Shams-Borhan & Harrison, 1981; Fraser, 1982). Maintenance of Ca^{2+} homeostasis in the tail is therefore extremely important for successful function.

Our observation that Ca^{2+} -ATPase is more likely to play a role during capacitation than in initiating the acrosome reaction is consistent with an earlier study that provided evidence for the presence of Ca^{2+} channels in mouse spermatozoa, these channels playing a role in acrosomal exocytosis and not in capacitation *per se* (Fraser & McIntyre, 1989). Since capacitation requires relatively little extracellular Ca^{2+} and acrosomal exocytosis requires high Ca^{2+} , it is not unreasonable that different mechanisms might be involved in controlling Ca^{2+} fluxes. We would therefore like to propose that mouse spermatozoa leave the epididymis coated with decapacitation factor which maintains a high level of Ca^{2+} -ATPase activity and, consequently, low intracellular Ca^{2+} ; Ca^{2+} channels are present but inactive. In an appropriate environment, capacitation is initiated. This involves, at least in part, a gradual loss of decapacitation factor and hence a decrease in Ca^{2+} -ATPase activity. Intracellular Ca^{2+} rises until it reaches a critical threshold, at which point the Ca^{2+} channels are activated and can function. Their opening, either spontaneously as a result of even higher intracellular Ca^{2+} levels or in a directed manner, as a consequence of sperm interaction with oocyte-associated initiators of acrosomal exocytosis, would permit a rapid influx of a high concentration of Ca^{2+} and trigger the acrosome reaction.

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