A biphasic pattern of $^{45}\text{Ca}^{2+}$ uptake by mouse spermatozoa *in vitro* correlates with changing functional potential

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Mouse sperm capacitation *in vitro*, leading to hyperactivated motility, acrosomal exocytosis and rapid fertilization, takes approximately 120 min in a medium containing sufficient Ca^{2+} . During that period, spermatozoa incubated in $^{45}\text{Ca}^{2+}$ exhibited a biphasic pattern of Ca^{2+} uptake, with the first and lower peak occurring from 10 to 50 min and the second and higher peak from 60 to 90 min. When the exogenously supplied glucose was reduced from 5.56 mmol l^{-1} to $5.56 \mu\text{mol l}^{-1}$, the latter supporting capacitation but not fertilization, only the first peak of $^{45}\text{Ca}^{2+}$ uptake was observed. Increasing the glucose to a millimolar concentration produced a second peak of uptake. We therefore propose that the first phase of $^{45}\text{Ca}^{2+}$ uptake is associated with capacitation and the second phase with acrosomal exocytosis, which are both necessary prerequisites for fertilization. In micromolar glucose the rate of $^{45}\text{Ca}^{2+}$ uptake during the first 30 min was 47% higher than in millimolar glucose, suggesting that the former conditions might promote a precocious rise in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and hence accelerate capacitation. This hypothesis was confirmed by demonstrating both significantly accelerated transition from the uncapacitated F pattern of chlortetracycline (CTC) fluorescence to the capacitated B and AR patterns and significantly higher fertility *in vitro* in suspensions preincubated for 30 min in micromolar glucose, compared with those maintained continuously in millimolar glucose. These results suggest that an ATP-dependent mechanism, for example a Ca^{2+} -ATPase, may be involved in maintaining a low $[\text{Ca}^{2+}]_i$. In micromolar glucose, available ATP would be limited and hence the ATPase activity would decline, allowing $[\text{Ca}^{2+}]_i$ to rise. The possibility that such an ATPase might be calmodulin-sensitive was investigated by incubating sperm suspensions in the presence of trifluoperazine (TFP), a calmodulin antagonist. TFP significantly accelerated both the initial uptake of $^{45}\text{Ca}^{2+}$ and the transition from uncapacitated to capacitated CTC patterns, suggesting a role for a calmodulin-sensitive Ca^{2+} -ATPase during capacitation. Finally, inclusion of the mitochondrial inhibitor azide had little effect on $^{45}\text{Ca}^{2+}$ uptake, indicating that most of the observed uptake in this study was occurring in the sperm head.

Introduction

When mammalian spermatozoa are released from the male reproductive tract they must complete capacitation (Austin, 1951; Chang, 1951), exhibit hyperactivated motility and undergo acrosomal exocytosis before they can fertilize an oocyte (Fraser, 1984; Yanagimachi, 1988). The biochemical requirements for these events have been investigated in several species (Fraser, 1984; Fraser and Ahuja, 1988) and it has been observed that spermatozoa from most species require the presence of calcium ions in their extracellular environment to undergo capacitation and to express full functional ability (e.g. cattle: Singh *et al.*, 1978; hamsters: Yanagimachi, 1982; humans: Irvine and Aitken, 1986; Stock and Fraser, 1989; mice: Fraser, 1982, 1987).

In mouse spermatozoa it has been demonstrated that different concentrations of extracellular Ca^{2+} , some of which are internalized, are required to support capacitation and acrosomal exocytosis (Fraser, 1987), with relatively little Ca^{2+} sufficing during capacitation but much more being needed for fertilization itself. This result suggests that different mechanisms may be involved in regulating the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in the sperm cell. In mammalian spermatozoa there are several mechanisms that could contribute to the regulation of $[\text{Ca}^{2+}]_i$; these include a Ca^{2+} -ATPase (Bradley and Forrester, 1980a; Breitbart *et al.*, 1983), a Na^{+} - Ca^{2+} exchanger (Bradley and Forrester, 1980b; Ashraf *et al.*, 1982) and Ca^{2+} channels (Babcock and Pfeiffer, 1987; Fraser and McIntyre, 1989). The role that each mechanism might play in the events leading to successful sperm-oocyte fusion is poorly understood. In mouse spermatozoa it has been postulated that a Ca^{2+} -ATPase may play a role in capacitation (Fraser and McDermott, 1992), and that voltage-sensitive Ca^{2+} ion channels are involved in

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exocytosis (Fraser and McIntyre, 1989; Florman *et al.*, 1992; Fraser, in press).

Apart from the requirement for extracellular Ca^{2+} , in mouse spermatozoa it has been observed that the presence of a sufficient amount of glycolysable substrate is essential for acrosomal exocytosis (Fraser and Quinn, 1981). The preferred substrate is glucose, although mannose can substitute (Fraser and Herod, 1990). However, capacitation can be achieved in the absence of glucose or any glycolysable substrate and once sufficient glucose is supplied, cells are immediately fertile (Fraser and Quinn, 1981). Hence, manipulation of the glucose concentration in the sperm environment enables investigations to be focussed on capacitation with or without acrosomal exocytosis.

This study was designed to investigate the pattern of Ca^{2+} uptake, using $^{45}\text{Ca}^{2+}$ as a tracer, over the period during which mouse spermatozoa undergo capacitation and acquire demonstrable fertilizing ability. In particular, the possible role played by a Ca^{2+} -ATPase in capacitation was explored. A preliminary report of some of this work has been made (Adeoya-Osiguwa and Fraser, 1992).

Materials and Methods

Media

The culture medium used in all experiments was a modified Tyrode's medium (Fraser, 1983) containing 4 mg crystalline BSA ml^{-1} (Sigma, Poole, Dorset). Media contained either 5.56 mmol glucose l^{-1} (standard medium) or 5.56 μmol glucose l^{-1} (low glucose medium). The CaCl_2 concentration in all media was 1.80 mmol l^{-1} with only a small contribution from $^{45}\text{CaCl}_2$ ($< 50 \mu\text{mol l}^{-1}$).

Sperm suspension preparation

Spermatozoa were extruded from the cauda epididymides of mature (> 8 weeks) male TO mice (Tuck, Battlesbridge, Essex), using two pairs of sterile watchmakers' forceps, into medium (1 ml per each pair of epididymides) in a sterile culture dish overlaid with autoclaved liquid paraffin (Boots, Nottingham). Sperm suspensions, with a mean concentration of 2×10^7 cells ml^{-1} (range of 1.5 – $2.5 \times 10^7 \text{ ml}^{-1}$), were left to disperse for 5 min before incubations commenced.

$^{45}\text{Ca}^{2+}$ uptake assay

After 5 min dispersal, approximately $50 \mu\text{Ci } ^{45}\text{CaCl}_2 \text{ ml}^{-1}$ (specific activity 2 mCi ml^{-1} , Amersham, Bucks) was added to the sperm suspensions. After gentle agitation, the dishes were placed in an anaerobic culture jar, gassed with a mixture of 5% CO_2 –5% O_2 –90% N_2 and incubated for up to a total of 120 min at 37°C . Suspensions were sampled as detailed for individual experimental series. To permit gassing and to allow a short equilibration time, the earliest point at which an accurate determination of $^{45}\text{Ca}^{2+}$ uptake could be obtained was 10 min. When establishing this assay, we observed that the amount of radioactivity obtained increased linearly with the volume of cells filtered; to obtain sufficient radioactivity above the background value, 100 μl of the sample was used throughout.

At each time point, duplicate 100 μl aliquots of sperm suspension were rapidly filtered on to Whatman GF/C filters (Whatman International Ltd, Maidstone) and washed three times with 5 ml phosphate-buffered saline (PBS) at 4°C . Filters were transferred into scintillation vials; 2 ml scintillant was added (Ultima Gold, Packard Canberra, Pangbourne) and the $^{45}\text{Ca}^{2+}$ content was determined (Packard Tri-Carb scintillation counter). Motility was maintained throughout incubations, i.e. there was no evidence that cells died as a result of exposure to $^{45}\text{Ca}^{2+}$.

To ensure that the $^{45}\text{Ca}^{2+}$ associated with the cells was not just bound to an extracellular site, in preliminary experiments the $^{45}\text{Ca}^{2+}$ content of cells was determined after washing with either PBS or PBS plus 1 mM EGTA at 4°C . Since values were very similar, we used PBS. In other experiments we demonstrated a monensin-stimulated rise and fall in $^{45}\text{Ca}^{2+}$ content that follows the time-course of induced acrosomal exocytosis (Fraser, in press). The fact that both exocytosis and the initial increase were essentially totally inhibited by the Ca^{2+} channel blocker nifedipine provides clear evidence that the $^{45}\text{Ca}^{2+}$ is being internalized during the incubations.

CTC fluorescence assay

A modification of Ward and Storey's (1984) chlortetracycline (CTC) fluorescence method was used (Fraser and McDermott, 1992); this involved staining and fixing cells in suspension, an approach that enables precise timing of assessment. The CTC solution was prepared daily and contained 750 $\mu\text{mol CTC l}^{-1}$ (Sigma) 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). The solution was kept wrapped in foil to exclude light and stored at 4°C until use. Samples were prepared by adding 45 μl sperm suspension to a small (0.5 ml) foil-wrapped microcentrifuge tube, followed by 45 μl CTC solution (room temperature to avoid cold shock). After mixing well, 8 μl of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris-HCl l^{-1} buffer (final pH 7.4) was added. Ten microlitres of this suspension was placed on a clean slide and one drop of 0.22 mol 1,4-diazabicyclo [2.2.2] octane (Sigma) in glycerol:PBS (9:1, v/v) was carefully mixed in to retard fading of fluorescence. After adding a coverslip, the slide was firmly compressed between tissues to remove excess fluid. This is an important step, helping to maximize the number of cells that are lying flat; this orientation is required for unambiguous assessment. Slides were sealed with colourless nail varnish and stored in the dark at 4°C . They were examined either the same or the next day using an Olympus BHS microscope fitted with phase-contrast and epifluorescence optics. The excitation beam was passed through a 405 nm band filter and CTC fluorescence emission was observed through a DM 455 dichroic mirror.

In each aliquot, 100 sperm cells (50 on each of two slides) were classified as having one of three staining patterns: (1) 'F', with fluorescence over the entire head, characteristic of acrosome-intact, uncapacitated spermatozoa; (2) 'B', with a fluorescence-free band in the post-acrosomal region, characteristic of acrosome-intact, capacitated spermatozoa; (3) 'AR', with fluorescence absent from the head, characteristic of an acrosome-reacted spermatozoa. The presence or absence of the acrosomal cap was confirmed by phase contrast assessment (Fraser, 1987).

Fig. 1. $^{45}\text{Ca}^{2+}$ uptake by mouse spermatozoa incubated *in vitro* for 120 min in standard Tyrode's medium with $5.56 \text{ mmol glucose l}^{-1}$. Data presented as means $\pm$ SEM ($n = 5$).

Fig. 2. $^{45}\text{Ca}^{2+}$ uptake by mouse sperm cells incubated *in vitro* for 120 min in Tyrode's medium with $5.56 \mu\text{mol glucose l}^{-1}$. Data presented as means $\pm$ SEM ($n = 4$).

Experimental Details and Results

Series I. What is the pattern of $^{45}\text{Ca}^{2+}$ uptake in spermatozoa incubated in millimolar glucose medium?

Spermatozoa were released into medium containing the standard glucose concentration, 5.56 mmol l^{-1} , and incubated for 120 min in the presence of $^{45}\text{Ca}^{2+}$. At intervals of 10 min up to 90 min and at 120 min duplicate aliquots were taken and filtered as described earlier. Five replicates were carried out ($n = 5$).

Spermatozoa incubated under these conditions exhibited two phases of $^{45}\text{Ca}^{2+}$ uptake (Fig. 1). The first peak occurred over the first 50 min, followed by a second peak of $^{45}\text{Ca}^{2+}$ uptake from 60 to 90 min; values then reached a plateau (Fig. 1). Comparison of the two phases showed that the initial uptake of $^{45}\text{Ca}^{2+}$ was smaller than the second, suggesting that different amounts of Ca^{2+} were required for functional changes occurring in spermatozoa during the incubation period. In this and the following series, variation in the absolute values for $^{45}\text{Ca}^{2+}$ uptake was observed among the replicates, reflecting qualitative variations (e.g. percentage of motile cells) among suspensions. However, the same pattern of response, with a low point of 50 min, was seen in all the replicates and analysis using MANOVA (repeated measurements) showed significant ($P < 0.01$) changes in values with time.

Series II. What is the pattern of $^{45}\text{Ca}^{2+}$ uptake in spermatozoa incubated in micromolar glucose medium?

The standard modified Tyrode's medium was diluted 1000-fold with glucose-free medium to give a final glucose concentration of $5.56 \mu\text{mol l}^{-1}$ (low glucose). Sperm cells were released into this medium and incubated as described in Series I ($n = 4$).

In low glucose medium a time-dependent uptake of $^{45}\text{Ca}^{2+}$ occurred over the first 10–50 min after which no further uptake was observed (Fig. 2). Examination of spermatozoa by phase-contrast microscopy showed that under these conditions spermatozoa failed to exhibit hyperactivated motility, an observation consistent with earlier studies (Fraser and Quinn, 1981;

Table 1. Rate of $^{45}\text{Ca}^{2+}$ uptake by mouse spermatozoa during the first 30 min incubation *in vitro*

Glucose concentration	Rate of $^{45}\text{Ca}^{2+}$ uptake ^a
5.56 mmol l^{-1}	0.36 ± 0.09
$5.56 \mu\text{mol l}^{-1}$	0.53 ± 0.15

^aData are expressed as pmol $^{45}\text{Ca}^{2+} \text{ min}^{-1} 10^{-7}$ cells, mean $\pm$ SEM for four replicates.

Fraser and Herod, 1990). Comparison of the rates of $^{45}\text{Ca}^{2+}$ uptake between 10 and 30 min by sperm suspensions incubated in standard medium (Series I) and in low glucose medium (Series II) revealed that the mean rate of $^{45}\text{Ca}^{2+}$ uptake was 47% greater in cells incubated in micromolar glucose than that incubated in millimolar glucose (Table 1).

Since conditions that promote a more rapid rise in intracellular Ca^{2+} have been shown to accelerate capacitation, as evidenced by more rapid fertilization *in vitro* (Fraser, 1987), these results suggest that mouse spermatozoa incubated in micromolar glucose might be functionally superior to those incubated in millimolar glucose.

Series III. Does incubation in micromolar glucose medium affect the rate of capacitation as assessed by CTC fluorescence?

Sperm suspensions were prepared in glucose-free medium and then divided into two aliquots. Appropriate glucose stock solutions were added to produce micromolar ($5.56 \mu\text{mol l}^{-1}$) and millimolar (5.56 mmol l^{-1}) glucose suspensions. After incubation for 30 min, a high glucose stock solution was added to raise the glucose from a micromolar to a millimolar concentration (low G $\rightarrow$ + G). Both suspensions (continuous + G and low G $\rightarrow$ + G) were incubated further, with sampling after 10 and 40 min (total time elapsed was 40 and 70 min, respectively). Aliquots were filtered through short columns of Sephadex G-25 (medium) to remove non-motile cells (Fraser, 1983) and spermatozoa were then prepared for CTC assay ($n = 3$).

Fig. 3. Chlortetracycline (CTC) fluorescence patterns in mouse sperm suspensions incubated *in vitro* either for 30 min in micromolar glucose-containing medium followed by a rise in glucose in a millimolar concentration (low G → +G) or continuously in millimolar glucose (+G): (a) sampled 10 min and (b) sampled 40 min after increase in glucose. ▨, F; ■, B; ▩, AR. * $P < 0.05$, ** $P < 0.025$ compared with +G suspensions.

Assessment 10 min after raising the glucose concentration indicated that significantly more ($P < 0.05$ – Cochran's modification of the 2×2 contingency tables; Snedecor and Cochran, 1980) cells in the low G → +G treatment group had made the transition from the uncapacitated, acrosome-intact F pattern to the capacitated, acrosome-intact B pattern (Fig. 3a). After an additional 30 min, a similar decrease in F and an increase in B pattern cells was observed in the continuous +G suspensions. In the low G → +G group, the accelerated transition was maintained, with significantly ($P < 0.05$) fewer F and more AR pattern cells than in the +G controls (Fig. 3b). Thus, preincubation for 30 min in micromolar glucose accelerated capacitation as judged by CTC fluorescence patterns.

Series IV. Does incubation in micromolar glucose medium accelerate the acquisition of fertilizing ability *in vitro*?

Unfertilized cumulus-intact oocytes were obtained from mature (> 8 weeks) TO female mice induced to superovulate by i.p. injections of 7.5 iu PMSG (Folligon, Intervet, Cambridge) and, 48–54 h later, 5 iu hCG (Chorulon, Intervet). Fourteen hours after hCG, cumulus clots were released into 4 ml low glucose medium. Approximately equal numbers of oocytes

Table 2. Effect of preincubation of spermatozoa in micromolar glucose-containing medium on fertilization *in vitro*

Preincubation ^a	[Glucose] Fertilization	Oocytes fertilized/ total oocytes (%)	Range (%)
5.56 mmol l ⁻¹	5.56 mmol l ⁻¹	4/120 (3.3)	0–10
5.56 μmol l ⁻¹	5.56 mmol l ⁻¹	28/108 (25.9)*	7–60

Values are for four replicates.

^aPreincubated for 30 min, then diluted into medium containing millimolar glucose; * $P < 0.05$ compared with continuous 5.56 mmol glucose l⁻¹.

were then transferred to 400 μl of sperm suspensions which had been prepared as follows.

From one male, sperm suspensions in low glucose and standard glucose media (one epididymis per 0.5 ml each medium) were prepared and preincubated for 30 min. Both suspensions were then diluted 1/10 (final concentration of $2-3 \times 10^6$ cells ml⁻¹) in standard glucose medium and oocytes were added. After incubation for 65 min, oocytes were transferred to fresh droplets of medium and, at 65 min, fixed with buffered formalin (4% formaldehyde). Oocytes were stained with 0.75% aceto-orcein, mounted and assessed for the presence of a fertilizing sperm head (Fraser, 1983). Four replicates were performed.

Results (Table 2) indicated that spermatozoa preincubated in low (micromolar) glucose before functional assessment in millimolar glucose were significantly ($P < 0.05$; Cochran's test) more fertile than their counterparts incubated continuously in millimolar glucose. Thus, the cells incubated in low glucose capacitated more quickly, a result consistent with the CTC assessment of similarly treated suspensions in Series III.

Series V. Does the addition of millimolar glucose affect $^{45}\text{Ca}^{2+}$ uptake by spermatozoa preincubated in micromolar glucose medium?

Sperm suspensions prepared in low glucose medium were incubated as described in Series II. Aliquots for determination of $^{45}\text{Ca}^{2+}$ were taken at intervals of 10 min up to 50 min. The sperm suspension was then divided into two aliquots; to one half a concentrated stock of glucose ($\times 1000$) was added to produce a final glucose concentration of 5.56 mmol l⁻¹, and to the other an equivalent volume of low glucose medium was added. Suspensions were then incubated for a further 70 min (total of 120 min). Aliquots for the determination of $^{45}\text{Ca}^{2+}$ uptake were taken at 10 min intervals up to 90 min, then at 120 min ($n = 4$).

Suspensions incubated in low glucose medium for 50 min exhibited only one phase of $^{45}\text{Ca}^{2+}$ uptake. When the concentration of glucose was increased to millimolar levels, an increase in $^{45}\text{Ca}^{2+}$ uptake was observed within 10 min (Fig. 4). We suggest that this uptake in $^{45}\text{Ca}^{2+}$ represents the second peak of Ca^{2+} observed in Series I (Fig. 1).

Series VI. Does the presence of trifluoperazine affect $^{45}\text{Ca}^{2+}$ uptake by spermatozoa?

Indirect evidence has suggested that a Ca^{2+} -ATPase may play a role in maintaining a low $[\text{Ca}^{2+}]_i$ in mouse spermatozoa

Fig. 4. $^{45}\text{Ca}^{2+}$ uptake by mouse sperm cells incubated for 50 min in medium containing $5.56 \mu\text{mol glucose l}^{-1}$ (low G, —○—), then either having glucose increased to 5.56 mmol l^{-1} (low G $\rightarrow$ +G, --●--) or maintained at a micromolar concentration for the next 70 min. Data presented as means $\pm$ SEM ($n = 4$).

Fig. 5. $^{45}\text{Ca}^{2+}$ uptake by mouse sperm cells incubated *in vitro* for 120 min in the absence (—○—) or presence (—●—) $20 \mu\text{mol}$ trifluoperazine l^{-1} . Data presented as means $\pm$ SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$ compared with control suspensions.

(Fraser and McDermott, 1992). Since somatic cell Ca^{2+} -ATPase is generally calmodulin-sensitive (e.g. Carafoli, 1987), we investigated the effect of the calmodulin antagonist trifluoperazine (TFP) on $^{45}\text{Ca}^{2+}$ uptake by mouse sperm cells. Sperm suspensions were prepared in standard medium, and after 5 min dispersal, they were divided between two dishes. To one dish a concentrated stock solution of TFP (dissolved in absolute ethanol before diluting in medium) was added to give a final concentration of $20 \mu\text{mol TFP l}^{-1}$ (containing 0.4% ethanol). To the second dish an equivalent volume of standard medium containing 0.4% ethanol was added. Suspensions were then incubated in the presence of $^{45}\text{Ca}^{2+}$, and aliquots were taken for assay from both dishes at intervals of 10 min up to 90 min incubation, and then at 120 min ($n = 3$).

Incubation of sperm cells in the presence of $20 \mu\text{mol TFP l}^{-1}$ resulted in a significant ($P < 0.05$, 0.01; paired t test) stimulation of $^{45}\text{Ca}^{2+}$ uptake during the first 30 min, after which no difference in the pattern of uptake was observed in the absence or presence of this calmodulin antagonist (Fig. 5). Examination

Fig. 6. Chlortetracycline (CTC) fluorescence patterns in mouse sperm suspensions incubated in the absence (control) or presence of $20 \mu\text{mol}$ trifluoperazine l^{-1} (TFP) for 30 min ($n = 3$). ▨, F; ■, B; ▩, AR. *** $P < 0.001$ compared with control suspensions.

of sperm cells under phase-contrast microscopy showed that the concentration of TFP used in these experiments had no adverse effects on sperm motility. The stimulation of $^{45}\text{Ca}^{2+}$ uptake over the first 30 min suggests that a calmodulin-dependent mechanism (e.g. Ca^{2+} -ATPase) could play a role in regulating $[\text{Ca}^{2+}]_i$.

Series VII. Does TFP affect the rate of capacitation, as assessed by CTC fluorescence?

Sperm suspensions were prepared in standard medium and, after 5 min to allow dispersal, they were filtered through Sephadex columns as detailed in Series IV. The filtered suspension was divided into two aliquots; TFP (final concentration of $20 \mu\text{mol l}^{-1}$) was added to one aliquot and ethanol-containing medium to the other. After incubation for 30 min, cells were prepared for CTC assessment. Results indicated that capacitation was accelerated in the presence of TFP (Fig. 6). Significantly ($P < 0.001$; Cochran's test) fewer F and more AR pattern cells were observed in the TFP-treated cells.

Series VIII. Does azide affect $^{45}\text{Ca}^{2+}$ uptake by spermatozoa?

Spermatozoa were incubated in the presence of azide (a potent inhibitor of mitochondrial activity) to obtain an estimate of the proportion of $^{45}\text{Ca}^{2+}$ taken up by the flagellum via the mitochondria. Sperm suspensions were prepared and divided as described in Series III. A concentrated stock solution of azide was added to one dish to give a final concentration of 5 mmol l^{-1} and an equivalent volume of standard medium was added to the other. Suspensions were incubated in the presence of $^{45}\text{Ca}^{2+}$ and aliquots were removed at 10, 40, 60 and 120 min for assay ($n = 5$).

Results (Fig. 7) indicate that azide had essentially no effect on $^{45}\text{Ca}^{2+}$ uptake at 10 and 40 min. At 60 and 120 min, there was a slight inhibition in the azide-treated cells compared with azide-free controls. These differences were not significant, indicating that the $^{45}\text{Ca}^{2+}$ uptake reported in this study occurred primarily in the sperm head. In preliminary experiments, sperm motility was observed to be unaffected by azide concentrations between

Fig. 7. $^{45}\text{Ca}^{2+}$ uptake by mouse sperm cells incubated *in vitro* for 120 min in the absence (■) or presence (▨) of azide. Data are presented as means $\pm$ SEM ($n = 5$).

0.1 and 10 mmol l^{-1} . Examination of sperm motility in the suspensions showed no noticeable differences and spermatozoa were capable of exhibiting hyperactivity motility in both the absence and presence of azide. In further exploratory experiments, sperm suspensions were incubated in the presence of other mitochondrial inhibitors, namely Antimycin A and oligomycin; results obtained were similar to those observed with azide.

Discussion

The results reported here reveal a distinctive pattern of Ca^{2+} uptake from the extracellular environment by epididymal mouse spermatozoa over 120 min, during which time they acquire full functional competence. This uptake of Ca^{2+} is not continuous but biphasic and occurs at specific time intervals; furthermore, the amount of Ca^{2+} taken up in each phase is different. Previous studies (Fraser, 1987) have shown that the minimum concentration of extracellular Ca^{2+} ions required for capacitation *per se* to occur is 90 $\mu\text{mol l}^{-1}$, whereas for expression of hyperactivated motility and acrosomal exocytosis it is 1.80 mmol l^{-1} (the concentration used throughout this study). Observations by Fraser and McDermott (1992), using CTC fluorescence, showed that both capacitation and the acrosome reaction were Ca^{2+} - and time-dependent. When sperm cells are incubated in an environment that provides sufficient Ca^{2+} , capacitation generally takes more than 30 min to complete, with significant levels of spontaneous acrosomal exocytosis occurring by 120 min (Fraser and McDermott, 1992). The above studies, as well as many others using spermatozoa from a variety of species, have indicated that the responses require internalization of Ca^{2+} . Given that we used culture conditions similar to those used for functional assessments and that the time course of Ca^{2+} uptake correlates with demonstrable functional changes, we suggest that the first peak of Ca^{2+} uptake is associated with capacitation and the second peak with acrosomal exocytosis.

A more detailed examination of Ca^{2+} uptake during capacitation was undertaken in this study by incubating spermatozoa in a medium containing low glucose (5.56 $\mu\text{mol l}^{-1}$). Under these conditions spermatozoa exhibited only the first phase of

Ca^{2+} uptake and therefore we would conclude that the cells could undergo capacitation but not exocytosis. This conclusion is consistent with earlier studies demonstrating that capacitation could proceed in the presence of very low or even in the absence of exogenously supplied glycolysable substrate, but that millimolar concentrations of glucose were required for hyperactivation, acrosomal exocytosis and fertilization *in vitro* (Fraser and Quinn, 1981; Fraser and Herod, 1990). An important observation arising from these experiments on $^{45}\text{Ca}^{2+}$ uptake came from comparing the rate of uptake in sperm suspensions incubated in micromolar glucose with that of cells incubated in millimolar glucose during the first 30 min: the rate of Ca^{2+} uptake was 47% greater in spermatozoa incubated in low glucose than in those incubated in standard glucose. The possibility that this increased rate of Ca^{2+} uptake reflected accelerated capacitation was investigated by preincubating sperm suspensions in low glucose, then increasing the glucose to millimolar concentrations and assessing their *in vitro* fertilizing ability and their CTC fluorescence patterns. The results confirmed our hypothesis: spermatozoa preincubated in low glucose were significantly more fertile and a significantly higher proportion of cells expressed the B and AR CTC patterns characteristic of capacitated cells. Thus the increased rate of $^{45}\text{Ca}^{2+}$ uptake observed in sperm cells incubated in low glucose correlated with demonstrable changes in sperm function. Furthermore, the addition of millimolar glucose to a sperm suspension preincubated in low glucose resulted in an increase in $^{45}\text{Ca}^{2+}$ uptake which we suggest is that required for acrosomal exocytosis. This result correlates with previously reported observations that sufficient glycolysable substrate is essential for mouse spermatozoa to reach complete fertilizing potential (Fraser and Quinn, 1981; Fraser and Herod, 1990).

From these results, we suggest that incubation in low glucose limits the amount of available ATP which in turn modulates the activity of a Ca^{2+} -ATPase that regulates $[\text{Ca}^{2+}]_i$. An accelerated decrease in activity of this enzyme would allow more rapid increase in $[\text{Ca}^{2+}]_i$, resulting in the observed accelerated capacitation. We therefore propose that a Ca^{2+} -ATPase plays an important role during capacitation, its activity declining as capacitation proceeds. In support of this hypothesis are the observations of Fraser and McDermott (1992), who incubated mouse sperm suspensions in the presence of quercetin and ethacrynic acid, both inhibitors of somatic cell Ca^{2+} -ATPases (Fewtrell and Gomperts, 1977; Wüthrich and Schatzmann, 1980). The presence of these antagonists in sperm suspensions accelerated the transition from the uncapacitated F pattern of CTC fluorescence to the capacitated B and AR patterns. In the B pattern, fluorescence is absent from the post-acrosomal region and fluorescence from the quercetin itself showed that it bound particularly to the post-acrosomal region (Fraser and McDermott, 1992), suggesting that the Ca^{2+} -ATPase was abundant there. This also appears to be the region where surface-associated decapacitation factor (DF) molecules that modulate fertilizing ability are localized. Fraser *et al.* (1990) showed that adding either a crude or partially purified preparation of mouse sperm DF to capacitated suspensions inhibited fertilizing ability *in vitro*. It also inhibited any rise in AR pattern cells, while causing a marked decrease in capacitated, acrosome-intact B pattern cells and a marked increase in uncapacitated, acrosome-intact F pattern cells. We suggest that the DFs, in

reassociating with the sperm head, exert their inhibitory effect on sperm function by interacting with the Ca^{2+} -ATPase: when DFs are present, enzyme activity is high and $[\text{Ca}^{2+}]_i$ remains low; as DFs are lost during capacitation, Ca^{2+} -ATPase activity declines, allowing $[\text{Ca}^{2+}]_i$ to rise. The inhibition of fertilizing ability observed when DFs are added back to cells would reflect activation of the Ca^{2+} pump and a consequent lowering of $[\text{Ca}^{2+}]_i$. Further experiments to address this directly are underway.

The Ca^{2+} -ATPase present in all somatic cells except skeletal muscle is calmodulin-sensitive (Schatzmann, 1982; Carafoli, 1987), and the presence of high concentrations of this calcium-binding protein in mammalian spermatozoa (Brooks and Siegel, 1973; Jones *et al.*, 1980; Sidhu and Guraya, 1989) suggests that the enzyme present in sperm cells could also be calmodulin dependent. If so, experimental interference with calmodulin should decrease Ca^{2+} -ATPase activity and allow $[\text{Ca}^{2+}]_i$ to rise. In the presence of the calmodulin antagonist TFP (Levin and Weiss, 1977; Vincenzi, 1982), we observed a significant stimulation of $^{45}\text{Ca}^{2+}$ uptake, a result consistent with our hypothesis that a decrease in Ca^{2+} -ATPase may play a role in capacitation by increasing the rate at which the $[\text{Ca}^{2+}]_i$ rises. Furthermore, incubation with TFP significantly accelerated capacitation as assessed by CTC fluorescence, with many of the cells having completed capacitation and undergone acrosomal exocytosis. Leclerc *et al.* (1992) reported a decline in the intracellular concentration of calmodulin during heparin-induced capacitation of bull spermatozoa; they noted that the decline in calmodulin correlated with an increase in the fertilizing ability of sperm suspensions. These observations are consistent with a naturally occurring capacitation-related decrease in Ca^{2+} -ATPase activity and hence add support to our experimentally derived conclusions.

Hyperactivated motility is Ca^{2+} -dependent (Fraser, 1977, 1982) and hence it was not unreasonable to expect that some proportion of the observed $^{45}\text{Ca}^{2+}$ uptake would be associated with the flagellum, particularly the mitochondria. Incubation of spermatozoa in the presence of mitochondrial inhibitors, including azide, at a concentration that had no detectable effect on motility, showed that the majority of $^{45}\text{Ca}^{2+}$ uptake was occurring in the sperm head and that any $^{45}\text{Ca}^{2+}$ uptake by mitochondria was relatively constant over 120 min. Since fertilizing spermatozoa must express hyperactivated motility, our results suggest that Ca^{2+} requirements for this pattern of motility are satisfied primarily by Ca^{2+} stored in the mitochondrial matrix and that most of the $^{45}\text{Ca}^{2+}$ uptake observed in this study was related to capacitation and acrosomal exocytosis.

In conclusion, our data indicate that mouse epididymal spermatozoa exhibit a biphasic pattern of $^{45}\text{Ca}^{2+}$ uptake during 120 min incubation under conditions that support capacitation and the acquisition of demonstrable fertilizing ability *in vitro*. The first and smaller peak appears to be related to capacitation *per se*, while the second and larger peak appears to be related to acrosomal exocytosis. Our data suggest that the rate of Ca^{2+} uptake is modulated by the availability of intracellularly generated ATP; when limited, Ca^{2+} uptake is greater and cells undergo accelerated capacitation. We suggest that a Ca^{2+} -ATPase is responsible for modulating $[\text{Ca}^{2+}]_i$ during capacitation, its activity normally declining and thus allowing $[\text{Ca}^{2+}]_i$ to rise. That this Ca^{2+} -ATPase may be regulated by calmodulin

is suggested by the observed effects of the calmodulin antagonist, TFP: in the presence of TFP, the rate of $^{45}\text{Ca}^{2+}$ uptake during the first phase was significantly stimulated and the rate of transition from the uncapacitated to the capacitated patterns of CTC fluorescence was significantly accelerated.

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