Divalent cations, capacitation and the acrosome reaction in human spermatozoa*

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Summary. The extracellular Ca^{2+} requirements for support of capacitation and the spontaneous acrosome reaction (AR) in human spermatozoa have been evaluated. Motile suspensions were prepared using a swim-up method, incubated for up to 24 h in media of various Ca^{2+} concentrations, fixed and assessed for occurrence of the AR using transmission electron microscopy. Results indicated that the AR response was significantly lower after incubation in Ca^{2+} -deficient medium (generally <10% reacting cells) than in 1.80 mM- Ca^{2+} -containing medium (~15%). In the latter the majority of cells were fully reacted, while in Ca^{2+} -deficient conditions the majority were at intermediate stages of the AR. Subsequent experiments indicated that a maximum AR response required the continuous presence of millimolar Ca^{2+} ; pre-incubation in the presence of micromolar Ca^{2+} did not prepare the spermatozoa to undergo rapid AR upon increase of Ca^{2+} to millimolar concentrations, suggesting that capacitation requires relatively high concentrations of extracellular Ca^{2+} . Incubation in elevated Ca^{2+} (3.60 mM) promoted an even greater response (mean of 24–35% reacting cells compared with 12% for 1.80 mM- Ca^{2+}). The ability of the divalent cations Ba^{2+} , Mg^{2+} and Sr^{2+} (each at 1.80 mM) to substitute for 1.80 mM- Ca^{2+} in promoting the AR was also assessed. Of these, only Sr^{2+} provided a response greater than that observed in unsupplemented Ca^{2+} -deficient medium. In Sr^{2+} the proportion of responding cells after 24 h (~13%) was similar to that obtained in Ca^{2+} (~15%), although a majority of those in Sr^{2+} were at intermediate stages. In 3.60 mM- Sr^{2+} the response was significantly higher than that observed in both 1.80 mM- Ca^{2+} and 1.80 mM- Sr^{2+} , but significantly lower than that in 3.60 mM- Ca^{2+} . Under all conditions motility was maintained at >90% for 24 h. The introduction of the Ca^{2+} ionophore ionomycin, in the presence of 1.80 mM- Ca^{2+} , induced the AR in a concentration-dependent but preincubation time-independent manner, with the maximum response of ~60% being obtained with 30 μM -ionomycin. Finally, incubation in the presence of 1.80 mM- Ca^{2+} and verapamil, generally considered to be a calcium channel antagonist, resulted in a concentration- and incubation time-dependent increase in the AR, the maximum response in all groups being observed only after 24 h incubation. Recent evidence from other species suggests that this may represent an agonistic interaction with calcium channels. We conclude that optimal conditions for capacitation and the AR in human spermatozoa require extracellular Ca^{2+} at ≥ 1.80 mM. An influx of Ca^{2+} is associated with AR initiation, as indicated by the ionomycin-induced response, and calcium channels may play a role in the entry of Ca^{2+} into capacitated cells just before the AR. While Sr^{2+} and Ca^{2+} both appear able to support capacitation and initiation of the AR in human spermatozoa, Sr^{2+} is less effective in promoting completion of the

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AR. This may have relevance to other reports that zona binding and gamete fusion in the human are lower in the presence of Sr^{2+} than Ca^{2+} .

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Introduction

Extracellular calcium ions are obligatory for successful fertilization in invertebrates (Loeb, 1915) and mammals (mouse: Iwamatsu & Chang, 1971). In all species examined, an influx of Ca^{2+} is required to trigger the acrosome reaction (AR) which results in release of lytic enzymes and membrane alterations required for sperm-egg interaction. In mammalian spermatozoa, with a requirement for capacitation which prepares them for the acrosome reaction, Ca^{2+} may play a role during both these initial stages as well as the terminal ones associated with the acrosome reaction. Current evidence suggests this to be true for hamster (Yanagimachi, 1982) and mouse (Fraser, 1982, 1987b) spermatozoa, although guinea-pig spermatozoa appear to undergo capacitation in the absence of added Ca^{2+} : if incubated for a sufficient time in Ca^{2+} -deficient medium, the introduction of millimolar concentrations of Ca^{2+} triggers a rapid AR response (Yanagimachi & Usui, 1974). Similar treatment of hamster (Yanagimachi, 1982) and mouse (Fraser, 1982) spermatozoa, using incubation times which support complete capacitation in the presence of extracellular Ca^{2+} , does not evoke an immediate AR in the majority of cells and does not promote immediate expression of optimal fertilizing ability; these cells therefore appear to be only partly capacitated. For mouse spermatozoa, incubation for a further 60 min after addition of millimolar Ca^{2+} leads to complete capacitation: such cells are able to fertilize rapidly a majority of eggs (Fraser, 1987b). Furthermore, the minimum Ca^{2+} concentrations required to support mouse sperm capacitation *per se* and optimal fertilization differ markedly, being 90 μM and 1.80 mM, respectively (Fraser, 1987b).

Considerable interest is now being focussed on human spermatozoa and their specific requirements for capacitation, the AR and fertilization. We have recently described the time-dependent pattern of spontaneous AR in human spermatozoa, from men of proven fertility, during incubation under conditions which support fertilization *in vitro* (Stock & Fraser, 1987). In the present study we have compared the incidence of spontaneous AR in suspensions of human spermatozoa incubated in the presence or absence of added Ca^{2+} . We have also examined the response to addition of ionomycin, a divalent cation ionophore which promotes the entry of Ca^{2+} , and verapamil, a calcium channel antagonist (Triggle & Janis, 1987).

We have also examined the possibility that divalent cations other than Ca^{2+} might be able to support capacitation and/or the AR. In other studies, Sr^{2+} has proved to be the most effective ion to replace Ca^{2+} , being able to support both the AR and hyperactivated motility in guinea-pig spermatozoa (Yanagimachi & Usui, 1974), sperm-egg fusion in the hamster (Yanagimachi, 1978), and capacitation, AR, hyperactivated motility, zona penetration and sperm-egg fusion in the mouse (Fraser, 1987c). There have also been reports that human spermatozoa preincubated in Sr^{2+} were more successful in fertilizing zona-free hamster eggs in the presence of Ca^{2+} than control cells incubated continuously in Ca^{2+} (Mortimer, 1986; Mortimer *et al.*, 1986). These latter results suggested that perhaps Sr^{2+} -treated spermatozoa were able to undergo capacitation but not the AR. In the present study we have evaluated the AR response in human spermatozoa incubated continuously in Sr^{2+} , Ba^{2+} or Mg^{2+} and compared this with counterparts incubated in Ca^{2+} . Because of the existing evidence, we have focussed primarily on Sr^{2+} .

Materials and Methods

Media. The standard medium was Earle's medium, with added gentamicin, which contained 1.80 mM- CaCl_2 . Modifications involved alterations in the concentration of Ca^{2+} : Ca^{2+} -deficient (no added CaCl_2), low Ca^{2+}

(0.45–0.9 mM) and elevated Ca^{2+} (3.60 mM). Where necessary, extra NaCl was added to maintain osmolality at 275–285 mosmol (measured on an osmometer). To study other divalent cations, 1.80 mM- BaCl_2 , - MgCl_2 or - SrCl_2 was added to Ca^{2+} -deficient medium; elevated Sr^{2+} at 3.60 mM ($\times 2$) was also evaluated. In some experiments initial incubation in Ca^{2+} -deficient (with or without other divalent cations) or low Ca^{2+} -media was followed by an increase in Ca^{2+} concentration to 1.80 mM; appropriate concentrated CaCl_2 stocks were used to accomplish this. All media contained Fraction V human serum albumin (hSA; Sigma, Poole, Dorset, UK) at 5 mg/ml.

Stocks of the ionophore ionomycin (Calbiochem, Cambridge BioScience, Cambridge, UK) were prepared in DMSO; the final concentration of DMSO in sperm suspensions was 0.5% for all concentrations of ionomycin examined. Verapamil (Sigma) stocks were prepared in 0.9% NaCl; the final dilution factor for each was $\times 100$.

Sperm preparation. Semen was obtained from fertile donors and from individuals undergoing assessment in the Semiology Laboratory, Chelsea Hospital for Women. Semen (0.5 ml) was gently pipetted under 1 ml of the appropriate medium in 14-ml plastic tubes (Falcon Plastics, Becton Dickinson UK Ltd, Cowley, Oxford, UK) and allowed to swim up for 1–1.5 h at 37°C. Motile cells were carefully aspirated from the upper interface with a Pasteur pipette. A small drop on a microscope slide was assessed for proportion of spermatozoa exhibiting progressive motility; this was $\geq 95\%$. Sperm concentration was determined with a haemocytometer and adjusted to 5×10^6 cells/ml. Suspensions were gassed with 5% CO_2 –5% O_2 –90% N_2 and incubated at 37°C.

Series I. Spermatozoa were allowed to swim up into Ca^{2+} -containing (+Ca) and Ca^{2+} -deficient (–Ca) media. Suspensions were incubated for a total of 24 h, with aliquants being removed at 6 h and 24 h, fixed, processed for transmission electron microscopy (details below) and assessed. Twenty samples were examined ($n = 20$).

Series II. Motile suspensions ($n = 8$) were prepared in –Ca and +Ca media and incubated as for Series I. At 6 h, an aliquant of the –Ca suspension was removed and CaCl_2 stock added to raise the Ca^{2+} concentration to 1.80 mM (–Ca $\rightarrow$ +Ca group). After 15 min, this sample plus an aliquant from both the +Ca and –Ca suspensions were fixed and assessed. The procedure was repeated at 24 h.

Series III. Motile suspensions ($n = 3$) were prepared in –Ca, low Ca (0.45–0.9 mM- CaCl_2) and +Ca media and incubated. At 6 h, an aliquant of the low Ca suspension was removed and the Ca^{2+} concentration raised to 1.80 mM. After 15 min this sample plus one from each of the other suspensions were fixed. This resulted in 4 treatment groups (–Ca, low Ca, low Ca $\rightarrow$ +Ca and +Ca). The procedure was repeated at 24 h.

Series IV. Motile suspensions were prepared in standard medium (1.80 mM- CaCl_2 ; +Ca) and medium with elevated Ca^{2+} (3.60 mM- CaCl_2 ; $\times 2$ Ca) and incubated. Aliquants were removed from both suspensions at 6 h, if sufficient suspension was available, and at 24 h and fixed ($n = 3$ for 6 h; $n = 6$ for 24 h).

Series V. Motile suspensions ($n = 6$) were prepared in standard medium. Aliquants were removed at 0, 3 and 6 h and received either DMSO (0.5% final concentration) or ionomycin (final concentration of 3, 10 and 30 μM in 0.5% DMSO). After 15 min, motility was assessed and the suspensions were fixed. A drug-free control was also included at each time point.

Series VI. Motile suspensions ($n = 4$) were prepared in standard Earle's medium, diluted to 5×10^6 cells/ml and 4 aliquants prepared. One of these served as the drug-free control; verapamil stock solutions were added to the remaining aliquants to give final concentrations of 25, 50 and 100 μM -verapamil. Samples were removed from each at 3, 6 and 24 h, assessed for motility and fixed.

Series VII. Motile suspensions ($n = 5$) were prepared in the following media: –Ca, +Ca, +Ba (1.80 mM- BaCl_2) and +Mg (1.80 mM- MgCl_2). After incubation for 24 h, aliquants were removed from the +Ba and +Mg suspensions and Ca^{2+} stock solution was added to give a final concentration of 1.80 mM- Ca^{2+} . After 15 min, all 6 samples (–Ca, +Ba, +Ba $\rightarrow$ +Ca, +Mg, +Mg $\rightarrow$ +Ca, +Ca) were fixed and assessed.

Series VIII. Motile suspensions ($n = 8$) were prepared in the following media: –Ca, +Ca and +Sr (1.80 mM- SrCl_2). These were incubated for a total of 24 h. At 6 h, an aliquant of the +Sr suspension was removed, centrifuged at 600 g for 5 min and resuspended in +Ca medium. After incubation for 15 min, this and aliquants from the –Ca, +Ca and +Sr samples were fixed. The 4 treatments were therefore –Ca, +Ca, +Sr and +Sr $\rightarrow$ +Ca. The procedure was repeated at 24 h.

Series IX. Motile suspensions ($n = 9$) were prepared in the following media: –Ca, +Ca, +Sr, +2 $\times$ Ca (3.60 mM- CaCl_2) and +2 $\times$ Sr (3.60 mM- SrCl_2). After incubation for 24 h, aliquants from the +Sr and +2 $\times$ Sr suspensions were centrifuged as in Series VIII and resuspended in +Ca medium for 15 min. These and the remaining samples were fixed, giving a total of 7 treatment groups: –Ca, +Ca, +Sr, +Sr $\rightarrow$ +Ca, +2 $\times$ Ca, +2 $\times$ Sr and +2 $\times$ Sr $\rightarrow$ +Ca. With this increase in treatments, some samples had insufficient spermatozoa to assess all 7 conditions. The minimum number of replicates for any one set of conditions was 6.

Processing and assessment. All samples were fixed in 3% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.2, for 45 min at room temperature. They were then centrifuged at 600 g for 5 min and the pellet was resuspended in fresh 0.1 M-cacodylate buffer. After 24 h samples were post-fixed in 1% osmium tetroxide, washed in 50% alcohol, 'blocked up' in molten agar (Ryder & McKenzie, 1981), dehydrated in graded alcohols and embedded in araldite resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with either a Philips 301G or Hitachi HU12A electron microscope.

A minimum of 200 sperm heads was assessed in each sample. Each cell was classified into one of the 6 stages of the AR which we have previously described (Stock & Fraser, 1987; Stock *et al.*, 1989): Stage I cells are acrosome-intact; Stages 2–4 are at intermediate stages at which initiation of the AR can be seen (swelling of the acrosomal cap, formation of intra-acrosomal vesicles from the outer acrosomal membrane, loss of acrosomal matrix despite retention of plasma and outer acrosomal membranes); Stages 5 and 6 are acrosome-reacted with exposed inner acrosomal membrane except in the equatorial segment (vesicles may still be associated with the sperm head). Only morphologically normal heads were counted.

Data were analysed using Cochran's modification of the 2×2 contingency tables (Snedecor & Cochran, 1967). This allows for comparison within and between replicates.

Results

Series I: is extracellular Ca^{2+} required for the AR?

The total mean proportion of spermatozoa which had initiated (Stages 2–4) or completed (Stages 5 & 6) the AR was significantly lower in the $- \text{Ca}$ suspensions than in their $+ \text{Ca}$ counterparts at 6 h (5.8% vs 10.0%, respectively; $P < 0.01$) and 24 h (8.4% vs 14.7%, respectively; $P < 0.01$). Most of the cells in $- \text{Ca}$ medium were at intermediate stages rather than having completed the reaction (Fig. 1). In the $+ \text{Ca}$ suspensions, the opposite pattern was observed and at 24 h about two-thirds of all positive cells were fully reacted. Motility was maintained at a mean of $\geq 90\%$ in both treatment groups. From this we conclude that extracellular Ca^{2+} is required for the AR in human spermatozoa.

Fig. 1. AR response in human sperm suspensions incubated for (a) 6 h and (b) 24 h in calcium-deficient medium ($- \text{Ca}$) and calcium-containing medium ($+ \text{Ca}$; 1.80 mM-Ca^{2+}). The stippled bars represent the proportion of cells at intermediate Stages 2–4 and reacted Stages 5 + 6. The solid bars (T) represent the total mean reacted cells (Stages 2–6). Values are mean % $\pm$ s.e.m. ($n = 20$). ** $P < 0.01$ compared with $+ \text{Ca}$ controls.

Series II: will Ca^{2+} added at the end of incubation trigger the AR?

In this series responses in 3 groups were compared: $- \text{Ca}$, $- \text{Ca} \rightarrow + \text{Ca}$ and $+ \text{Ca}$. Results (Fig. 2) indicate that the mean proportion of cells at Stages 2–6 was significantly lower ($P < 0.05$) in the $- \text{Ca}$ and $- \text{Ca} \rightarrow + \text{Ca}$ suspensions when compared with their $+ \text{Ca}$ counterparts at 6 h ($- \text{Ca}$, 5.0%; $- \text{Ca} \rightarrow + \text{Ca}$, 4.9%; $+ \text{Ca}$, 9.4%) and 24 h ($- \text{Ca}$, 8.3%; $- \text{Ca} \rightarrow + \text{Ca}$, 7.9%; $+ \text{Ca}$, 13.2%). There were no detectable differences between $- \text{Ca}$ and $- \text{Ca} \rightarrow + \text{Ca}$ suspensions. In both groups, as in $- \text{Ca}$ in Series I, most cells were at intermediate stages. Motility at $\geq 95\%$ was maintained in all groups. We conclude that extracellular Ca^{2+} cannot trigger the AR in human spermatozoa incubated for up to 24 h in Ca^{2+} -deficient medium; this would suggest that extracellular Ca^{2+} needs to be present during capacitation.

Fig. 2. AR response in human sperm suspensions incubated for (a) 6 h and (b) 24 h in calcium-deficient medium ($-Ca$), calcium-containing medium ($+Ca$) and calcium-deficient medium with 1.80 mM-Ca^{2+} being added for the final 15 min ($-Ca \rightarrow +Ca$). Values are mean $\% \pm \text{s.e.m.}$ ($n = 8$). * $P < 0.05$ compared with $+Ca$ controls.

Series III: will low Ca^{2+} concentrations present during incubation permit terminal addition of high Ca^{2+} to trigger the AR?

Four treatment groups were compared: $-Ca$, low Ca , low $Ca \rightarrow +Ca$ and $+Ca$. In one replicate 0.45 mM-CaCl_2 was used for low Ca and in two, 0.9 mM-CaCl_2 was used. Responses were similar and therefore have been combined. As can be seen in Fig. 3, responses in $+Ca$ were higher than those in the other 3 groups at 6 h ($-Ca$, 5.3%; low Ca , 4.3%; low $\rightarrow +Ca$, 5.6%; $+Ca$, 9.6%) and 24 h ($-Ca$, 8.0%; low Ca , 9.4%; low $Ca \rightarrow +Ca$, 9.6%; $+Ca$, 12.7%). Again the patterns of response were consistent with those observed in the earlier series: in $+Ca$ suspensions, the majority of cells were fully reacted while in the 3 with reduced Ca^{2+} present during initial incubation the reverse was true. Because the responses were consistent in the 3 replicates, further ones were not carried out. Motility was maintained at $\geq 95\%$ in all groups. We suggest that human spermatozoa require continuous exposure to high (millimolar range) Ca^{2+} concentrations in order to complete capacitation and to undergo the AR.

Series IV: what is the effect of elevated extracellular Ca^{2+} on the AR response?

The continuous presence of $2 \times Ca$ (3.60 mM) resulted in a significantly higher ($P < 0.001$) incidence of AR (34.7%) at 24 h in comparison with the standard $+Ca$ (11.9%) (Fig. 4). One of the $2 \times Ca$ 24 h group exhibited a massive proportion of reacted cells (91%) with most at Stages 5 and 6. Despite the unusually high value, the mean responses in this $2 \times Ca$ sample at 6 h and in both the 6 and 24 h $+Ca$ samples were unremarkable and we have no firm basis for rejecting this value. If it is ignored, however, the mean response is still elevated (24.2%; $P < 0.01$). In all samples motility was maintained at $\geq 95\%$ throughout the 24-h incubation period. We conclude that elevated extracellular Ca^{2+} concentrations stimulate the AR.

Fig. 3. AR response in human sperm suspensions incubated for (a) 6 h and (b) 24 h in calcium-deficient medium ($-Ca$), calcium-containing medium ($+Ca$), low calcium medium (low Ca ; $0.45\text{--}0.90\text{ mM-Ca}^{2+}$) and low calcium medium with calcium raised to 1.80 mM for the final 15 min (low $Ca \rightarrow +Ca$). Values are mean $\% \pm$ s.e.m. ($n = 3$).

Fig. 4. AR response in human sperm suspensions incubated for (a) 6 h and (b) 24 h in standard calcium-containing medium ($+Ca$; 1.80 mM-Ca^{2+}) and elevated calcium medium ($+2 \times Ca$; 3.60 mM-Ca^{2+}). Values are mean $\% \pm$ s.e.m. The broken lines indicate the mean value, including the unusually high response in one aliquot; the solid bars indicate the mean excluding that sample (total $n = 3$ for 3 h and 6 for 24 h). *** $P < 0.001$ compared with $+Ca$ controls for all samples (** $P < 0.01$ excluding the high response).

Series V: what is the effect of ionomycin on the AR in suspensions incubated in Ca^{2+} -containing medium?

A significant increase ($P < 0.05$ – $P < 0.001$, depending on treatment) in the incidence of AR was observed in all ionomycin-treated suspensions, compared with the drug-free controls (Fig. 5).

Fig. 5. AR response in human sperm suspensions preincubated for (a) 0 h, (b) 3 h and (c) 6 h in calcium-containing medium (1.80 mM-Ca^{2+}) and then incubated in ionomycin ($0\text{--}30 \mu\text{M}$) for 15 min. Values are mean $\% \pm \text{s.e.m.}$ ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with appropriate drug-free controls.

The magnitude of the response was concentration-dependent, the greatest response being elicited by $30 \mu\text{M}$ -ionophore, but the responses were similar at all 3 time points for each drug concentration evaluated. The total mean proportions of responding cells at 0, 3 and 6 h were: $3 \mu\text{M}$ —18.8%, 21.8%, 25.6%; $10 \mu\text{M}$ —35.7%, 43.6%, 34.8%; $30 \mu\text{M}$ —53.8%, 63.0%, 59.8%. Although there was an increase in the mean % reacted cells from 0 h to 3 h in all groups, this was only significant ($P < 0.05$) in $30 \mu\text{M}$ -ionomycin. Since an increase was not observed in all suspensions in any of the treatment groups, a much larger sample size would be needed to determine whether the increase was of biological relevance. In general, half or more of the responding cells were at intermediate stages (2–4), indicating that ionomycin did not necessarily induce a completed AR within the 15 min allowed for response. DMSO itself had no detectable effect on the response (data not presented). Motility at $>95\%$ was maintained in the ionomycin-free samples throughout the 6-h incubation period. No obvious effect was observed with $3 \mu\text{M}$ -ionomycin and values of $\geq 90\%$ were maintained with $10 \mu\text{M}$. The highest concentration, $30 \mu\text{M}$, caused a marked decline during the

Fig. 6. AR response in human sperm suspensions incubated for (a) 3 h, (b) 6 h and (c) 24 h in increasing concentrations of verapamil (0–100 μM). Values are mean % $\pm$ s.e.m. ($n = 4$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with appropriate drug-free controls.

15-min treatment period to values of 0–70%. We conclude that ionomycin induces an influx of extracellular Ca^{2+} and thereby triggers the AR.

Series VI: what effect does verapamil, a calcium channel antagonist, have on the AR?

The effects of verapamil treatment were dependent on both the drug concentration and the length of incubation (Fig. 6). After 3 h no significant differences were detected among the 4 treatment groups, with the mean total % of reacting/reacted cells ranging from 10.3 to 14.1%, but after 6 h a significantly higher AR response (mean of 20.6%; $P < 0.01$) was observed in the 100 μM -verapamil group than in the others (12.3–15.5%). After 24 h, a significantly elevated response was detected in all 3 verapamil-treated groups: 25 μM (22.0%) and 50 μM (22.8%), $P < 0.05$; 100 μM (57.0%), $P < 0.001$, when compared with drug-free controls (15.3%). Motility was maintained at $\geq 90\%$ with the exception of one suspension after 24 h in 100 μM -verapamil; this was not felt to be significant since pilot studies revealed no obvious effect on motility. The dependence of the drug effects on extended incubation suggests that only capacitated spermatozoa can respond.

Fig. 7. AR response in human sperm suspensions incubated for 24 h in calcium-deficient medium ($-Ca$), media containing 1.80 mM-CaCl_2 ($+Ca$), $-BaCl_2$ ($+Ba$) and $-MgCl_2$ ($+Mg$), and $+Ba$ and $+Mg$ media with suspensions receiving 1.80 mM-CaCl_2 for the final 15 min ($+Ba \rightarrow +Ca$, $+Mg \rightarrow +Ca$). The stippled bars represent the proportion of cells at intermediate Stages 2-4 and reacted Stages 5 + 6. The solid bars (T) represent the total mean reacted cells. Values are mean $\% \pm$ s.e.m. ($n = 5$).

Fig. 8. AR response in human sperm suspensions incubated for (a) 6 h and (b) 24 h in calcium-deficient medium ($-Ca$), calcium-containing medium ($+Ca$; 1.80 mM-CaCl_2), strontium-containing medium ($+Sr$; 1.80 mM-SrCl_2) and strontium-containing medium, centrifuged and resuspended in $+Ca$ medium for final 15 min ($+Sr \rightarrow +Ca$). Values are mean $\% \pm$ s.e.m. ($n = 8$). * $P < 0.05$ compared with $+Ca$ controls.

Series VII: can Ba^{2+} and Mg^{2+} substitute for Ca^{2+} during capacitation and the AR?

The total mean proportion of sperm cells which had initiated (Stages 2-4) or completed (Stages 5 & 6) the AR after 24-h incubation was lower in all 5 treatment groups ($-Ca$, 5.6%; $+Ba$, 6.4%; $+Ba \rightarrow +Ca$, 6.8%; $+Mg$, 7.7%; $+Mg \rightarrow +Ca$, 8.0%) compared with the control group, $+Ca$ (10.2%). Again, relatively few cells in $-Ca$ medium were at Stages 5 and 6 and a similar pattern was observed in all $+Ba$ and $+Mg$ samples. This contrasts with the $+Ca$ group in which the

Fig. 9. AR response in human sperm suspensions incubated for 24 h in the following media: calcium-deficient ($-Ca$), calcium-containing ($+Ca$), strontium-containing ($+Sr$), elevated calcium ($+2 \times Ca$; 3.60 mM-CaCl_2) and elevated strontium ($+2 \times Sr$; 3.60 mM-SrCl_2). Aliquants of the strontium-incubated suspension were centrifuged and resuspended in $+Ca$ medium ($+Sr \rightarrow +Ca$; $+2 \times Sr \rightarrow +Ca$). Values are mean $\% \pm \text{s.e.m.}$ ($n = 6-9$). * $P < 0.05$, ** $P < 0.01$ compared with $+Ca$ controls; † $P < 0.05$ compared with $+2 \times Ca$ suspensions.

majority of cells were fully reacted (Fig. 7). Motility was maintained at $\geq 95\%$ in all groups except those with $+Mg$, in which a slightly lower level of $\sim 90\%$ was observed. From these results we conclude that millimolar concentrations of barium and magnesium cannot substitute for calcium in supporting or fully preparing for the AR.

Series VIII: can Sr^{2+} substitute for Ca^{2+} during capacitation and the AR?

At 6 and 24 h, the mean proportion of cells at Stages 2–6 was significantly lower ($P < 0.05$) in the $-Ca$ group (5.8% and 10.4%, respectively) than in the $+Ca$ controls (12.1% and 14.7%, respectively). Although the equivalent figures for the $+Sr$ group were slightly lower (6 h, 9.6%; 24 h, 12.5%) than the controls, the difference was not significant (Fig. 8). Likewise, the values for $+Sr \rightarrow +Ca$ (6 h, 11.1%; 24 h, 14.1%) did not differ significantly from the controls. At both time points the total response in $+Sr$ and $+Sr \rightarrow +Ca$ suspensions was higher than that observed in $-Ca$; this difference was statistically significant ($P < 0.05$) for $+Sr \rightarrow +Ca$ at 6 h and nearly so at 24 h ($\chi^2 = 3.82$). The majority of Sr -treated cells at both times were at Stages 2–4, in a pattern similar to that seen in $-Ca$; in contrast, at 24 h the majority of cells in $+Ca$ were fully reacted. Motility was maintained at $\geq 95\%$ in all groups. Therefore, while it would appear that Sr^{2+} promotes a greater response than that observed in the absence of Ca^{2+} , it does not support completion of the reaction as successfully as does Ca^{2+} .

Series IX: does elevated Sr^{2+} increase the AR in a manner similar to elevated Ca^{2+} ?

The effect on the AR of incubating suspensions in medium containing increased Sr^{2+} (3.60 mM; $+2 \times \text{Sr}$), with and without the addition of Ca^{2+} ($+2 \times \text{Sr}$, $+2 \times \text{Sr} \rightarrow +\text{Ca}$) was compared with that obtained in media containing different amounts of Ca^{2+} or Sr^{2+} : $-\text{Ca}$, $+\text{Ca}$, $+\text{Sr}$, $+\text{Sr} \rightarrow +\text{Ca}$, $+2 \times \text{Ca}$ (Fig. 9). When all responses were compared with $+\text{Ca}$ suspensions (mean of 13.4% cells at Stages 2–6), a significantly lower ($P < 0.01$) AR response was observed in $-\text{Ca}$ medium (8.8%). In the $+\text{Sr}$ suspensions, 10.4% of cells had responded; while this value was significantly lower ($P < 0.05$) than the controls, the similar but very slightly higher response in $+\text{Sr} \rightarrow +\text{Ca}$ of 10.9% was not significantly different.

The AR in the presence of elevated concentrations of divalent cation was significantly higher compared with controls (13.4%): $+2 \times \text{Ca}$, 22.6% ($P < 0.01$); $+2 \times \text{Sr}$, 16.8% ($P < 0.05$); $+2 \times \text{Sr} \rightarrow +\text{Ca}$, 17.8% ($P < 0.05$). At the same time, the response to $+2 \times \text{Sr}$ was significantly lower ($P < 0.05$) when compared with the $+2 \times \text{Ca}$ response. In 5 suspensions (data not presented in Fig. 9) we also evaluated the conditions $+2 \times \text{Sr} \rightarrow +2 \times \text{Ca}$; with the addition of elevated Ca^{2+} , the corresponding AR figure was 19.2%, with about half the cells fully reacted. Thus, while elevated Sr^{2+} evokes a greater response than $+\text{Ca}$, it is less effective than elevated Ca^{2+} . Moreover, ~70% of responding cells in $+2 \times \text{Ca}$ were fully reacted compared with only 47–50% in the two $+2 \times \text{Sr}$ -treated groups. Therefore, even elevated Sr^{2+} concentrations are less able to support the complete reaction. Again, motility was maintained at $\geq 95\%$ in all groups.

Discussion

Consistent with observations made in numerous other species, invertebrate and vertebrate, our results indicate that the presence of extracellular Ca^{2+} permits a significantly higher proportion of human spermatozoa to undergo the spontaneous AR than is observed in Ca^{2+} -deficient conditions. Furthermore, Ca^{2+} also appears to be required during capacitation, with effective concentrations being in the millimolar range. Extended preincubation in micromolar amounts of Ca^{2+} does not prepare human spermatozoa to respond rapidly to millimolar Ca^{2+} and undergo the AR. In having a Ca^{2+} requirement during capacitation, human spermatozoa are similar to mouse spermatozoa, but the concentrations required for the human cells (≥ 1.80 mM) are much higher than comparable ones for the mouse (90 μM ; Fraser, 1987b). The greatest human sperm AR response was seen when samples were incubated continuously in elevated Ca^{2+} (3.60 mM). The fact that differences between 1.80 and 3.60 mM- Ca^{2+} -incubated suspensions were detectable at 6 h and significantly higher in 3.60 mM at 24 h suggests that these conditions may both accelerate/promote capacitation and trigger the AR in a greater proportion of spermatozoa. This would be consistent with results obtained for the mouse in which elevated Ca^{2+} concentrations (3.60 mM) precociously stimulated the AR and in-vitro fertilizing ability (Fraser, 1987b). Whether elevated Ca^{2+} would promote enhanced functional ability in human spermatozoa cannot be predicted. Extended incubation in ≥ 3.60 mM- Ca^{2+} irreversibly damaged mouse sperm fertilizing ability and even a short incubation in 7.20 mM- Ca^{2+} was deleterious; these effects appeared to be associated with qualitative effects on motility (Fraser, 1987b).

Our data also provide evidence that while the divalent cations Ba^{2+} and Mg^{2+} cannot effectively substitute for Ca^{2+} , Sr^{2+} can do so: the mean total of responding cells was not significantly different from that obtained in Ca^{2+} -containing medium (Fig. 8). However, a consistent difference in the distribution of cells between intermediate and completed stages of the AR was observed, with the majority of cells in Ca^{2+} being completely reacted and the majority in Sr^{2+} at intermediate stages (Fig. 8, 9). This latter pattern is also seen in Ca^{2+} -deficient and low Ca^{2+} conditions (Figs 1–3 and 7–9) and in Ba^{2+} or Mg^{2+} (Fig. 7), suggesting that they are less able to promote the complete AR. Motility was maintained at $\geq 90\%$ for 24 h. The total of reacting and reacted cells in Ca^{2+} -deficient medium increased slightly from 6 h to 24 h. It is likely that this increase is due in part to

some cells dying during incubation and losing the acrosome. There is also the possibility that during the swim-up preparation the cells bound sufficient Ca^{2+} from seminal plasma to promote the early stages of the AR. Certainly completion of the AR in such spermatozoa would appear to require millimolar concentrations of Ca^{2+} .

Current evidence (reviewed by Fraser, 1987a) indicates that capacitation involves a rise in the intracellular Ca^{2+} concentrations (e.g. White & Aitken, 1989) which leads ultimately to triggering of the AR. Divalent cation ionophores, in the presence of extracellular Ca^{2+} , increase the uptake of Ca^{2+} into spermatozoa (Babcock *et al.*, 1976; Peterson & Freund, 1976) and can effectively bypass the temporal requirements of capacitation, resulting in precocious AR (e.g. guinea-pig and hamster: Talbot *et al.*, 1976; boar: Peterson *et al.*, 1978; mouse: Fraser, 1982; ram: Shams-Borhan & Harrison, 1981; human: Jamil & White, 1981). Using the Ca^{2+} ionophore ionomycin we have confirmed that ionophores can eliminate the need for extended incubation of human spermatozoa normally required to obtain the characteristic incidence of $\sim 15\%$ spontaneous AR (Stock & Fraser, 1987). A 15-min exposure to ionomycin at 0 h was essentially as effective in inducing the AR as a similar exposure following a 6-h drug-free preincubation. In this, our results contrast markedly with those of Byrd *et al.* (1989), in which the greatest response to ionophore was only observed in suspensions preincubated for 6 h before treatment. Lee *et al.* (1987) have also reported that ionophore treatment of fresh suspensions failed to stimulate the AR. The reason for these discrepancies is unclear. The media differed, being Earle's in our study and BWW in the others, but Cross *et al.* (1986) and Aitken *et al.* (1984) have provided evidence of rapid responses to ionophore in BWW medium. Indeed, the majority of similar studies have observed rapid responses of uncapacitated sperm suspensions to ionophore, both in terms of AR (e.g. Shams-Borhan & Harrison, 1981; Fraser, 1982; Cross *et al.*, 1986) and increased fertilizing ability (e.g. Fraser, 1982; Aitken *et al.*, 1984).

In agreement with Byrd *et al.* (1989) we observed a concentration-dependent response to ionomycin over the range examined (3–30 μM). The mean maximum response in the 30 μM -treated group was approximately 60%, with a high proportion ($\sim 50\%$) of cells at intermediate stages probably reflecting the short incubation after ionophore addition; a longer incubation would have allowed completion of the AR. While it is possible that a longer exposure to ionophore would have increased the proportion of reacted cells, this level of response is similar to that in other studies of human spermatozoa in the presence of ionophore, commonly A23187 but occasionally ionomycin. The maximum response noted, in some instances after several hours of incubation in ionophore, has consistently fallen within the range of ~ 40 – 70% reacting cells rather than approaching 100% (Russell *et al.*, 1979; Jamil & White, 1981; Aitken *et al.*, 1984; Tesařik, 1985; Cross *et al.*, 1986; Lee *et al.*, 1987; Thomas & Meizel, 1988; Byrd *et al.*, 1989; Mortimer & Camenzind, 1989). This suggests that some of the cells, even in selected and highly motile suspensions, are unable to respond to this exogenous stimulus and therefore may be inherently defective. The presence of a smaller proportion of reacting cells could be associated with basic cellular dysfunction. Aitken *et al.* (1984) have presented evidence that while A23187 treatment of sperm suspensions from men of proven fertility was associated with an increase in successful penetration of zona-free hamster eggs, similar treatment of samples from oligozoospermic men failed to alter markedly the poor penetration pattern observed. These same workers have suggested recently that at least one type of dysfunction in such suspensions is an excessive production of reactive oxygen species (Aitken & Clarkson, 1987).

We failed to observe any correlation between the concentration of extracellular Ca^{2+} and maintenance of sperm motility. Motility was maintained equally well for 24 h whether the medium was Ca^{2+} -deficient ($\sim 15 \mu\text{M}$ -free Ca^{2+} ; Fraser, 1987b) or contained Ca^{2+} at 1.80–3.60 mM. There have been numerous conflicting reports regarding Ca^{2+} and human sperm motility (see Hong *et al.*, 1984). In suspensions which have been removed from seminal plasma and then incubated in prepared media, the Ca^{2+} concentration is more likely to affect qualitative features of motility which we did not evaluate, particularly hyperactivated motility (e.g. in mouse: Fraser, 1982; Cooper,

1984). A preliminary study has suggested that a correlation exists between the extracellular Ca^{2+} concentration and the proportion of human spermatozoa exhibiting hyperactivated motility (Robertson & Mortimer, 1988). Our observation that exposure to a high concentration of ionophore ($30\text{ }\mu\text{M}$ -ionomycin) resulted in fairly rapid loss of motility is consistent with similar effects noted in other studies using ionophores (e.g. Shams-Borhan & Harrison, 1981; Fraser, 1982; Aitken *et al.*, 1984; Tesafik, 1985; Thomas & Meizel, 1988). This response is due to ionophore-related effects on mitochondrial Ca^{2+} concentrations (Reed & Lardy, 1972). There are ways of manipulating treated suspensions to maintain motility (Shams-Borhan & Harrison, 1981; Fraser, 1982; Thomas & Meizel, 1988), particularly important for demonstrating fertilizing ability. However, we terminated all experiments 15 min after introduction of ionomycin and hence made no attempt to maintain or re-establish full motility.

Using ultrastructural characteristics as the basis for assessment, we have consistently observed features of the human AR which differ from the generally accepted pattern of the mammalian sperm AR. In particular, the intermediate AR stages in human cells appear to involve the formation of intra-acrosomal vesicles, primarily derived from the outer acrosomal membrane (Nagae *et al.*, 1986; Stock & Fraser, 1987), rather than vesicles formed from fusion between plasma and outer acrosomal membranes (see Bedford, 1983). In contrast, however, another recent study (Yudin *et al.*, 1988) has reported that the human AR conforms to the general mammalian pattern. Clearly these discrepancies need to be addressed. The possibility that human spermatozoa considered to be at intermediate stages of the reaction might simply be degenerating has been considered. The consistency with which these stages have been observed under a variety of conditions in which very high levels of motility are maintained, an indication that cells are not moribund, would appear to negate this (e.g. Stock & Fraser, 1987; Stock *et al.*, 1988, 1989). Furthermore, the observation of increased numbers of cells at intermediate stages in the present ionomycin experiments, in which recently prepared suspensions were treated for 15 min and fixed while motility was high (particularly in 3 and $10\text{ }\mu\text{M}$ -ionomycin treated groups), would argue strongly against an association with degenerative changes.

Our data indicate that human sperm capacitation and the spontaneous AR require the presence of millimolar concentrations of extracellular Ca^{2+} . The fact that a brief exposure to ionophore induces the AR suggests that at least some of the Ca^{2+} is internalized before the reaction and Irvine & Aitken (1986) have demonstrated that ionomycin treatment of human spermatozoa results in a rise in intracellular Ca^{2+} . Thomas & Meizel (1988) have recently presented evidence that a transient increase in intracellular free Ca^{2+} occurs concomitantly with the AR. Because both this rise and the AR can be blocked by addition of chelators or La^{3+} , a Ca^{2+} -antagonist, these authors also conclude that the AR requires an influx of extracellular Ca^{2+} . However, the mechanisms controlling such an influx have yet to be identified. In sea urchin spermatozoa, the ability of drugs which antagonize voltage-sensitive calcium channels in somatic cells (Triggle & Janis, 1987) to block a Ca^{2+} -induced AR suggests that these channels play an important role in Ca^{2+} influx (Schackmann *et al.*, 1978; Kazazoglou *et al.*, 1985). The evidence from mammalian spermatozoa is more equivocal since treatment of spermatozoa with drugs (e.g. verapamil, D600 and various dihydropyridines) usually regarded as antagonists of such calcium channels has generally had no effect or has enhanced the AR response (e.g. Singh *et al.*, 1980; Roldan *et al.*, 1986). However, recent studies have provided support for the existence of similar channels in ram (Babcock & Pfeiffer, 1987) and mouse spermatozoa (Fraser & McIntyre, 1989). We have demonstrated that verapamil and the dihydropyridine nifedipine do not block capacitation *per se* (Fraser & McIntyre, 1989) but can block a Ca^{2+} -stimulated AR in capacitated mouse sperm cells. The blocking of the AR was only observed when spermatozoa were capacitated in low Ca^{2+} ($90\text{ }\mu\text{M}$) medium. While not supporting the AR, these conditions do support capacitation since introduction of millimolar Ca^{2+} results in rapid triggering of the AR and fertilizing ability (Fraser, 1987b); however, the introduction of calcium channel antagonists before the millimolar Ca^{2+} significantly inhibited the AR response. In contrast, addition of these same drugs to suspensions capacitated in millimolar

Ca^{2+} resulted in a significantly increased AR response. This suggested that these cells were in an altered state, possibly due to accumulation of intracellular Ca^{2+} to critical threshold levels, such that these same drugs, usually observed to have antagonistic effects on calcium channels, had an agonistic effect. Studies in somatic cells have demonstrated that 'antagonists' can have agonistic effects under appropriate conditions (Scott & Dolphin, 1987).

Given that human spermatozoa appear to require prolonged incubation in millimolar Ca^{2+} , the capacitation phase presumably requires or allows the intracellular Ca^{2+} to rise to a level approximating such an altered state. If drugs such as verapamil did not affect calcium channels during capacitation *per se* in human spermatozoa, as is the case for their mouse counterparts, then one would predict that verapamil could have an agonistic effect on these channels in capacitated cells, leading to an influx of Ca^{2+} and the AR. Both our observation of a concentration- and time-dependent increase in the proportion of reacted cells and the enhanced responses observed in studies of other mammalian spermatozoa cited above are consistent with this hypothesis. The fact that at a given concentration the response was highest in suspensions incubated for 24 h argues strongly that the AR was elicited in capacitated spermatozoa. This pattern of response differs markedly from the ionophore-induced AR, for which maximal responses could be evoked at the very start of sperm incubation (see Figs 5 & 6). Also consistent with our hypothesis is the observation of Irvine & Aitken (1986) that neither verapamil nor diltiazem affected either the resting level of intracellular Ca^{2+} or the passage of Ca^{2+} into uncapacitated human spermatozoa when the extracellular Ca^{2+} concentration was increased. We therefore propose that calcium channels may exist in human spermatozoa. While not controlling Ca^{2+} influx during capacitation, they could function at the end of capacitation with agonistic interactions between channels and oocyte-associated molecules resulting in a Ca^{2+} influx and triggering of the AR.

This study and many others have provided evidence that Ca^{2+} can support capacitation and the AR, and this would be the most abundant divalent cation *in vivo*. Of the various other divalent cations assessed, only Sr^{2+} supported responses similar to Ca^{2+} (Figs 7, 8 & 9). Although Mortimer (1986) and Mortimer *et al.* (1986) suggested that Sr^{2+} -incubated spermatozoa were able to undergo capacitation but not the AR, our data do not support this proposal and Mortimer *et al.* (1988) have reached the same conclusion since they were unable to detect any significant differences in AR in the presence of 2.4 mM- Sr^{2+} and 2.4 mM- Ca^{2+} . They did observe, however, that sperm penetration of zona-free hamster eggs was considerably lower in Sr^{2+} than Ca^{2+} . In an earlier study, Yanagimachi (1978) reported that, while 1.71 mM- Sr^{2+} supported little penetration of human spermatozoa into similar hamster eggs, 3.0 mM- Sr^{2+} was nearly as effective as 1.71 mM- Ca^{2+} plus 1.19 mM- Mg^{2+} . Whether these discrepancies possibly reflect differences in the Sr^{2+} concentration or the presence of EGTA in the Mortimer *et al.* (1988) study is uncertain. Our results indicate that the proportion of responding cells is certainly higher in 3.60 mM- than in 1.80 mM- Sr^{2+} , both for fully reacted cells as well as all reacting cells. Higher fusion rates in elevated Sr^{2+} would therefore be consistent with earlier reports that only acrosome-reacted spermatozoa can fuse with the egg plasma membrane (Yanagimachi, 1981).

Additionally, Mortimer *et al.* (1988) examined the ability of suspensions incubated in either Sr^{2+} - or Ca^{2+} -containing medium to bind to salt-stored human zonae pellucidae. Noting that approximately twice as many motile cells bound in the presence of Ca^{2+} as in Sr^{2+} , they concluded that sperm-zona interactions in the human are less effectively supported by Sr^{2+} . It is therefore suggestive that we observed fewer spermatozoa that were fully acrosome-reacted in Sr^{2+} -containing medium. Perhaps a completed AR is a prerequisite for zona binding in the human. This apparent inability of Sr^{2+} to substitute fully for Ca^{2+} in zona binding is in direct contrast to observations in the mouse: mouse spermatozoa were able to interact with the zona and achieve normal penetration in Sr^{2+} -containing medium as evidenced by the presence of numerous spermatozoa in the perivitelline space (Fraser, 1987c). Fusion with zona-intact eggs was minimal because of Sr^{2+} -induced egg activation, but when zona-free eggs were used essentially all were penetrated and exhibited a high incidence of polyspermy. The report by Heffner *et al.* (1980) that Sr^{2+} did not

support sperm–zona binding in the mouse would appear to reflect the use of a minimal, possibly non-physiological, medium, containing only Tris–HCl and NaCl in addition to Sr^{2+} .

The ability of Sr^{2+} to replace Ca^{2+} in the changes undergone by spermatozoa which are required for fertilization would therefore appear to be species-specific. Sr^{2+} can completely substitute for Ca^{2+} in mouse spermatozoa, supporting capacitation, the AR, sperm–zona binding and gamete fusion. In human spermatozoa Sr^{2+} can replace Ca^{2+} during capacitation and initiation of the AR, but it is less effective in supporting completion of the AR. This may explain, in part, the apparent inability of Sr^{2+} to substitute for Ca^{2+} during zona binding and gamete fusion.

In conclusion, our results indicate that the continuous presence of millimolar Ca^{2+} is required to ensure that a maximal spontaneous AR is obtained, suggesting that Ca^{2+} is needed during both capacitation and the AR. In either the absence of added Ca^{2+} or in low Ca^{2+} , a significantly lower proportion of spermatozoa initiate, and few complete, the AR compared with their counterparts incubated in $\geq 1.80 \text{ mM-Ca}^{2+}$. They are unable to respond rapidly to millimolar Ca^{2+} , even after 24 h incubation. Of the other divalent cations assessed, Sr^{2+} was able to substitute for Ca^{2+} although it proved less effective than Ca^{2+} in promoting completion of the AR. That some of the Ca^{2+} must enter the cell is indicated by the ability of the ionophore ionomycin to remove the time-dependent features of the spontaneous response and induce an immediate AR. Finally, calcium channels, capable of modulating Ca^{2+} influx prior to the AR, may exist in human spermatozoa.

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References

- Aitken, R.J. & Clarkson, J.S. (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fert.* **81**, 459–469.
- Aitken, R.J., Ross, A., Hargreave, T., Richardson, D. & Best, F. (1984) Analysis of human sperm function following exposure to the ionophore A23187. *J. Androl.* **5**, 321–329.
- Babcock, D.F. & Pfeiffer, D.R. (1987) Independent elevation of cytosolic $[\text{Ca}^{2+}]$ and pH of mammalian sperm by voltage-dependent and pH-dependent mechanisms. *J. biol. Chem.* **262**, 15041–15047.
- Babcock, D.F., First, N.L. & Lardy, H.A. (1976) Action of ionophore A23187 at the cellular level. Separation of the effects at the plasma and mitochondrial membranes. *J. biol. Chem.* **251**, 3881–3886.
- Bedford, J.M. (1983) Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol. Reprod.* **28**, 108–120.
- Byrd, W., Tsu, J. & Wolf, D.P. (1989) Kinetics of spontaneous and induced acrosomal loss in human sperm incubated under capacitating and noncapacitating conditions. *Gamete Res.* **22**, 109–122.
- Cooper, T.G. (1984) The onset and maintenance of hyperactivated motility of spermatozoa from the mouse. *Gamete Res.* **9**, 55–74.
- Cross, N.L., Morales, P., Overstreet, J.W. & Hanson, F.W. (1986) Two simple methods for detecting acrosome reacted human sperm. *Gamete Res.* **15**, 213–226.
- Fraser, L.R. (1982) Ca^{2+} is required for mouse sperm capacitation and fertilization *in vitro*. *J. Androl.* **3**, 412–419.
- Fraser, L.R. (1987a) Extracellular calcium and fertilization-related events. In *The Role of Calcium in Biological Systems*, Vol. IV, pp. 163–190. Ed. L. J. Anghileri. CRC Press, Boca Raton.
- Fraser, L.R. (1987b) Minimum and maximum extracellular Ca^{2+} requirements during mouse sperm capacitation and fertilization *in vitro*. *J. Reprod. Fert.* **81**, 77–89.
- Fraser, L.R. (1987c) Strontium supports capacitation and the acrosome reaction in mouse sperm and rapidly activates mouse eggs. *Gamete Res.* **18**, 363–374.
- Fraser, L.R. & McIntyre, K. (1989) Calcium channel antagonists modulate the acrosome reaction but not capacitation in mouse spermatozoa. *J. Reprod. Fert.* **86**, 223–233.
- Heffner, L.J., Saling, P.M. & Storey, B.T. (1980) Separation of calcium effects on motility and zona binding ability in mouse spermatozoa. *J. exp. Zool.* **212**, 53–59.
- Hong, C.Y., Chiang, B.N. & Turner, P. (1984) Calcium ion is the key regulator of human sperm function. *The Lancet* **ii**, 1449–1451.
- Irvine, D.S. & Aitken, R.J. (1986) Measurement of intracellular calcium in human spermatozoa. *Gamete Res.* **15**, 57–71.
- Iwamatsu, T. & Chang, M.C. (1971) Factors involved in the fertilization of mouse eggs *in vitro*. *J. Reprod. Fert.* **26**, 197–208.
- Jamil, K. & White, I.G. (1981) Induction of acrosomal reaction in sperm with ionophore A23187 and calcium. *Archs Androl.* **7**, 283–292.
- Kazazoglou, T., Schackmann, R.W., Fosset, M. & Shapiro, B.M. (1985) Calcium channel antagonists inhibit the

- acrosome reaction and bind to plasma membranes of sea urchin sperm. *Proc. natn. Acad. Sci. USA* **82**, 1462–1464.
- Lee, M.A., Trucco, G.S., Bechtol, K.S., Wummer, N., Kopf, G.S., Blasco, L. & Storey, B.T. (1987) Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay. *Fert. Steril.* **48**, 649–658.
- Loeb, J. (1915) On the nature of the conditions which determine or prevent the entrance of the spermatozoa into the egg. *Am. Nat.* **49**, 257–285.
- Mortimer, D. (1986) Comparison of the fertilizing ability of human spermatozoa preincubated in calcium- and strontium-containing media. *J. exp. Zool.* **237**, 21–24.
- Mortimer D. & Camenzind, D. (1989) The role of follicular fluid in inducing the acrosome reaction of human spermatozoa incubated *in vitro*. *Human Reprod.* **4**, 169–174.
- Mortimer, D., Curtis, E.F. & Dravland, J.E. (1986) The use of strontium-substituted media for capacitating human spermatozoa: an improved sperm preparation method for the zona-free hamster egg penetration test. *Fert. Steril.* **46**, 97–103.
- Mortimer, D., Chorney, M.J., Curtis, E.F. & Trounson, A.O. (1988) Calcium dependence of human sperm fertilizing ability. *J. exp. Zool.* **246**, 194–201.
- Nagae, T., Yanagimachi, R., Srivastava, P.N. & Yanagimachi, H. (1986) Acrosome reaction in human spermatozoa. *Fert. Steril.* **45**, 701–707.
- Peterson, R.N. & Freund, M. (1976) Relationship between motility and the transport and binding of divalent cations to the plasma membrane of human spermatozoa. *Fert. Steril.* **27**, 1301–1307.
- Peterson, R.N., Russell, I., Bundman, D. & Freund, M. (1978) Presence of micro filaments and tubular structures in boar spermatozoa after chemically inducing the acrosome reaction. *Biol. Reprod.* **19**, 459–466.
- Reed, P.N. & Lardy, H.A. (1972) A23187: A divalent cation ionophore. *J. biol. Chem.* **247**, 6970–6977.
- Robertson, L. & Mortimer, D. (1988) The effect of extracellular free Ca^{2+} on the development of hyperactivated motility in capacitating human spermatozoa. *J. Reprod. Fert., Abstr. Ser.* **1**, 65, abstr.
- Roldan, E.R.S., Shibata, S. & Yanagimachi, R. (1986) Effect of Ca^{2+} channel antagonists on the acrosome reaction of guinea pig and golden hamster spermatozoa. *Gamete Res.* **13**, 281–292.
- Russell, L., Peterson, R.N. & Freund, M. (1979) Morphologic characteristics of the chemically induced acrosome reaction in human spermatozoa. *Fert. Steril.* **32**, 87–92.
- Ryder, T.A. & Mackenzie, M.L. (1981) Routine preparation of seminal fluid specimens for transmission electron microscopy. *J. clin. Pathol.* **34**, 1006–1009.
- Schackmann, R.W., Eddy, E.M. & Shapiro, B.M. (1978) The acrosome reaction of *Strongylocentrotus purpuratus* sperm. Ion requirements and movements. *Devl Biol.* **65**, 483–495.
- Scott, R.H. & Dolphin, A.C. (1987) Activation of a G protein promotes agonist responses to calcium channel ligands. *Nature, Lond.* **330**, 760–762.
- Shams-Borhan, G. & Harrison, R.A.P. (1981) Production, characterization and use of ionophore-induced, calcium-dependent acrosome reaction in ram spermatozoa. *Gamete Res.* **4**, 407–432.
- Singh, J.P., Babcock, D.F. & Lardy, H.A. (1980) Induction of accelerated acrosome reaction in guinea pig sperm. *Biol. Reprod.* **22**, 566–570.
- Snedecor, G. & Cochran, W. (1967) *Statistical Methods*, 6th edn. Iowa State University Press, Ames.
- Stock, C.E. & Fraser, L.R. (1987) The acrosome reaction in human sperm from men of proven fertility. *Hum. Reprod.* **2**, 109–119.
- Stock, C.E., Bates, R., Lindsay, K.S., Edmonds, D.K. & Fraser, L.R. (1988) Human oocyte cumulus complexes enhance the human acrosome reaction. *J. Reprod. Fert., Abstr. Ser.* **2**, 10, abstr.
- Stock, C.E., Bates, R., Lindsay, K.S., Edmonds, D.K. & Fraser, L.R. (1989) Extended exposure to follicular fluid is required for significant stimulation of the acrosome reaction in human spermatozoa. *J. Reprod. Fert.* **86**, 401–411.
- Talbot, P., Summers, R.G., Hylander, B., Keough, E.M. & Franklin, L.E. (1976) The role of calcium in the acrosome reaction: an analysis using ionophore A23187. *J. exp. Zool.* **198**, 383–392.
- Tesařík, J. (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability *in vitro*. *J. Reprod. Fert.* **74**, 383–388.
- Thomas, P. & Meizel, S. (1988) An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid. *Gamete Res.* **20**, 397–411.
- Triggle, D.J. & Janis, R.A. (1987) Calcium channel ligands. *Ann. Rev. Pharmacol. Toxicol.* **27**, 347–369.
- White, D.R. & Aitken, R.J. (1989) Relationship between calcium, cyclic AMP, ATP and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. *Gamete Res.* **22**, 163–177.
- Yanagimachi, R. (1978) Calcium requirement for sperm-egg fusion in mammals. *Biol. Reprod.* **19**, 949–958.
- Yanagimachi, R. (1981) Mechanisms of fertilization in mammals. In *Fertilization and Embryonic Development in vitro*, pp. 81–182. Eds L. Mastroianni & J. D. Biggers. Plenum Publishing, New York.
- Yanagimachi, R. (1982) Requirements of extracellular calcium ions for various stages of fertilization and fertilization-related phenomena in the hamster. *Gamete Res.* **5**, 323–344.
- Yanagimachi, R. & Usui, N. (1974) Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Expl Cell Res.* **89**, 161–174.
- Yudin, A.I., Gottlieb, W. & Meizel, S. (1988) Ultrastructural studies of the early events of the human sperm acrosome reaction as initiated by human follicular fluid. *Gamete Res.* **20**, 11–24.

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