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Minimum and maximum extracellular Ca^{2+} requirements during mouse sperm capacitation and fertilization *in vitro*

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Summary. The minimum and maximum extracellular Ca^{2+} concentrations required to promote capacitation, the acrosome reaction, hyperactivated motility, zona penetration and gamete fusion in the mouse have been established. The traces of free calcium in Ca^{2+}-deficient medium were shown not to enhance capacitation since the inclusion of EGTA to chelate free ions during a 120 min preincubation failed to alter the kinetics of capacitation from those observed in the absence of EGTA; 1 h after addition of 1·80 mM-Ca^{2+}, both suspensions were highly fertile. Complete capacitation, when suspensions were immediately functional upon the addition of 1·80 mM-Ca^{2+}, required the presence of $\geq$90 μM-Ca^{2}. Considerably higher concentrations were required to initiate optimal sperm responses: acrosome reaction, 900 μM; gamete fusion, 900 μM; hyperactivated motility, 1·80 mM; zona penetration, 1·80 mM. None of these changes was effected when Ca^{2+} was $<$450 μM. The responses to elevated Ca^{2+} were dependent on the length of incubation, being initially positive and then negative. A short (30 min) exposure to 3·40 mM-Ca^{2+} ($\times$2 the standard) accelerated capacitation, as evidenced by significantly increased acrosome loss, precocious expression of hyperactivated motility and enhanced fertilizing ability when Ca^{2+} was reduced to 1·80 mM. However, extended (120 min) preincubation irreversibly damaged sperm function. In the presence of 7·20 mM-Ca^{2+} ($\times$4), fertilizing ability was inhibited at both 30 and 120 min, despite a high incidence of acrosome loss. The primary deleterious effect appeared to be on motility which was judged to be more erratic than in 1·80 mM-Ca^{2+}, possibly due to elevated intracellular Ca^{2+}. Because of the considerable difference in threshold Ca^{2+} concentrations, it is now possible to dissociate the Ca^{2+}-dependent events of capacitation from those of the acrosome reaction and motility changes.

Introduction

It has long been known that extracellular free calcium ions are required for successful fertilization in invertebrates (Loeb, 1915) and mammals (mouse: Iwamatsu & Chang, 1971). In all systems examined an influx of Ca^{2+} is required to initiate the acrosome reaction, with its attendant release of enzymes and membrane alterations necessary for sperm–egg interaction (reviewed by Fraser, 1987). However, after leaving the male reproductive tract mammalian spermatozoa must first undergo capacitation (Austin, 1951; Chang, 1951) which then enables them to undergo the acrosome reaction and to express hyperactivated motility. Both are required for spermatozoa to penetrate the egg investments and fuse with the egg plasma membrane (Yanagimachi, 1981; Bedford, 1983; Fraser, 1984) and hyperactivated motility, as well as the acrosome reaction, is Ca^{2+}-dependent (guinea-pig: Yanagimachi & Usui, 1974; mouse: Fraser, 1977; hamster: Yanagimachi, 1982). The fact that these are terminal events raises the possibility that Ca^{2+}-dependent steps may also occur during capacitation. Experiments to date suggest this to be true in the hamster and the mouse, although guinea-pig spermatozoa appear to undergo capacitation in

the absence of Ca^{2+}. If incubated in Ca^{2+}-deficient medium sufficiently long to promote capacitation in complete medium, they will undergo the acrosome reaction within minutes of introduction of extracellular Ca^{2+} (Yanagimachi & Usui, 1974). Similar manipulations of hamster (Yanagimachi, 1982) and mouse spermatozoa (Fraser, 1982) have shown them not to be functionally equivalent to fully capacitated suspensions, based on acrosome loss, hyperactivated motility expression and fertilizing ability, but rather to be partially capacitated. If Ca^{2+} is added at the end of preincubation, fertilizing ability is acquired gradually. Mouse spermatozoa require extracellular Ca^{2+} during the final 30–60 min of a 120-min preincubation phase in order to equal the functional abilities of spermatozoa preincubated in the continuous presence of Ca^{2+} (Frazer, 1982).

Since Ca^{2+} plays such a central role in fertilization-related events, this study was designed to examine in greater detail the concentrations required to support capacitation, the acrosome reaction, hyperactivated motility and fertilization. While the emphasis has been placed on minimum concentrations, values higher than the control have also been examined. Once these limits have been established, it may prove possible to manipulate individual steps during fertilization in specific ways.

Materials and Methods

Media. The standard medium was a modified Tyrode's solution lacking pyruvate and lactate and containing 5·56 mM-glucose and 1·80 mM-$CaCl_2$ (Fraser, 1983b). All media were supplemented with crystalline BSA (4 mg/ml; Sigma, Poole, Dorset, U.K.). Calcium-deficient medium was prepared by omitting $CaCl_2$; although containing trace amounts of Ca^{2+} contributed by the other salts, this medium supports neither complete capacitation nor fertilization *in vitro* (Fraser, 1982). The amount of free Ca^{2+} in BSA-supplemented complete and Ca^{2+}-deficient media was measured using an Orion Model 932000 calcium electrode in conjunction with a Model 900100 single junction reference electrode (Orion Research Ltd, Cambridge, MA, U.S.A.). This electrode is reliable, $\pm 10\%$, down to 10^{-7} M-Ca^{2+}. To introduce 1·80 mM-Ca^{2+} as noted below, a stock of Tyrode's containing 22·5 mM-$CaCl_2$ (higher concentrations led to precipitation of the salt) was prepared and 20 µl of this were added to each 230 µl Ca^{2+}-deficient medium. To prepare media containing 90–900 µM-$CaCl_2$, substocks of the 22·5 mM solution were prepared and added in the same ratio as above, e.g. 1/12·5. For Series I, a stock solution of 2·0 mM-ethyleneglycol-bis-(b-aminoethyl ether)-*N,N,N',N'*,-tetra-acetic acid (EGTA) in Tyrode's was prepared and added to suspensions to give a final concentration of 20 µM.

In-vitro fertilization. Mature female outbred TO mice (age >8 weeks) were induced to superovulate with i.p. injections of 7·5 i.u. PMSG (Gestyl: Organon, Morden, Surrey, U.K.) and, 54 h later, 5 i.u. hCG (Pregnyl: Organon). Eggs were recovered 14 h after hCG by releasing cumulus clots directly into sperm suspensions. When sperm preparations were sampled at 2 times, hCG was injected asynchronously to avoid using aged eggs. Zona-free eggs were prepared by sequential treatment with hyaluronidase and pronase (Fraser, 1983a).

Sperm suspensions were prepared by releasing the contents of 1 cauda epididymidis from each of 2 mature (age >8 weeks) TO males into 1 ml medium. By using this ratio of epididymides to medium, a maximum of 4 samples can be prepared from each pair of males. Suspensions were preincubated for a total of 120 min at 37°C and fertilizing ability was assessed at 30 and/or 120 min. Aliquants were diluted ~10-fold to yield a final concentration of 1–2 × 10^6 spermatozoa/ml; eggs were released into 300 µl droplets of diluted suspension.

Incubations were carried out in 30-mm plastic tissue culture dishes (Sterilin, Teddington, Middlesex, U.K.) and medium was overlaid with autoclaved liquid paraffin (Boots, Nottingham, U.K.). A gas mixture of 5% CO_2–5% O_2–90% N_2 was used throughout.

Routine assessments. At 65–70 min after mixing of gametes in all fertilization experiments, eggs were transferred from sperm suspensions to droplets of fresh medium and, at 75 min, fixed by flooding the dishes with neutral buffered formalin (4% formaldehyde). Eggs were then stained, mounted and assessed for fertilization (Fraser, 1983a, b). They were considered to be fertilized if they had resumed the second meiotic division and contained a decondensing sperm head. As a measure of rapid sperm penetration, the proportion of eggs at telophase II/second polar body which had a fully decondensed sperm head was calculated; this is the most advanced stage achieved within 75 min (Fraser, 1983a).

Diluted sperm suspensions were assessed for proportion of cells exhibiting motility. In addition, a subjective estimate was made of the proportion of motile spermatozoa exhibiting the very fluid flagellar undulations, including the figure-of-eight pattern, that characterize hyperactivated motility as illustrated in Fraser (1977).

Acrosome loss was evaluated after filtering sperm suspensions to select motile cells. Short columns of Sephadex G-25 (Medium) were equilibrated and then, after application of sperm suspensions, eluted with appropriate media; this procedure yielded ~90% motile cells. Eluted spermatozoa were fixed in formalin, drops were placed on clean slides and coverslips were added. These preparations were allowed to dry completely and then rehydrated by introducing a minimal volume of formalin under the coverslips. A minimum of 100 cells in each sample were assessed

under phase contrast for the presence or absence of the acrosome. Using this method of preparation, the great majority of spermatozoa appear in profile and the acrosome, if present, can be detected easily as a dark band along the convex rim of the sperm head (see Fig. 2). If it is absent, the only dark structure is at the tip of the head; this is the so-called perforatorium, formed by the inner acrosomal membrane.

Data were analysed using Cochran's test for the combination of 2 × 2 contingency tables (Snedecor & Cochran, 1967).

Results

Series I: are trace amounts of Ca^{2+} required for partial capacitation?

Measurement of free Ca^{2+} in BSA-supplemented Ca^{2+}-containing (+Ca) and Ca^{2+}-deficient (−Ca) media gave values of 1·30 mM and 13 μM, respectively. To chelate the traces of Ca^{2+} in the latter, EGTA at a final concentration of 20 μM was used (−Ca + EGTA). A 2-ml sperm suspension was prepared in −Ca medium and after 5 min for dispersal, 6 droplets of equal volume were prepared. To 2 of these, +Ca stock was added to give 1·80 mM-$CaCl_2$ (+Ca) and to 2 more EGTA stock was added (−Ca + EGTA); to the final pair, an appropriate amount of −Ca medium was added to duplicate the slight dilution in other droplets. After 120 min preincubation, the suspension in 1 of each pair of droplets was diluted into +Ca medium and assessed for fertilizing ability. To the remaining −Ca and −Ca + EGTA droplets, +Ca stock was added to give 1·80 mM-$CaCl_2$ and all 3 suspensions were incubated an additional 60 min, then diluted in +Ca medium and assessed for fertilizing ability. Three replicates were performed (N = 3).

Table 1. Fertilizing ability of mouse sperm suspensions preincubated for 120 min in Ca^{2+}-deficient (−Ca) medium with and without 20 μM-EGTA

Medium			Eggs fertilized			Maximal nuclear development†, %
Preincubation						
0–120 min	120–180 min	Fertilization	No.	%	Range (%)	
+Ca	−	+Ca	71/93	76·3	71–83	78·9
+Ca	+Ca	+Ca	63/87	72·4	69–76	77·8
−Ca	−	+Ca	10/72	13·9**	11–18	10·0
−Ca	+Ca	+Ca	75/93	80·6	65–92	64·0
−Ca + EGTA	−	+Ca	9/57	15·8**	14–18	33·3
−Ca + EGTA	+Ca	+Ca	73/87	83·9	67–90	63·0

After addition of 1·80 mM-Ca^{2+} at 120 min, suspensions were assessed both immediately and following an additional 60 min preincubation. Control suspensions were maintained continuously in 1·80mM-Ca^{2+} medium (+Ca).

†Fertilized eggs at telophase – second polar body with fully decondensed sperm heads.

**$P < 0·01$ compared with 120 min +Ca suspensions.

Suspensions preincubated in +Ca medium for both 120 and 180 min were highly fertile and the majority of fertilized eggs had fully decondensed sperm heads (Table 1). In contrast, suspensions preincubated for 120 min in −Ca or −Ca + EGTA medium were poorly fertile (~15% of eggs fertilized and few of these had fully decondensed sperm heads). However, the introduction of 1·80 mM-$CaCl_2$ at 120 min and further preincubation until 180 min resulted in high rates of fertilization with both suspensions (80–85% with a majority of advanced nuclear stages). Functionally, there was no difference between the spermatozoa incubated continuously for 180 min in +Ca and those incubated for the final 60 min in +Ca; furthermore, the inclusion of EGTA during the initial phase did not alter the rate of capacitation and subsequent response to extracellular Ca^{2+}.

Series II: the minimum Ca^{2+} concentration required to support complete capacitation

In this series, media containing various concentrations of Ca^{2+} were prepared by diluting +Ca medium with −Ca medium. After preincubation for 120 min in reduced Ca^{2+}, all suspensions were diluted in +Ca medium and assessed for fertilizing ability. The Ca^{2+} concentrations examined included: 1·80 mM, 180 μM, 90 μM, 9 μM, 900 nM and 0 (i.e. Ca^{2+}-deficient medium).

When preincubated in +Ca or −Ca medium, and evaluated in +Ca, spermatozoa exhibited fertilizing abilities similar to those obtained in Series I: +Ca, a mean of 91% eggs fertilized and >90% at advanced stages of development; −Ca → +Ca, a mean of 20% eggs fertilized and most of these at early stages. Essentially all sperm suspensions preincubated in low concentrations of Ca^{2+} were functionally superior to those maintained in −Ca medium, but below a concentration of 90 μM-$CaCl_2$ the fertilization response was highly variable and significantly reduced, compared with 1·80 mM-Ca^{2+} controls (Fig. 1). The calculated amount of exogenous $CaCl_2$ in the 2 lowest concentrations (9 μM and 900 nM) was less than the measured amount of free Ca^{2+} in −Ca medium; therefore the true values will be slightly greater than 13 μM. From these results, 90 μM-$CaCl_2$ is the minimum amount of added Ca^{2+} that will consistently support complete capacitation. Since not all media could be examined simultaneously, each replicate included +Ca, −Ca and two others (N = 7 for +Ca and −Ca).

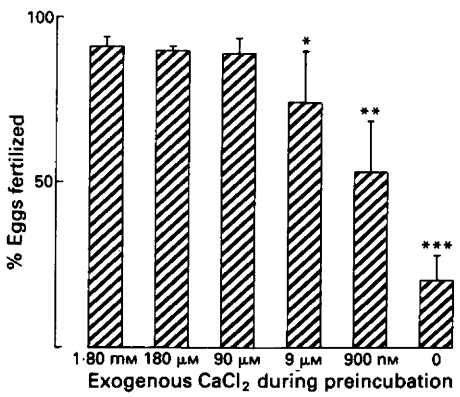

Fig. 1. Fertilizing ability of mouse spermatozoa preincubated for 120 min in medium containing 1·80 mM-$CaCl_2$ or less and then assessed in 1·80 mM-Ca^{2+}. Data are presented as mean % eggs fertilized ±s.e.m. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ compared with 1·80 mM controls.

Series III: the minimum Ca^{2+} concentration required for fertilization of zona-intact eggs

From a single sperm suspension prepared in −Ca medium, 2 aliquants were taken and the appropriate $CaCl_2$ stock was added to give final Ca^{2+} concentrations of 1·80 mM and 90 μM. After preincubation for 120 min, aliquants of the 90 μM-Ca^2 suspension were diluted into media containing 90 μM, 180 μM, 450 μM, 900 μM and 1·80 mM-Ca^{2+}; the control (+Ca) suspension was diluted into +Ca medium (N = 3).

The results indicate that spermatozoa preincubated in 90 μM-Ca^{2+}, which promotes complete capacitation, can only express their full fertilizing potential in the presence of 1·80 mM-Ca^{2+} (Table 2). While no eggs were fertilized in 90 μM-Ca^{2+} medium, there was a concentration-related response with the other media: 180 μM, ~4%, 450 μM, ~20%; 900 μM, ~70%. All responses differed significantly from that obtained with 1·80 mM-Ca^2 ($P < 0{\cdot}05$–$0{\cdot}001$, depending on Ca^{2+} concentration). A similar series was carried out with suspensions preincubated in 180 μM-Ca^{2+}

Table 2. Fertilizing ability with zona-intact eggs of mouse sperm suspensions preincubated in medium containing 90 μM-Ca^{2+} and assessed in increasing concentrations of Ca^{2+}

[Ca^{2+}]		Eggs fertilized			Maximal nuclear development, %
Preincubation	Fertilization	No.	%	Range (%)	
90 μM	90 μM	0/46	0***	–	–
	180 μM	3/79	3·8***	0–8	33·3
	450 μM	19/91	20·9**	18–26	63·2
	900 μM	60/84	71·4*	54–91	76·7
	1·80 mM	62/69	89·9	59–100	98·4
1·80 mM	1·80 mM (controls)	52/52	100	–	100

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with suspensions assessed in 1·80 mM-Ca^{2+}.

Table 3. Fertilizing ability with zona-free eggs of mouse sperm suspensions preincubated for 120 min in medium containing 90 μM-Ca^{2+} and assessed in increasing concentrations of Ca^{2+}

[Ca^{2+}]		Eggs fertilized			Polyspermic eggs, %
Preincubation	Fertilization	No.	%	Range (%)	
90 μM	90 μM	0/59	0***	–	–
	180 μM	0/56	0***	–	–
	450 μM	34/64	53·1*	17–80	14·7
	900 μM	81/82	98·8	93–100	35·8
	1·80 mM	72/73	98·6	93–100	33·3
1·80 mM	1·80 mM (controls)	46/46	100	–	67·4

*$P < 0.05$, ***$P < 0.001$ compared with 1·80 mM-Ca^{2+} control suspensions.

(data not shown) and the responses were completely consistent with those presented here: only 1·80 mM-Ca^{2+} promoted maximal fertilization.

Series IV: the minimum Ca^{2+} concentration required for sperm–egg fusion

Sperm suspensions containing 90 μM and 1·80 mM-Ca^{2+} were prepared, preincubated and diluted as described for Series III. Zona-free eggs were prepared using 90 μM-Ca^{2+} medium and added to the 6 different suspensions. While no eggs were fertilized in the presence of 90 or 180 μM-Ca^{2+}, approximately 50% were fertilized in 450 μM-Ca^{2+} and essentially all were fertilized in 900 μM and 1·80 mM-Ca^{2+} (Table 3). The incidence of polyspermy was lower in the suspensions preincubated in reduced calcium.

Series V: acrosome loss in response to increasing concentrations of Ca^{2+}

Two sets of experiments were performed. In the first, a sperm suspension was prepared in −Ca medium and from this 3 droplets were made. The appropriate $CaCl_2$ stock was added to give final

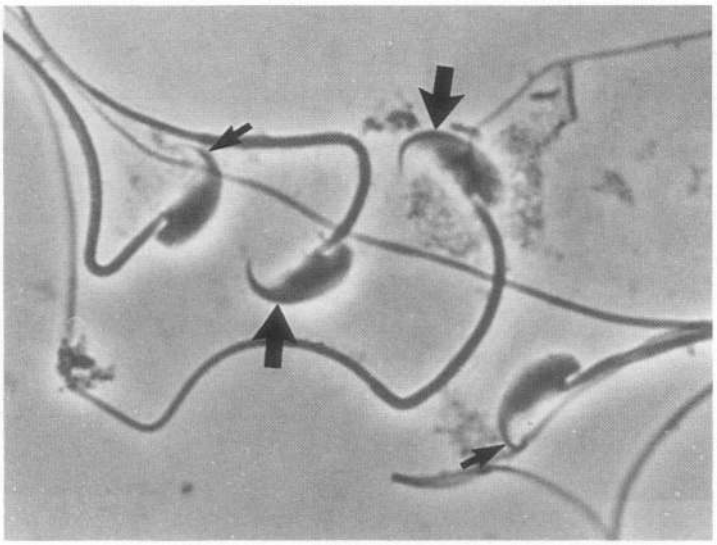

Fig. 2. Phase contrast micrograph of acrosome-intact and acrosome-free mouse spermatozoa. Two cells have an acrosome (large arrow) and two do not; the perforatorium at the anterior tip of the latter is indicated by a small arrow. × 1600.

concentrations to 90 μM- and 1·80 mM-$CaCl_2$ in 2 of the droplets; the third received −Ca medium only. After incubation for 120 min, aliquants taken from the −Ca and 90 μM-Ca^2 droplets received $CaCl_2$ stock to give a final concentration of 1·80 mM. After 10 min, all 5 suspensions were filtered, fixed and assessed for presence of the acrosome (N = 3). Typical acrosome-intact and acrosome-free spermatozoa are illustrated in Fig. 2. In the second set, a sperm suspension was prepared in 90 μM-Ca^{2+} medium and preincubated for 120 min, at which time 4 aliquants were removed. To each was added the appropriate $CaCl_2$ stock to give final concentrations of 180 μM, 450 μM, 900 μm and 1·80 mM. After 10 min suspensions were filtered, fixed and assessed (N = 4).

The acrosome reaction response was consistent with the fertilization data obtained in Series I and II. After preincubation in 90 μM-$CaCl_2$, few spermatozoa had undergone the acrosome reaction but the introduction of 1·80 mM-Ca^2 immediately triggered a response which did not differ statistically from that observed in the +Ca suspension (Fig. 3). In contrast, acrosome loss was low in the −Ca suspension, irrespective of whether 1·80 mM-Ca^{2+} was added. This confirms the conclusion drawn from Series II that 90 μM-Ca^{2+} supports full capacitation.

In the second set of experiments, the minimum concentration of Ca^{2+} required to trigger the maximal acrosome reaction response in capacitated sperm suspensions was 900 μM, i.e. half that found in the standard medium (Fig. 4). Although no response was evoked by 180 μM-Ca^{2+}, a significantly increased incidence of the acrosome reaction was promoted by 450 μM-Ca^{2+}.

Series VI: the effect of reduced Ca^{2+} concentrations on motility

In general, the proportion of motile cells was similar in all suspensions whether the Ca^{2+} concentration was 1·80 mM or lower; the most striking differences were in the expression of hyperactivated motility. For example, in Series I essentially no spermatozoa exhibited hyperactivated motility when Ca^{2+} was introduced after a 120 min incubation in −Ca (±EGTA) medium, but after an additional 60 min in Ca^{2+} the suspensions were indistinguishble from those incubated continuously in the presence of Ca^{2+}. When spermatozoa were incubated in 90 μM-Ca^{2+} to capacitate and then increasing amounts of Ca^{2+} were introduced, there was a concentration-dependent

increase in the proportion of motile cells exhibiting hyperactivated motility: 90 μM-Ca^2, none; 180 μM-Ca^2, none; 450 μM, ~5–10%; 900 μM, ~25–30%; 1·80 mM, ~30–40%. The value for spermatozoa incubated continuously in 1·80 mM-$CaCl_2$ was ~40–50%. The response was therefore greatest in the presence of 900 μM and 1·80 mM-Ca^{2+}, the concentrations which promoted the highest fertilization rates.

Series VII: the effect of elevated Ca^{2+} concentrations on capacitation and fertilization

Medium containing elevated Ca^{2+} concentrations was prepared by adding appropriate volumes of 22·5 mM-$CaCl_2$ stock to the standard +Ca (1·80 mM-$CaCl_2$) medium to obtain 3·60 and 7·20 mM-Ca^{2+}; these represent ×2 and ×4, respectively, the standard concentrations. No attempt was made to adjust the osmolality since the increase due to additional $CaCl_2$ was a maximum of only 16·2 mosmol. Sperm suspensions were prepared in media containing 1·80, 3·60 and 7·20 mM-Ca^{2+}. After 30 min preincubation, aliquants were diluted into the same medium or +Ca to assess fertilizing ability (N = 4 except for 7·20 mM-Ca^{2+} for which N = 1). After a further 90 min preincubation, suspensions were again sampled as above. In addition, +Ca suspensions were diluted

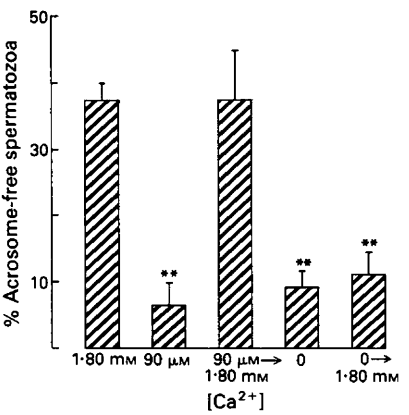

Fig. 3. Acrosome loss in mouse sperm suspensions incubated for 120 min in the presence of 1·80 mM, 90 μM and 0 (Ca^{2+}-deficient) Ca^{2+}; assessment of the latter two was made before and after addition of 1·80 mM-Ca^{2+}. Data are presented as mean % ±s.e.m. $^{**}P < 0·01$ compared with 1·80 mM-Ca^{2+} controls.

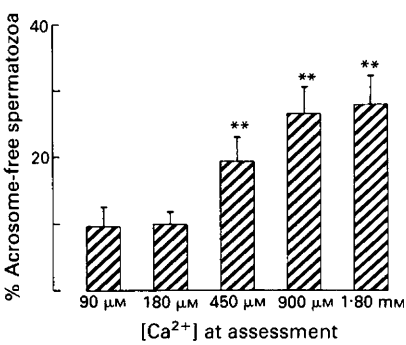

Fig. 4. Acrosome loss in mouse sperm suspensions incubated in medium containing 90 μM-Ca^{2+} for 120 min; increasing amounts of Ca^{2+} were then added. Data are presented as mean % ±s.e.m. $^{**}P < 0·01$ compared with 90 μM-Ca^{2+} suspensions.

into 3·60 and 7·20 mM-Ca^{2+} (N = 3). In a separate set of experiments, sperm suspensions incubated as above and diluted into the same medium were assessed with zona-free eggs (N = 2).

Suspensions preincubated 30 min in +Ca medium were moderately fertile with only a minority of eggs reaching advanced stages (Table 4) and a similar pattern was observed in the 3·60 mM-Ca^{2+} suspensions, although values were slightly elevated. However, when the Ca^{2+} was reduced in the latter before assessment, a significantly higher ($P < 0·05$) proportion of eggs was fertilized compared with the 1·80 and 3·60 mM-Ca^{2+} suspensions. In the single replicate in which 7·20 mM-Ca^{2+} was evaluated, fertilization in the presence of both 7·20 and 1·80 mM-Ca^{2+} was reduced: 10/25, 37% and 5/21, 24%, respectively (data not tabulated). These results therefore suggest that increasing the normal Ca^{2+} by a factor of 2 accelerates capacitation while even higher values of Ca^{2+} are deleterious; despite this, expression of fertilizing potential is more fully realized in the presence of lower Ca^{2+} (i.e. 1·80 mM).

Table 4. Fertilizing ability of mouse sperm suspensions preincubated and assessed in Ca^{2+} concentrations ranging from 1·80 to 7·20 mM

Preincubation time, min	[Ca^{2+}]		Eggs fertilized			Maximal nuclear development, %
	Preincubation	Fertilization	No.	%	Range (%)	
30	1·80	1·80	57/101	56·4	50–70	43·9
	3·60	3·60	86/130	66·2	20–80	52·3
		1·80	157/186	84·4†	76–90	61·8
120	1·80	1·80	81/87	93·1	87–100	92·6
		3·60	42/56	75·0*	56–96	83·3
		7·20	42/57	73·7*	65–90	97·6
	3·60	3·60	19/49	38·8**	23–62	73·7
		1·80	12/35	34·3**	6–45	66·7
	7·20	7·20	3/35	8·6***	0–14	66·7
		1·80	2/38	5·3***	0–11	50·0

†$P < 0·05$ compared with 30 min 1·80 mM-Ca^{2+} suspensions.
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with 120 min 1·80 mM-Ca^{2+} suspensions.

Consistent with results in earlier series, preincubation of spermatozoa for 120 min in 1·80 mM-Ca^{2+} promoted complete capacitation and rapid penetration of the fertilizing spermatozoa (93%, Table 4). When aliquants of these same suspensions were diluted into both 3·60 and 7·20 mM-Ca^{2} there was a slight but significant ($P < 0·05$) reduction in fertilizing ability. Although 3·60 mM-Ca^{2+} had a positive effect on spermatozoa within 30 min, suspensions preincubated for 120 min in elevated Ca^{2+} were significantly less fertile ($P < 0·01$), when assessed in either 3·60 or 1·80 mM-Ca^{2+}, than control +Ca suspensions. Even fewer eggs (<10%) were fertilized with suspensions preincubated in 7·20 mM-Ca^{2+}. Preincubation for 120 min in Ca^{2+} concentrations above 1·80 mM therefore significantly inhibits fertilizing ability when zona-intact eggs are used.

Despite the above observations no significant differences in the ability of suspensions preincubated for 120 min in 1·80 or 3·60 mM-Ca^{2+} to fertilize zona-free eggs could be detected (Table 5): all eggs were fertilized and the incidence of polyspermy was similar. Even in 7·20 mM-Ca^{2+}, ~75% of eggs were fertilized; the much reduced incidence of polyspermy in this treatment group is consistent with the apparently reduced functional ability of the sperm population.

Series VIII: the effect of elevated Ca^{2+} concentrations on acrosome loss

Suspensions were prepared in 1·80, 3·60 and 7·20 mM-Ca^{2+} and incubated. At 30 and 120 min, aliquants were removed and assessed for acrosome loss.

Table 5. Fertilizing ability with zona-free eggs of mouse sperm suspensions preincubated for 120 min and assessed in media containing 1·80 mM- to 7·20 mM-Ca^{2+}

	Eggs fertilized			
[Ca^{2+}]	No.	%	Range (%)	Polyspermic eggs, %
1·80	42/42	100	–	52·3
3·60	59/59	100	–	40·6
7·20	50/65	76·9	57–94	16·0

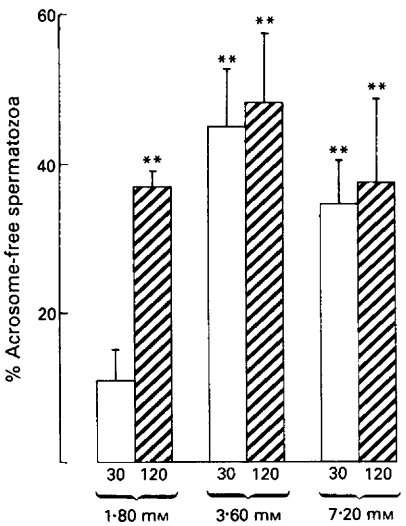

Fig. 5. Acrosome loss after 30 and 120 min in mouse sperm suspensions incubated in medium containing 1·80, 3·60 or 7·20 mM-Ca^{2+}. Data are presented as mean % ±s.e.m. $^{**}P < 0·01$ compared with 1·80 mM-Ca^{2+} at 30 min.

The pattern of acrosome loss in 1·80 mM-Ca^{2+} medium was typical of that usually seen (Fraser, 1983a, b): few acrosome-reacted spermatozoa at 30 min (mean of 11%; Fig. 5) but significantly more at 120 min (mean of 37%). In contrast, a significantly higher ($P < 0·01$) proportion of acrosome-reacted cells was observed in both 3·60 and 7·20 mM-Ca^{2+} at 30 min (45% and 35%, respectively). These values did not alter appreciably during the next 90 min.

Series IX: the effect of elevated Ca^{2+} concentrations on motility

Diluted suspensions in the various media were evaluated at 30 and 120 min for proportion motile and expression of hyperactivated motility. At 30 min, spermatozoa were highly motile in both 1·80 and 3·60 mM-Ca^{2+}; hyperactivated motility was regularly observed in about 10–15% of motile cells in the presence of elevated Ca^{2+} but not in the standard concentration of 1·80 mM-Ca^{2+}. This is consistent with the acceleration of capacitation and enhanced fertilizing ability observed in the former suspensions. At 120 min, suspensions incubated in 1·80 mM-Ca^{2+} were judged to express hyperactivated motility to the degree expected from previous evaluations of highly fertile populations (e.g. Series VI, this study; Fraser, 1983b) and while motility in the elevated

Ca^{2+} concentrations was often less good, it was never markedly reduced. In 3·60 mM-Ca^{2+}, the proportion of motile cells was always equivalent to that in control +Ca medium but the proportion expressing hyperactivated motility was frequently reduced, ranging from ~50% as seen in controls down to ~15%. Occasionally the movements had a somewhat jerky appearance, but suspensions were never judged to be severely affected. In contrast, the suspensions incubated in 7·20 mM-Ca^{2+} consistently had fewer (usually by ~10%) motile cells than did control suspensions, hyperactivated motility was seen in only about 10–15% of cells and movement patterns were usually erratic. Despite these differences, the spermatozoa in elevated Ca^{2+} did not appear to be severely compromised by the treatment when assessed by light microscopy.

Discussion

Data from both this study (Table 1) and an earlier one (Fraser, 1982) clearly indicate that mouse sperm suspensions preincubated in medium from which $CaCl_2$ has been omitted are functionally incompetent when millimolar Ca^{2+} subsequently is introduced, along with eggs. A criticism often levelled at the use of such 'Ca^{2+}-free' media is that they are not truly free of Ca^{2+} but have trace amounts contributed by other salts; the possibility has to be considered that even very low concentrations of Ca^{2+} may enhance cell function. This is not the case for mouse spermatozoa, since the inclusion of sufficient EGTA to chelate free calcium ions present in the Ca^{2+}-deficient medium (13 μM) did not affect the kinetics of capacitation. Whether preincubated for 120 min in −Ca medium in the presence or absence of EGTA, spermatozoa became functional at the same rate once millimolar Ca^{2+} was added; 1 h after such treatment, suspensions were highly fertile. This is consistent with the report by Fraser (1982) that complete capacitation of mouse spermatozoa required the presence of extracellular Ca^{2+} during the final 30–60 min of preincubation; with a shorter exposure suspensions proved to be only partially capacitated. In contrast, the introduction of Ca^{2+} to guinea-pig spermatozoa preincubated in Ca^{2+}-deficient medium promoted fertilization within a relatively short time (Yanagimachi & Usui, 1974), suggesting that the cells were completely capacitated and ready to respond to Ca^{2+}.

Further experiments established the minimum concentration required during preincubation to ensure a consistent maximal fertilization response when 1·80 mM-Ca^{2+} was added at 120 min. This proved to be 90 μM, i.e. only 5% of that included in standard Tyrode's. Interestingly, all lower concentrations examined generally improved fertilizing ability compared with that obtained with Ca^{2+}-deficient medium, although the response of individual suspensions varied considerably (Fig. 1). Following preincubation in 90 μM-Ca^{2+}, the minimum Ca^{2+} to ensure fertilization, including both zona penetration and sperm-egg fusion, was investigated using zona-intact eggs and zona-free eggs, respectively. Only 1·80 mM-Ca^{2+} supported maximal fertilization of zona-intact eggs. With zona-free eggs, no gamete fusion was observed until 450 μM-Ca^{2+} was added; then, 50% of eggs were fertilized, a figure considerably higher than the 20% of zona-intact eggs. The fact that a very few zona-intact (3/79, Table 2) but no zona-free eggs were fertilized in 180 μM-Ca^{2+} probably indicates the presence of some Ca^{2+} associated with the cumulus cell complex. There were no detectable differences in fusion between suspensions in 900 μM and 1·80 mM-Ca^{2+}. The fact that polyspermy was considerably higher with suspensions incubated continuously in 1·80 mM-Ca^{2+} suggests that the numbers of competent spermatozoa are greater under these conditions. On the basis of the above results, maximum fusion between mouse gametes requires 900 μM-Ca^{2+}, while maximum zona penetration requires 1·80 mM-Ca^{2+}. These results for zona penetration are consistent with those, also for the mouse, reported by Miyamoto & Ishibashi (1975). In most other species comparable optimal values have yet to be established, but gamete fusion in the hamster would appear to have a lower minimum since 250 μM-Ca^{2+} supported fusion in 83% of eggs and 1·00 mM, in 93% (Yanagimachi, 1978).

It has been reported that mouse spermatozoa require Ca^{2+} in order to bind the zona before penetration (Saling *et al.*, 1978; Heffner *et al.*, 1980). While binding was not specifically investigated in the present study, there was no noticeable decrease in numbers of spermatozoa remaining attached to zonae during the various manipulations which preceded microscopic evaluation, irrespective of the Ca^{2+} concentration used for sperm–egg incubations. Indeed, unfertilized eggs are usually readily distinguishable from fertilized ones because the latter have few spermatozoa associated with the zona, presumably reflecting changes in the zona which follow the cortical granule reaction. Yanagimachi (1982) has reported that capacitated hamster spermatozoa can attach to zonae in Ca^{2+}-deficient medium, even in the presence of EGTA.

The immediate high fertility upon introduction of 1·80 mM-Ca^{2+} to suspensions preincubated in 90 μM-Ca^{2+} suggests the ability to undergo the acrosome reaction. Assessment of acrosome loss showed this to be the case: introduction of millimolar Ca^{2+} at 120 min triggered the acrosome reaction in the same proportion of cells (38%) as observed in suspensions maintained continuously in +Ca medium, while few cells (7%) in low Ca^{2+} were acrosome-free (Fig. 3). When increasing concentrations of Ca^{2+} were introduced to similarly preincubated suspensions, the maximum acrosome reaction response was evoked by both 900 μM- and 1·80 mM-Ca^{2+} and a response still significantly above background was initiated by 450 μM-Ca^{2+}. It is generally accepted that spermatozoa must undergo the acrosome reaction before fusion with the egg (Yanagimachi, 1981) and these results for triggering of the acrosome reaction are completely consistent with the minimum and optimal Ca^{2+} concentrations required for gamete fusion. Few comparable data are available for other species, although in the study of Yanagimachi (1982) means of >30% motile hamster spermatozoa had undergone the acrosome reaction in media containing 200 and 400 μM-Ca^{2+} and >50% in 1·80 mM-Ca^{2+}. Again, the minimum values appear to be lower in the hamster than the mouse.

The most logical explanation for the differences in Ca^{2+} concentration required to elicit maximal zona penetration (1·80 mM) and the acrosome reaction/gamete fusion (900 μM) is that maximal hyperactivated motility requires higher extracellular Ca^{2+}. This proved to be the case, with 1·80 mM-Ca^{2+} evoking the highest response. Motility *per se* was maintained in all media assessed; earlier reports that mouse sperm motility is reduced in Ca^{2+}-deficient media (e.g. Heffner *et al.*, 1980; Heffner & Storey, 1981) have not been confirmed in either this or previous studies when appropriate energy substrates have been included in the media (Fraser, 1982; Cooper, 1984). Maximal fertilization of zona-intact eggs was only achieved when the Ca^{2+} concentration was sufficiently high (1·80 mM) to support both maximal acrosome reaction and motility responses. The calcium ion concentration in the mouse ampulla, when measured post-coitally, is 1·71–1·94 mM (Borland *et al.*, 1977) and thus should provide optimal Ca^{2+} concentrations for fertilization *in vivo*.

Responses to elevated Ca^{2+} proved to be strongly dependent on length of incubation in such conditions, with extended exposure irreversibly damaging fertilizing potential. The concentrations used, 3·60 and 7·20 mM, were ×2 and ×4 the concentration found in standard Tyrode's medium. The higher of these was very detrimental to sperm function after both brief (30 min) and extended (120 min) preincubations and these effects could not be reversed by lowering the Ca^{2+} concentration. In contrast, suspensions preincubated for 30 min in 3·60 mM-Ca^{2+} proved to be functionally superior, when Ca^{2+} was reduced to 1·80 mM, compared with counterparts maintained in 1·80 mM-Ca^{2+}; significantly more eggs were fertilized and more were at advanced stages of nuclear development (Table 4). Continued preincubation until 120 min, however, irreversibly damaged sperm fertilizing ability. Given that these spermatozoa underwent the acrosome reaction and could exhibit hyperactivated motility, the effect of high Ca^{2+} is on expression of fertilizing ability and not on capacitation *per se*.

The explanation for these somewhat paradoxical responses presumably lies in the effects that elevated extracellular Ca^{2+} has on intracellular Ca^{2+}. Since spermatozoa exist in environments with millimolar extracellular Ca^{2+} but maintain nanomolar concentrations of intracellular calcium, they must have efficient mechanisms for removing excess intracellular Ca^{2+}. Current

evidence suggests that Ca^{2+}-ATPases and/or Na^+/Ca^{2+} exchangers may fulfil this role (reviewed by Fraser, 1987). It is thought that during capacitation spermatozoa become more permeable to Ca^{2+} until a critical threshold is reached; increases beyond this will then trigger changes in the acrosome and motility pattern (see Yanagimachi, 1981; Harrison, 1982; Fléchon *et al.*, 1986). From the present study, it seems probable that in media with elevated Ca^{2+} the rate of Ca^{2+} influx exceeded the rate of Ca^{2+} efflux, leading to intracellular Ca^{2+} above the critical value. This would explain the significantly elevated incidence of the acrosome reaction and the precocious onset of hyperactivated motility after 30 min in high Ca^{2+}. However, normal function requires a stable intracellular Ca^{2+} concentration; motility in particular is inhibited if a species-dependent critical level is exceeded (Tash & Means, 1983). Continued incubation in high Ca^{2+} might well lead to intracellular Ca^{2+} levels, beyond those needed to trigger the acrosome reaction/hyperactivated motility, which would ultimately interfere with these responses. For example, although the initial incidence of acrosome loss was high, very little increase was seen from 30 to 120 min. While motility was affected, more noticeably in 7·20 than 3·60 mM-Ca^{2+}, this was expressed as a more erratic pattern rather than as a cessation of movement. The fact that zona penetration was inhibited more than sperm–egg fusion is consistent with a primary effect on motility; inappropriate or inadequate motility will prevent passage through the zona (Fraser, 1981).

Just as little is known about minimum Ca^{2+} concentrations and mammalian sperm function, the same is true for maximum values. Earlier studies of mouse spermatozoa *in vitro* had indicated an inhibition of fertilization in the presence of elevated Ca^{2+} but specific functional defects were not identified (Iwamatsu & Chang, 1971; Miyamoto & Ishibashi, 1975); in these, concentrations of approximately $\times 2{\cdot}5$ and $\times 3$ the standard were used, respectively. Less consistency in response has been obtained with rat spermatozoa. Davis (1978) and Kaplan & Kraicer (1978) found that elevated Ca^{2+} (up to 3·40–6·80 mM; $\times 2$–$\times 4$) enhanced fertilizing ability *in vitro*, while Miyamoto & Ishibashi (1975) reported that these concentrations were as inhibitory in the rat as in the mouse. Such discrepancies are possibly due to strain differences, but might also reflect the ability or inability of the standard culture media to promote Ca^{2+} entry.

The present study is the first to evaluate critically the minimal and maximal Ca^{2+} concentrations required to support capacitation, the acrosome reaction, hyperactivated motility, zona penetration and sperm–egg fusion. The optimal concentration to promote all steps in leading to successful fertilization is 1·80 mM. As Ca^{2+} is lowered, first motility and then the acrosome reaction/gamete fusion are impaired; as Ca^{2+} is raised, the primary effect appears to be on motility. Despite the fact that extracellular Ca^{2+} must be present to ensure complete capacitation, capacitation can now be dissociated from the acrosome reaction and motility changes since the Ca^{2+} requirements are so different. This will enable specific sperm functions to be examined more rigorously than hitherto possible by manipulation of Ca^{2+} and will not require use of various drugs to induce responses.

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